Pathologic Observations of Mouse Liver Infected with Tyzzer's Organisms Using Immunoperoxidase Method

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ABSTRACT. Using immunoperoxidase method the production of liver lesions and propagation of organisms were observed in mice intravenously inoculated with MSK strain of Tyzzer's organisms and treated with cortisone. Microbial antigen was first detected on Day 2 postinoculation (p.i.) within very small necrotized foci. On Day 3 p.i. or later when necrotic foci with neutrophil infiltration increased in size and number, many immunoperoxidase-positive bacteria were present within seemingly living hepatocytes at the periphery of lesions but not within the necrotized foci. Apart from large-sized necrotized foci some hepatocytes carrying abundant bacterial antigen were singly present without inducing any mesenchymal response. At the moribund stage, neutrophils as well as Kupffer cells were found to carry bacterial antigen in the sinusoidal spaces without showing morphologic evidence of bacterial growth. Electron microscopy revealed that antibody specifically combined with the flagella and bacterial surface.—**KEY WORDS:** immunoperoxidase, mouse, Tyzzer's disease.

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Tyzzer's disease is characterized by multiple focal necrosis in the liver [3, 22] due to "*Bacillus piliformis*" named by Tyzzer [22], which is known to be an obligate intracellular parasite being present in large number within the cytoplasm of liver cells around necrotic foci. The intracellular organisms are so difficult to stain with hematoxylin and eosin (HE) that Giemsa, periodic acid Schiff or Gomori's methenamine silver (GMS) method [9] has been used to detect the organisms in infected tissues. These stain procedures, however, seem to reveal only small number of morphologically intact organisms. The demonstration of lesser amount of microbial antigen might be rather of importance for studying pathogenesis of the disease caused by the extremely fragile bacterium [2]. Immunofluorescence is applicable to detection of organisms or their antigenic materials in lesions [13, 17], but it is not useful enough to see the localization of bacterial antigen in infected tissues.

In this study, liver tissues of experimental-

ly infected mice were observed by light and electron microscopy after application of immunoperoxidase method.

MATERIALS AND METHODS

Animals: Four- to 5-week-old ICR female mice weighing 17 to 21 g were obtained from Charles River Japan (Atsugi, Kanagawa) and maintained on pellets (Type MF, Oriental Yeast, Tokyo) and water *ad libitum*. The breeder colony was serologically checked for being Tyzzer's disease-free [7].

Inoculation: The MSK strain [8] of Tyzzer's organisms were passaged in mice by intravenous (i.v.) inoculation with severely infected mouse liver homogenate plus cortisone. Inoculation was made with 0.2 ml-infected mouse liver homogenate (1:200) in phosphate-buffered saline, pH 7.4 (PBS) containing more than 10^5 organisms. Shortly after the inoculation, 2.5 mg hydrocortisone acetate (HCA) (Scherosone F, Nippon Shering, Osaka) was given subcutaneously.

Histopathology: Five animals each were killed on Days 1 to 5 postinoculation (p.i.). Tissues of the liver, spleen, kidney and heart were taken and they were fixed in 10% neutral buffered formalin or Bouin's fluid without acetic acid and embedded in paraffin. Two to 4 μm serial sections were made and stained with HE, GMS method and avidin-biotin-complex (ABC) immunoperoxidase method [10, 11].

Immunoperoxidase procedures: After deparaffinization endogenous peroxidase activity of each tissue was blocked by treating with 0.3% H_2O_2 in absolute methanol. The sections were then incubated sequentially with (1) normal goat serum, (2) anti-MSK rabbit antibody (1:16,000) showing an indirect immunofluorescence staining titer [4] of 1:16,000, (3) biotinylated anti-rabbit IgG goat antibody (1:200), (4) ABC reagent and (5) 0.05% 3,3'-diaminobenzidine tetrahydrochloride and 0.1% H_2O_2 in Tris-HCl buffer, pH 7.6 (DAB- H_2O_2). Then, they were counterstained with hematoxylin. The primary antiserum was of rabbits immunized with organisms grown on the primary culture of mouse hepatocytes [12] and absorbed twice

with normal mouse liver acetone powder (100 mg/ml) before use. The secondary biotinylated antibody and ABC reagent were obtained commercially (Vectastain, Vector Lab., Burlingame, U.S.A.). Specificity of the immunostain was checked by incubating some sections with the anti-MSK serum previously absorbed with MSK-infected mouse liver powder, resulting in complete blocking.

Immunoelectron microscopy: Liver tissue blocks $4 \times 3 \times 1$ mm in size taken on Day 3 or 4 p.i. were fixed in a modified Zamboni's solution [18] for 6 hr. After washing in 0.1 M phosphate buffer (pH 7.6), they were immersed in 10% and 20% sucrose in the same buffer for 2 hr and 1 hr, respectively. The tissue blocks were introduced into n-hexane in acetone-dry ice and 20 μm cryostat sections were prepared. After washing in 0.1 M phosphate buffer, pH 7.6, sections were incubated with reagents (1) to (4) as described already, and they were fixed in 2% glutaraldehyde in PBS at 4°C for 20 min. After washing in the same buffer and incubated with DAB- H_2O_2 solution, the sections were washed in Tris-HCl buffer, postfixed in 1% OsO_4 in 0.1 M phosphate buffer, pH 7.4,

Fig. 1. Bacterial antigen within and around necrotized foci. Day 3 p.i. ABC method. $\times 45$.

dehydrated and embedded in Spurr resin [19]. Ultrathin sections were made by Sovall MT-5000 ultramicrotome and were examined under a JEM-100S electron microscope at 60 or 80 kV.

Fig. 2. Organisms within liver cells on two serial sections stained with GMS-HE (a) and ABC (b) methods. Immunoperoxidase-positive rods and granules were more numerous on the ABC rather than those stained with GMS. Day 3 p.i. $\times 460$.

RESULTS

On Day 2 p.i., small necrotized lesions were first demonstrated and a few degenerated liver cells were surrounded with some neutrophils. The cytoplasm of liver cells at the periphery of the lesions was shown to combine with anti-MSK antibody by immunoperoxidase method, but no bacterial anti-

Fig. 3. Liver cell carrying bacterial antigen and a few immunoperoxidase-positive free rods. Day 3 p.i. ABC method. $\times 460$.

Fig. 4. Bacterial antigen distributed at the periphery of a necrotic focus and infiltration of neutrophils and macrophages at the center. Day 3 p.i. ABC method. $\times 230$.

gen was detected in other parts of the liver.

On Day 3 p.i., multiple necrotic foci appeared within acini and perivascularly in the liver, and they increased in size and number (Fig. 1). On GMS-stained preparations, organisms were found as slender rods within the cytoplasm of liver cells, and individual organisms were located separately in parallel each other (Fig. 2a). On immunoenzymatically stained preparations, organisms colored dark-brown appeared to be thicker than GMS-stained ones in host cells. And many specifi-

cally stained granules were found within liver cells, some of which were located apart from necrotized lesions without any inflammatory response around it (Fig. 2b). Within the cytoplasm of adjacent liver cells rod shaped microbes were immunoperoxidase-positive (Fig. 3). In large necrotized lesions, the infiltration of neutrophils and macrophages was observed, and microbial antigen was located around or near necrotic foci but not present within them (Fig. 4).

On Day 4 p.i., necrotic foci further in-

Fig. 5. HE (a)- and ABC (b)-stained adjacent sections, showing bacterial antigen distributed in multiple necrotic foci. Day 4 p.i. $\times 45$.

creased in size and number, and they mostly fused each other (Fig. 5a). In extensively developed lesions, antigen was detected mostly at the periphery and very few at the centrum (Fig. 5b). Organisms were seen to be liberated into either the sinusoids or central veins, causing bacteremia. The antigen-positive granules were sometimes detected within the cytoplasm of sinusoidal lining cells as well as Kupffer cells without showing

multiplication of organisms (Fig. 6). Also antigen was present in renal glomerular capillaries and the splenic red pulp (Figs. 7 and 8).

By electron microscopy a few organisms were observed on Day 3 p.i. within the cytoplasm of liver cells without being surrounded by any host limiting membrane (Fig. 9). There was no degenerative changes in the organelles of host cells. The flagella and cell surface of organism were shown to combine

Fig. 6. Bacterial antigen within the cytoplasm of Kupffer cells. Day 4 p.i. ABC method. $\times 460$.

Figs. 7 and 8. Bacterial antigen in renal glomerular capillaries and the splenic red pulp seemingly without microbial propagation nor host inflammatory response. Day 4 p.i. ABC method. $\times 460$.

Fig. 9. Two organisms combining with antibody on their surface within the cytoplasm of a liver cell showing no changes in organelles. Day 3 p.i. ABC method. Electron microscopy (EM) $\times 9,000$.

Fig. 10. An organism with immunoperoxidase-positive flagella within the cytoplasm of a host cell. Day 3 p.i. ABC method. EM $\times 30,000$.

with anti-MSK antibody, and antigen-positive flagella intervened the bacterial body and cytoplasmic matrix (Fig. 10). The cytoplasmic matrix of some infected liver cells showed necrobiosis surrounded by a few flagellated organisms on Day 4 p.i. of infection (Fig.

11).

In severely infected liver cells, many organisms were located mostly in parallel and were separated each other (Fig. 12). The vacuolation of rough endoplasmic reticulum and deformation of mitochondria were seen in

Fig. 11. Four organisms with immunoperoxidase-positive flagella within a necrobiotic liver cell. Day 4 p.i. ABC method. EM $\times 12,000$.

Fig. 12. Organisms being positive for immunoperoxidase on their surface and located in the cytoplasmic matrix of an infected hepatocyte. Day 4 p.i. ABC method. EM $\times 10,000$.

infected liver cells. Occasionally antigen-positive materials contacted with the surface of neutrophils, and phagocytosis for the antigen occurred at the center of necrotized lesions (Figs. 13 and 14). Furthermore, frag-

mented organisms still combining with antibody were occasionally detected within the cytoplasm of Kupffer cells, which seemed to remain morphologically unstimulated (Fig. 15).

Fig. 13. Neutrophils phagocytizing immunoperoxidase-positive granules in the sinusoidal space. Day 4 p.i. ABC method. EM $\times 12,000$.

Fig. 14. Bacterial antigen within a phagosome of a neutrophil at the center of a necrotic focus. Day 4 p.i. ABC method. EM $\times 6,000$.

Fig. 15. Kupffer cell phagocytizing bacterial antigen. Day 4 p.i. ABC method. EM $\times 7,500$.

DISCUSSION

Tyzzar's disease due to a strict intracellular parasite has been difficult to study on its pathogenesis because of very rapid lysis of organism occurring following the death of

host cells [2, 5]. The propagation of the organisms was suggested to depend upon metabolic activities of host cells [15, 20]. For revealing the process of the production of a necrotic focus in the liver, it is of importance to see relationship between parasites and host

cells in more details using immunopathological method, which may be useful for detecting antigen or fragmented bacteria. In this study immunoperoxidase method was found to be very useful for detecting the bacterial specific antigen. It is more sensitive than other routine histopathologic stain, revealing a small number of organisms during either early or late stage of infection.

On Day 2 p.i. bacterial antigen was already detected within the cytoplasm of liver cells located at the periphery of the lesions. With the propagation of organisms necrotized lesions increased in size and number, and immunoperoxidase-positive organisms or their fragments were demonstrated in and/or around these lesions. For producing the initial liver lesions, there seems to be two possibilities; one is an accumulation of leucocytes phagocytizing the organisms in the sinusoid and the other a direct invasion of organisms into hepatocytes [6]. On Day 3 p.i. rod shaped bacteria enzymatically stained were detected within seemingly living liver cells connecting with each other, suggesting that organisms may infect directly the neighbouring liver cells forming necrotic foci.

The deep centrum of large-sized lesions consisted of degenerated or necrotized hepatocytes with infiltration of neutrophils and macrophages, but bacterial antigen could not be detected. By immunofluorescence microscopy Kraft and Thunert [13] observed that the disintegration of organisms in infected liver tissues started after 8 hr of storage *in vitro* while it was impossible to determine whether the bacteriolysis occurred during storage *in vitro* or already *in vivo*. The number of organisms as well as the infectivity in affected tissues were shown to be rapidly decreased *in vitro* [2, 5, 8], suggesting that *in vivo* microorganisms might be also disintegrated and lose their pathogenicity and antigenicity shortly after leaving necrotized host cells, when they failed to parasitize another host cells.

By electron microscopy, bacterial antigen was clearly demonstrated within the cytoplasmic matrix of host cells, even in degenerated or necrotized cells. Immunoperoxidase-positive organisms existed intracellularly without any host limiting membrane, being different from conventional parasitisms of other species of bacteria within the phagosome of host cells such as salmonellosis [21, 23], brucellosis [16] and tuberculosis [1].

In infected and severely degenerated hepatocytes, intracellular organisms showed cytoplasmic aggregation and lysis of the cell wall [14]. In earlier stage of infection, bacterial autolysis within host cells may start with the loss of flagella followed by lysis of the cell wall. The immunoperoxidase-positive fragments liberated from necrotized hepatocytes were mostly taken by locally appearing neutrophils, and some of them were liberated into the sinusoid. Fujiwara *et al.* [6] observed that Kupffer cells were activated at 4 to 8 hr p.i. peaking at 20 to 24 hr p.i. and that the endothelial lining cells never supported multiplication of organisms during the experimental infection. Some antigenic materials were taken by Kupffer cells as well as neutrophils, but these cells seemed to act as nonspecific scavenger cells.

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要 約

Tyzzer 菌感染マウス肝の免疫酵素抗体法による病理学的観察: 岡田信彦・鳥海 亘・坂本賢治・藤原公策 (東京大学農学部家畜病理学教室) —— Tyzzer 菌 MSK 株をマウス静脈内に接種し、肝病変の推移と菌増殖過程について免疫酵素抗体法による光顕および電顕的観察を行った。特異抗原は接種後 2 日に小壊死巣周囲の肝細胞内に見出された。壊死巣の拡大、増加とともに病巣周囲肝細胞に多量の菌抗原が認められ、壊死巣から離れた健常部においても、菌抗原を充満した肝細胞が見出されたが、周囲に炎症反応はみられなかった。大壊死巣では菌抗原は病巣周囲にのみ認められた。電顕的には、肝細胞内菌の細胞表面および周毛性鞭毛に特異抗体が結合し、菌体周囲に限界膜は認められなかった。菌増殖とともに、肝細胞基質の破壊、小胞体の空胞化、ミトコンドリアの変性が観察され、類洞内では好中球および Kupffer 細胞による菌抗原の貪食像がみられた。