Effects of the Components of *Fusobacterium necrophorum* in Experimental Liver Abscess Formation in Mice

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**ABSTRACT.** Hepatic lesions produced by intravenous inoculation of intact *Fusobacterium necrophorum* were compared with those produced by its extracted components in mice. Coagulative necrosis along with bacterial emboli in the sinusoid developed into true abscess within 7 days after the intact bacterial inoculation. Serositis was produced by the cell wall fraction. Hepatic degeneration and necrosis and abnormalities in blood coagulation were observed by inoculation with either the lipopolysaccharide or the cytoplasmic fraction. Fluorescent antigens corresponding to *F. necrophorum*, the lipopolysaccharide or the cytoplasmic fractions were observed in the sinusoid.—**KEY WORDS:** bacterial toxin, *Fusobacterium necrophorum*, hepatic necrosis, liver abscess.

Hepatic *Fusobacterium necrophorum* (*F. necrophorum*) infection has been studied by application of mice models [12], and histopathological changes of the abscess have been described [1, 10, 14]. Tiny foci of degenerated or necrotic hepatocytes, phagocytic reaction of macrophages against *F. necrophorum*, and liver abscess have been observed during the course of the infection [9]. *In vitro* productions of the toxins by *F. necrophorum* have also been investigated [2, 3, 6–8, 15, 18]. However, the process of the infection leading to abscess and the effects of toxins on the hepatic lesions are still obscure. In this paper, hepatic lesions produced by intact *F. necrophorum* were compared with those produced by extracted components from *F. necrophorum*. The bacteriological study of the results of these experiments has been published in part [17].

**MATERIALS AND METHODS**

*Inoculation of the intact bacteria to mice: F. necrophorum*, American type culture collection (ATCC) 25286, was grown using the roll tube method in the modified viande et de levures (VL) medium at 37°C for 18 hours [17]. The bacteria were washed once with the medium after centrifugation of 6,500×g for 20 minutes, and then suspended in the medium. The inoculum contained 2×10⁸ CFU/0.2 ml of viable bacteria. Sixty female outbred BALB/c mice (originated from NIH, Tokyo), 4 weeks of age, were used. Fifty-five mice were inoculated intravenously with 0.2 ml of the inoculum and 5 mice with 0.2 ml of the medium. The animals were autopsied at intervals of up to two weeks or immediately after death.

**Inoculation of the extracted components to mice: F. necrophorum**, ATCC 25286, was grown in VL medium at 37°C for 18 hours [17]. The organisms, 100 mg/ml wet weight, were obtained by centrifugation of 6,500×g for 20 minutes. Cell wall, cytoplasm and lipopolysaccharide (LPS) were prepared as follows. 1. Cell wall. The organisms were washed twice with 0.85% saline and disrupted with an ultrasonicator (Branson SONIFIER®, USA) for 20 minutes. After centrifugation at 20,000×g for 15 minutes, the sediments were washed twice with 1 M
NaCl and rewash five times with distilled water [3]. 2. Cytoplasm. Cytoplasmic fraction was prepared by precipitation with 50% saturated ammonium sulfate from the supernatant after the ultrasonication. After dialysis, the resulting solution was fractionated by gel filtration (Sephacryl: S-300 2.5 x 100 cm). The fraction eluted in the first peak was collected as the cytoplasmic fraction [8]. 3. LPS. LPS was extracted from the cell wall fraction with 45% phenol [7]. Finally, the volume of sugar and protein in each fraction was measured by the anthrone test and Lowry's method, respectively [13]. Sixty-five outbred BALB/c mice (originated from NIH, Tokyo), 6 weeks old, were divided into 6 experimental groups of 10 mice each and a control of 5 mice. In each group, mice were intraperitoneally inoculated with 0.2 ml of non-diluted or 1:5 dilution of the fractions or the medium. The mice inoculated with non-diluted fractions were autopsied after 24 hours or immediately after death, and the mice inoculated with 1:5 diluted fractions or the medium after 48 hours, respectively.

Histopathology: Samples taken from various organs were fixed in 10% buffered formalin and embedded in paraffin with routine method. Sections were stained with hematoxylin and eosin (H-E), Giemsa or phosphotungstic-acid hematoxylin (PTAH). The livers were also fixed in 95% cold ethanol and embedded in paraffin for application of the indirect immunofluorescence [17]. Sections were stained with anti-\textit{F. necrophorum} rabbit serum and fluorescein isothiocyanate labelled anti-rabbit IgG Fc goat serum. After observation using a fluorescent microscope, the same sections were stained with H-E.

RESULTS

Hepatic lesions produced by intact bacteria: Gross lesions in the liver were observed in 18 out of 40 mice which had manifested clinical signs from 24 hours on (Table 1). In addition, lumbar abscess was observed in a mouse after 7 days of inoculation and in two mice after 14 days, although these mice had no necrotic lesions in the liver.

Scattered macrophages and polymorphonuclear leukocytes were found in the sinusoid two and four hours after inoculation. A few fluorescent antigens showing a granular or tube-like appearance were observed in the sinusoids. The antigens slowly increased in number until 24 hours after inoculation. The antigens were observed in the cytoplasm of mononuclear cells or Kupffer’s cells with H-E staining (Fig. 1). Antigens in the polymorphonuclear leukocytes were hardly observed. Tiny foci composed of a few degenerated or necrotic hepatocytes and leukocytes were occasionally observed in the inoculated mice throughout the experiment except for the control mice (Fig. 2). Fluorescent antigens were not detected in such foci. After 24 hours, coagulatve necrosis were observed in the peripheral or subserosal parts of the liver. The structure of the hepatic cords was dis-
Fig. 1. The number of antigens increased slowly until 24 hours after intact bacterial inoculation. Most of the antigens were seen in Kupffer's cells or mononuclear cells (Arrows). Upper: Fluorescent microscopy. Lower: The same portion as that shown in the upper figure. Hematoxylin and eosin (H-E) stain ×400.

Fig. 2. Tiny necrotic foci consisted of a few hepatocytes at 2 hours after intact bacterial inoculation. H-E stain ×250.

Fig. 3. Circumscribed coagulative necrosis demarcated with cellular infiltrates at 24 hours after intact bacterial inoculation. H-E stain ×100.

Fig. 4. Focal serositis on the surface of the liver by inoculation with the cell wall fraction. H-E stain ×160.
Table 2. Chemical components of non-diluted fractions and their hepatic toxicity to mice

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Sugar</th>
<th>Protein</th>
<th>Mortality</th>
<th>Serositis</th>
<th>Focal necrosis</th>
<th>Disorder in blood coagulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell wall</td>
<td>186a)</td>
<td>3,600b)</td>
<td>1 10</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>288</td>
<td>11,000</td>
<td>6 10</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>L. P Sb)</td>
<td>80</td>
<td>10</td>
<td>8 10</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
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a) μg/ml.
b) Lipopolysaccharide.

cernible and filamentous bacteria filled the sinusoids adjacent to the necrotic hepatocytes. Slight phagocytic reaction was found around the necrotic foci or on the serosa near the necrotic area (Fig. 3). Deseminated granular fluorescent antigens were also seen in the other parts of the sinusoids of the liver. Irrespective of the presence of coagulative necrosis, leukocytes increased slightly in number and often accumulated as a mass in sinusoids until two weeks after inoculation. In mice without hepatic necrosis, fluorescent antigens slowly decreased in number. At 48 hours, the necrotic hepatocytes tended to lyse, their structure became obscure and their nuclei had completely disappeared. Fibrin thrombi were often formed around the necrotic foci. At 4 days, the necrotic foci consisted of a homogeneous mass with an annual ring-like structure in the center and a thin layer of cellular debris in the peripheral zone. Macrophages and polymophonuclear leukocytes also migrated around the necrotic foci. After 7 days, the necrotic foci involved adjacent tissues or organs and were encapsulated with immature granulation tissues.

Hepatic lesions produced by the components: As shown in table 2, the cytoplasmic fraction of the extracted components contain-
ed a larger amount of sugar and protein than those of the other fractions. Among the three fractions, the LPS fraction showed the highest mortality, followed by the cytoplasmic and cell wall fractions. The diluted fractions showed no mortality to mice.

Focal fibrinous serositis along with accumulation of polymorphonuclear leukocytes was commonly produced on the surface of the liver by non-diluted cell wall fraction (Fig. 4). Thrombosis and scattered single cell necrosis were infrequently seen in the liver, but the hepatic lesions by the non-diluted cell wall fraction were slight in severity as compared with those by the non-diluted other fractions (Table 2). The non-diluted LPS and cytoplasmic fractions produced similar hepatic lesions. Hepatocytes were diffusely swollen and contained eosinophilic granules or homogeneous masses in the cytoplasm. Tiny foci of coagulative necrosis or necrobiosis with slight leukocytic reaction were occasionally observed (Fig. 5). Pyknotic polymorphonuclear leukocytes and swollen macrophages were seen in the sinusoid. Hyaline droplets of coagulated blood plasma, tangled fibrils or fibrin thrombi were often observed in the sinusoids or blood vessels of the liver. The LPS and cytoplasmic antigens by fluorescent microscopy were observed disseminatedly in the cytoplasm of the sinusoidal cells, in the migrated leukocytes, or as scattered fine granules in the blood plasma in the sinusoid (Fig. 6). However, the LPS and cytoplasmic antigens were not observed in either the necrotic or the degenerated hepatocytes. Deposition of the LPS or cytoplasmic fluorescent antigens in the sinusoid had no correlation with the site of the necrotic foci, while the cell wall antigens were recognized mainly in the foci of the serositis and occasionally in the sinusoid. In mice inoculated with the diluted fractions, a few tiny foci of coagulative necrosis were seen in about one fourth of mice of each group. In addition, focal serositis was seen in four mice inoculated with the diluted cell wall fraction.

DISCUSSION

From our results, it was shown that the site of the multiplication of *F. necrophorum* was the sinusoids in the liver. Coagulative necrosis of the hepatocytes adjacent to the bacterial multiplication, thrombosis around the necrosis and encapsulation of the necrotic foci were considered to indicate the process of the infection. The tiny foci of the necrotic hepatocytes without bacterial multiplication, which resembled those described by García et al. [9], were regarded as being incapable of introducing abscess because there was no findings of bacterial multiplication. It has been suggested that intraperitoneally inoculated *F. necrophorum* may reach the liver through the blood stream [9], and that it takes more than 3 days for the formation of hepatic abscess [1, 9, 10]. Intravenous inoculation is more applicable to investigations of the pathological process in *F. necrophorum* infection as it demonstrates direct flow of the bacteria into the liver and induces formation of the lesions. Coagulative necrosis in the liver has been described as an early lesion of liver abscess in both natural and experimental infection of cattle and sheep [11, 16, 19]. The similarity of the lesions between mice and cattle suggests the utility of the mice as an experimental model in this infection.

The present results showed that three extracted components from *F. necrophorum* induced hepatic degeneration and necrosis with slight inflammatory response, focal serositis and abnormality in blood coagulation including thrombosis in mice. The cell wall fraction induced an inflammatory reaction, but its influence to the hepatic degeneration was less severe than that of the other fractions. Histo-pathological findings in both intact *F. necrophorum* infection and inoculation of its components were similar in nature except for bacterial multiplication and granulation. In
addition, the lesions produced by the cytoplasmic and the LPS fractions were similar. Vascular endothelial injury, blockage of the macrophage phagocytic system, induction of disseminated intravascular coagulation and hepatotoxic activity are well known as the activities of LPS [4, 5]. Biological activities of the LPS from F. necrophorum are similar to those of LPS from gram-negative bacteria [7, 18]. The cytoplasmic fraction of F. necrophorum has hemolytic activity in vitro [2]. The similarity of the histological findings by both LPS and cytoplasmic fractions suggested that the cytoplasmic fraction might affect hepatocytes similarly as does the LPS fraction. The deposition of antigens in the sinusoids suggested that F. necrophorum and its components might affect on the development of phagocytosis by macrophages, as was observed in an intact F. necrophorum infection with electron microscopy [9]. It was also considered that the changes in the sinusoids produced by the bacterial components, as the consequence of the disturbance of both blood circulation and activity of the macrophage phagocytic system in the liver, might be responsible for the degeneration of the hepatocytes.

Although quantity of toxins produced in natural infection and that of experimentally injected toxins may be differed, it is suggested that hepatic necrosis in F. necrophorum infection can be induced by the components of F. necrophorum, as well as by mechanical embolism resulting from multiplicated bacteria.

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REFERENCES

Fusobacterium necrophorum による肝膿瘍形成における菌体成分の影響：中島靖之・中村じん保・竹内正太郎（家畜衛生）——Fusobacterium necrophorum の菌体成分をマウスに接種して、形成される肝病変を検討した。生菌接種では、類洞の菌検査をもとになず固膜死が膿瘍となった。細胞壁分画を接種したマウスでは壁膜炎がみられ、リポ多糖体あるいは細胞質分画を接種したマウスでは肝臓の変性ないし壊死と血液凝固異常がみられた。生菌および各菌体成分に対応する抗がん抗体は類洞において観察された。