肝膿瘍の形成におけるFusobacterium necrophorumと他の細菌との相乗作用

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Pathogenic Synergism of *Fusobacterium necrophorum* and Other Bacteria in Formation of Liver Abscess in BALB/c Mice

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ABSTRACT. Experimental infection of *Fusobacterium necrophorum* biovar A in BALB/c mice was performed to approach the mechanism of liver abscess formation in cattle. Liver abscesses were formed in the mice inoculated intravenously or intraperitoneally with *F. necrophorum,* but not in those inoculated with *Corynebacterium pyogennes or Bacteroides oralis.* In such liver abscesses, a population of 3.6×10^9 viable cells of *F. necrophorum* was present. The bacterial counts in the lung, spleen, and kidney varied from 10^4 to 10^6 per gram. The liver abscess formation was enhanced when a mixture of *F. necrophorum* and *C. pyogenes* was inoculated into mice. When a mixture of *F. necrophorum* and *B. oralis* or *Staphylococcus aureus* was inoculated into mice, however, such pathogenic synergism was not recognized. In the mixed infections, a large number of *C. pyogenes* or *B. oralis* were recovered from the abscess portion where *F. necrophorum* had multiplied actively. From these results, it seems that *C. pyogenes,* often isolated from bovine liver abscess, may be a helper organism in development of liver abscess, and *B. oralis* and *S. aureus* are secondary invaders.

Recently, in Japan, such a problem has arisen in the field that there are frequent occurrences of liver abscess associated with *Fusobacterium necrophoum* in fattened dairy steers. Kanoe *et al.* [6] isolated *F. necrophorum* in pure culture from 22 of 66 liver abscesses. In other 35 specimens, *F, necrophorum* was discovered predominantly in association with *Corynebacterium* sp., *Bacteroides* sp., *Staphylococcus* sp., or other bacteria. Azuma and coworkers also isolated *Corynebacterium pyogenes* and *Bacteroides oralis* with *F. necrophorum* from some cases of bovine liver abscesses (unpublished data). The role of such other organisms on the formation of liver abscess has not sufficiently been investigated.

On the other hand, some investigators [1,3,5,] described a mouse model in which intraperitoneal or intravenous inoculation of *F. necrophorum* results in liver abscess formation.

To approach the mechanism of liver abscess formation in cattle, the authors carried out experimental infection of *F. necrophorum* biovar A in BALB/c mice. This paper is concerned with induction of liver abscess in BALB/c mice inoculated with *F. necrophorum* and with pathogenic synergism between *F. necrophorum* and *C. pyogenes, B. oralis,* or *Staphylococcus aureus* in the formation of liver abscess.

MATERIALS AND METHODS

Mice: Adult outbred mice of the BALB/c strain, weighing 20 to 25g, were used. They were originally obtained from National Institute of Health, Tokyo and have been bred in our colony.

Organisms and cultivation:. F. necrophorum strain ATCC 25286, a biovar A,

originated from American Type Culture Collection, was used throughout the present experiment. In addition,*C. pyogenes* strain S-2, *B. oralis* strain 14-5, and *S. aureus* strain Kitami 3-9D were used. They were isolated from porcine suppurative lesion, bovine liver abscess, and bovine mastitis, respectively.

The organisms except *S. aureus* were inoculated into the modified VL broth and cultivated anaerobically at 37°C for 18 hr by the CO_2 gas jet method described by Azuma and Suto [2]. The modified VL broth contained the followings: salt 1 [0.6% K_2HPO_4], 7.5 m*l*; salt 2 [0.6% KH_2PO_4, 1.2% $(NH_4)SO_4$, NaCl, 0.12% $MgSO_4 \cdot 7H_2O$, 0.12% $CaCl_2 \cdot 2H_2O$], 7.5 m*l*; 0.1% resazurin, 0.1 m*l*; distilled water, 78 m*l*; trypticase(BBL), 1.0 g; yeast extract(Difco), 0.2 g; glucose, 0.2 g; 0.07% hemin,0.1 m*l*; 8% Na_2CO_3, 5 m*l*; and 3% L-cysteine, 1 m*l*. *S. aureus* was cultured aerobically in heart infusion broth(Difco) at 37°c for 18 hr.

In most experiments, the organisms were harvested by centrifugation and resuspended in the modified VL broth. The cell suspension was incoculated into the caudal vein of mice in 0.2-m*l* doses. The bacrerial count of the suspension was made by the roll tube method [2].

Pathogenic synergism: The pathogenic synergism between *F. necrophorum* and other bacteria was examined in mice by three experiments. In experiments 1, 2, and 3, *C. pyogenes* (4.2×10^7 organisms), *B. oralis* (6.8×10^7 organisms), and *S. aureus* (7.5×10^7 organisms) were the admixture, respectively. The numbers of *F. necrophorum* inoculated in experiments 1, 2, and 3 were 1.4×10^7, 8.7×10^6, and 1.2×10^7 organisms per head, respectively.

In these experiments, BALB/c mice were divided into five groups. The mice of group 1 were given *F. necrophorum* alone. Those of group 2 were given a mixture of *F. necrophorum* and an other bacterium. Those of group 3 were given *F, necrophorum* and 8 hr later, another bacterium was inoculated. Those of group 4 were given another bacterium first and then *F. necrophorum*. The mice of group 5 were given *C. pyogenes*, *B. oralis*, or *S. aureus* alone. All experiments were carried out by intravenous inoculation.

Recovery of organisms: The mice were sacrificed and their livers, lungs, and kidneys were aseptically harvested. Each organ was weighed and triturated in 5 m*l* of sterile saline in a glass homogenizer. Blood was collected into 10 m*l* of sterile saline. The resulting samples were diluted tenfold serially with sterile saline. Then a 0.1-m*l* protion of each dilution was added to the modified VL agar in a glass tube and the tubes were cultivated anaeobically at 37°C for 2 to 4 days.

In the experiments with the mice inoculated with a combination of bacteria, *F. necrophorum* was distinguished from *C. pyogenes* or *B. oralis* by the size of the colonies formed in the modified VL agar. The colony of *F. necrophorum* was larger than those other bacteria. This was confirmed by Gram's staining and fluorescent antibody staining of the organisms isolated from the colonies.

Results

1. Pathogenicity of *F. necrophorum, C. pyogenes,* and *B. oralis* for mice

F. necrophorum, C. pyogenes, and *B. oralis* for mice were examined preliminarily for the pathogenicities to mice. The organisms were cultivated anaerobically at 37°C for 18 hr and 0.2 m*l* of the whole culture was inoculated intravenously into mice. All of the six mice inoculated with *F. necrophorum* or *C. pyogenes* succumbed to infection within 4 days after inocula-

tion, but no death was recognized in those inoculation with *B. oralis*. When 0.5 m*l* of each culture was inoculated intraperitoneally, death was recognized in all the mice receiving *F. necrophorum* and *C. pyogenes*, but not in those receiving *B. oralis*.

The cell suspensions of *F. necrophorum* and *C. pyognes* were each inoculated intravenously into mice. As a result, two of the six mice receiving *F. necrophorum* succumbed to the infection within 7 days after inoculation. In additon, macroscopic liver abscesses were observed in most dead and surviving mice (Fig. 1). Death was recognized in the mice inoculated with *C. pyogenes*; however, no liver abscess was observed in them.

2. *In vivo* growth of *F. necrophorum*

A *F. necrophorum* suspension containing 2×10^8 viable cells per 0.2 m*l* was inoculated intravenously into mice. The mice were sacrificed at regular intervals and viable bacterial counts were made from the lung, liver, spleen, kidney, and blood.

The results obtained are shown in Fig. 2. The viable bacterial counts in all organs in 8 hr were smaller than those in 2 hr after inoculation. Particulalry, no organisms were detected in the blood at this time. In one day after inoculation, abscesses were observed in the liver, but not in the lung, spleen, or kidney. A population of 3.6×10^9 viable cells of *F. necrophorum* was present in such liver abscesses. The viable bacterial counts in the lung, spleen, and kidney increased 2 days after inoculation and reached 10^4 to 10^6 organisms per gram of the organ. Some organisms were also detected in the blood at the same time. By 7 days after inoculation, decreased viable bacterial counts were seen in the lung, spleen, and kidney, but not in the liver abscesses. On the other hand, no organisms were

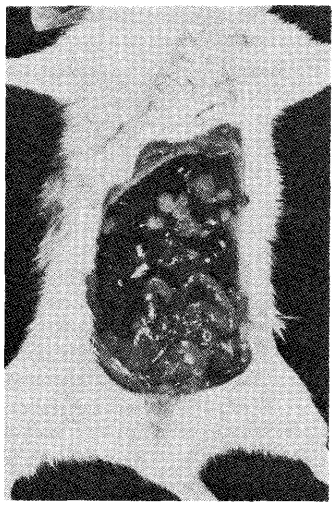

Fig. 1. Liver abscesses in the mouse inoculated intravenously with a washed cell suspension of *F. necrophorum*.

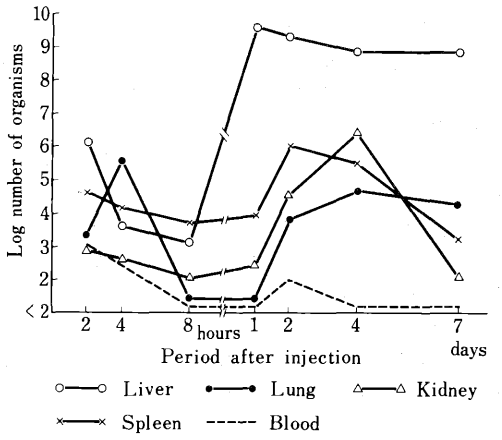

Fig. 2. Recovery of *F. necrophorum* from blood and organs of infected mice.

detected in any organ or blood of the mice, in which abscess was not observed, 4 or 7 days after inoculation.

3. Pathogenic synergism between *F. necrophorum* and other bacteria causing liver abscess formation

The pathogenic synergism between *F. necrophorum* and *C. pyogenes, B. oralis,* and *S. aureus* in mice was examined in experiments 1, 2, and 3, respectively. The mice of groups 1 and 5 in each experiment

Table 1. Pathogenic synergism of *F. necrophorum* and other bacteria in mice

Mouse group*1	Exp. 1		Exp. 2		Exp. 3	
	Fatality	Abscess*2	Fatality	Abscess	Fatality	Abscess
1	4/20*3	7/20	0/10	0/10	2/10	3/10
2	12/20	14/20	1/10	1/10	6/10	2/10(7/10)*4
3	2/10	4/20	0/10	0/10	2/10	0/10(8/10)
4	1/20	2/20	0/10	0/10	1/10	0/10(8/10)
5	0/20	0/20	0/10	0/10	3/10	0/10(9/10)

In experiments 1, 2, and 3, *C. pyogenes*, *B. oralis*, and *S. aureus* were used as the other bacteria, respectively.

*1 Group 1: *F. necrophorum* was injected into mice.
Group 2: The mixture of *F. necrophorum* and the other bacteria was injected.
Group 3: *F. necrophorum* was injected and then the other bacteria were injected.
Group 4: The other bacteria were injected and then *F. necrophorum* was injected.
Group 5: The other bacteria were injected.

*2 Liver abscess.

*3 Number of positive mice/Number of used mice.

*4 Kidney abscess.

were inoculated with *F. necrophorum* or another bacterium alone. The mice of the remaining groups were inoculated with combinations of *F. necrophorum* and another bacterium.

The results obtained are shown in Table 1. In experiment 1, death and liver abscesses were observed in four and seven of the 20 mice (Group 1), respectively, inoculated with a *F. necrophorum* suspension containing 1.4×10^7 viable cells. On the other hand, no death nor liver abscess was observed in the mice (Group 5) inoculated with 4.2×10^7 viable cells of *C. pyogenes*. When a mixture of *F. necrophorum* and *C. pyogenes* was inoculated into mice (Group 2), pathogenic synergism was recognized; deaths and liver abscesses were observed in 12 and 14 of the 20 mice, respectively. Such pathogenic synergism was not seen when *F. necrophorum* was inoculated into mice and then *C. pyogenes* was inoculated (Group 3) nor when *C. pyogenes* was inoculated and then *F. necrophorum* was inoculated (Group 4).

In experiment 2, no death nor liver abscess was observed in the mice (Group 1) inoculated with 8.7×10^6 viable cells of *F. necrophorum* alone. When a mixture of *F. necrophorum* and *B. oralis* was inoculated, however, deaths and liver abscess were observed only in one of the 10 mice.

In experiment 3, fatality increased when a mixture of *F. necrophorum* and *S. aureus* was inoculated into mice (Group 2), but no enhancement of liver abscess formation was seen.

4. Recovery of the organisms from the mice inoculated with a mixture of *F. necrophorum* and other bacterium

Recovery of the organisms was attempted from the mice having liver abscesses in group 2 of experiments 1 and 2. The results obtained are shown in Table 2. In experiment 1, a population of 3.2×10^8 viable cells of *F. necrophorum* was present in the abscess portion of the liver of the mice 2 days after inoculation. In the normal portion of the liver and other organs, however, the viable count of *F. necrophorum* ranged from 1×10^4 to 3×10^5 per gram of the tissue. On the other hand, the viable counts of *C. pyogenes* were from 3.7×10^3 to 1×10^6 in the normal portion of the liver and other or-

Table 2. Recovery of the organisms from mice injected with a mixture of *F. necrophorum* and *C. pyogenes* or *B. oralis*

Organ	Exp. 1*[1]		Exp. 2	
	F. necrophorum	*C. pyogenes*	*F. necrophorum*	*B. oralis*
Lung	1.3×10^{4}*[2]	3.7×10^{3}	1.2×10^{3}	3.7×10^{3}
Liver (A)*[3]	3.2×10^{8}	1.0×10^{8}	9.9×10^{7}	7.2×10^{8}
Liver (N)	3.0×10^{5}	2.0×10^{4}	1.6×10^{5}	1.3×10^{6}
Spleen	9.6×10^{4}	1.3×10^{4}	3.8×10^{4}	1.8×10^{4}
Kidney	1.0×10^{4}	1.0×10^{6}	8.1×10^{3}	7.1×10^{3}

*[1] Exp. 1: A mixture of *F. necrophorum* and *C. pyogenes* was injected into mice.
Exp. 2: A mixture of *F. necrophorum* and *B. oralis* was injected into mice.
*[2] Number of organisms recovered (CFU/g).
*[3] (A): Abscess portion.
(N): Normal portion.

gans, whereas the count was 1×10^{8} in the abscess portion of the liver.

In experiment 2, a population of 7.2×10^{8} viable *B. oralis* was present in the liver abscess portion, from where 9.9×10^{7} viable *F. necrophorum* were recovered.

The results demonstrated that *C. pyogenes* and *B. oralis* multiplied actively in the abscess portion of the liver.

Discussion

Several investigations have developed such mouse models that appear to be useful for studying *F. necrophorum* infection. Hill *et al.* [5] succeeded in inducing liver abscesses in about 60% of male inbred A/J mice with a human isolate of *F. necrophorum*. Abe *et al.* [1] demonstrated that chronic liver abscesses were formed in CF-1 mice after intraperitoneal inoculation of a bovine isolate of *F. necrophorum*. Thereafter, Swiss Wester, DBA/2Cr, Swiss Albino, and ICR mice have been used as laboratory animal models [3, 4, 10]. In the present experiment, BALB/c mice were used for experimental infection with *F. necrophorum* biovar A. As a result, liver abscesses were formed in most mice injected intravenously or intraperitoneally with 10^{8} viable cells of *F. necrophorum*. The percentage of liver abscess formation was nearly the same to that in A/J mice described by Hill *et al.* [5]. From these results, it seems that BALB/c mice may be useful as a laboratory animal model for studying experimental liver abscess formation. Therefore, the authors used BALB/c mice throughout the present experiments.

Liver abscesses were formed one day after intravenous inoculation of 2×10^{8} viable cells of *F. necrophorum* into mice. Bacteriological data show that a population of 3.6×10^{9} viable organisms was present in such liver abscesses, but the viable bacterial counts in the lung, spleen, and kidney were 10^{4} to 6^{6} organisms per gram of the organ. The results indicate that *F. necrophorum* multiplied more actively in the liver than in other organs of mice. Such a preferential growth in the liver has been described by other investigatiors [1, 3, 5]. In addition, Abe *et al.* [1] conjectured that such predilection for the liver may be due to a nutritonal and/or microenvironmental factor (s).

Hill *et al.* [5] demonstrated that the percentage of liver abscess formation was enhanced by a mixed infection with *F.*

necrophorum together with *Bacteroides melaninogenicus* or *Fusobacterium nucleatum*. In the present experiments, liver abscess formation was enhanced when a mixture of *F. necrophorum* and *C. pyogenes* was inoculated (Experiment 1), but no enhancement was recognized when *C. pyogenes* was inoculated into mice and then *F. necrophorum* 8 hr later. In experiment 2, the inoculum size of *F. necrophorum* was slightly smaller than those in experiments 1 and 3, and, therefore, liver abscess was not formed in the mice inoculated with *F. necrophorum* alone. No remarkable enhancement of liver abscess formation was recognized in mixed infection with *F. necrophorum* and *B. oralis*. In addition, no pathogenic synergism was seen between *F. necrophorum* and *S. aureus* (Experiment 3). On the other hand, in mixed infection with *F. necrophorum* and *C. pyogenes* or *B. oralis*, large numbers of *C. pyogenes* and *B. oralis* were recovered from the abscess portion of the liver in which *F. necrophorum* multiplied activity. It was conjectured that the multiplication of both organisms was attributable to the inhibition of phagocytosis by the exotoxin of *F. necrophorum* as described by Roberts [8, 9].

From these results, *C. pyogenes* may not be a primary invader but a helper organism in development of liver abscess. In addition, the authors conjectured that *B. oralis* and *S. aureus*, isolated from bovine liver abscesses, were a secondary invader.

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要　約

肝膿瘍の形成における *Fusobacterium necrophorum* と他の細菌との相乗作用: 竹内正太郎・中島靖之[1]・橋本和典[2] (農林水産省家畜衛生試験場, 北陸支場, [1], 東北支場, [2], 製剤研究部)——牛の肝膿瘍の発生機序を解析する一つのアプローチとして, BALB/c マウスを用いて, *Fusobacterium necrophorum* の感染実験を行った。肝膿瘍は *F. necrophorum* を腹腔内あるいは静脈内に接種したマウスで認められ, 膿瘍内には 3.6×10^9 個/g の *F. necrophorum* が存在していたが, 肺臓, 脾臓および腎臓における菌数は 10^4～10^6 個/g であった。*Corymebacterium pyogenes* あるいは *Bacteroides oralis* を接種したマウスでは膿瘍は認められなかったが, *F. necorphorum* と *C. pyogenes* との混合液を接種すると, 肝膿瘍形成は増強された。しかし, このような相乗作用は *F. necrophorum* と *B. oralis* あるいは黄色ブドウ球菌との間では認められなかったが, *F. necrophorum* の活発に増殖した肝臓の膿瘍部から多数の *C. pyogenes* と *B. oralis* が回収された。これらの成績から, しばしば牛の肝膿瘍から分離される *C. pyogenes* は肝膿瘍の形成においてヘルパー細菌であり, *B. oralis* と黄色ブドウ球菌は二次感染菌であると思われた。