RESEARCH LETTER – Pathogens & Pathogenicity

Vibrio vulnificus detected in the spleen leads to fatal outcome in a mouse oral infection model

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One sentence summary: It was found that clearance of Vibrio vulnificus from the spleen and liver greatly influenced the survival rate of the host.

Editor: Mark C. Enright

ABSTRACT

Vibrio vulnificus causes rapid disseminating septicemia by oral infection in infected individuals who have an underlying disease, especially chronic liver diseases. Although the elucidation of specific risk factors for V. vulnificus infection in patients with liver diseases is of urgent importance, no appropriate experimental animal model that mimics the liver diseases in this bacterial infection has been available so far. To discover these risk factors, we generated a liver disordered mouse by performing bile duct ligation (BDL). Hepatitis developed in the BDL mice; however, this did not affect mortality in mice after orogastric administration of V. vulnificus, suggesting that the liver disorders caused by the BDL were not risk factors for V. vulnificus septicemia. When the dead and surviving mice were compared, V. vulnificus could be detected from the spleen only in the dead group. Furthermore, significantly higher numbers of V. vulnificus were detected from the intestines in the dead group than in the surviving group (P < 0.001). These findings suggested that proliferation of the challenge inoculum in the intestine was needed for the oral infection with V. vulnificus, and that the elimination of V. vulnificus in the liver and/or spleen plays a critical role in survival of the host.

Keywords: Vibrio vulnificus; risk factor; liver diseases; sepsis

INTRODUCTION

Vibrio vulnificus, a Gram-negative estuarine bacterium, causes septicemia and wound infections in humans (Hlady and Klontz 1996; Linkous and Oliver 1999; Strom and Paranjpye 2000). Primary septicemia is caused by the ingestion of contaminated seafood and wound infections can result from exposure to seawater or through handling a marine product in liver disease patients (Koenig, Mueller and Rose 1991). Over 50% of patients with septicemia die within the first 48 h of admission. Septicemia caused by V. vulnificus is associated clinically with liver cirrhosis, hemochromatosis or alcoholism (Linkous and Oliver 1999; Strom and Paranjpye 2000). The elevation of serum iron levels is a risk factor for V. vulnificus infection in humans (Wright, Simpson and...
because high serum iron levels in liver disorders were found to stimulate growth of *V. vulnificus* in human serum (Wright, Simpson and Oliver 1981). It was also reported that the 50% lethal dose in iron dextran-overloaded mice was decreased from $10^6$ to 1.1 cells (Wright, Simpson and Oliver 1981). Thus, the iron-overloaded mouse model has been used to analyze the pathogenic mechanisms of *V. vulnificus*. However, iron-overload induced lymphocyte apoptosis without infection of *V. vulnificus* (Kashimoto et al., 2005), suggesting that the iron overload might affect the mouse immune system by generating reactive oxygen species via the Fenton reaction (Fenton 1894). In addition, the iron overload could not mimic any chronic liver diseases because the livers of iron-overloaded mice were healthy. The elevation of serum iron is clearly a risk factor for *V. vulnificus* infection in liver disease patients, but the iron-overloaded mouse is not an appropriate model for *V. vulnificus* infection in liver diseases patients.

On the other hand, many previous studies reported that translocation of intestinal bacteria from intestine to liver occurred in patients with liver disorders due to a breakdown in intestinal barrier function (Campillo et al., 1999; Ersöz et al., 1999; Pascual et al., 2003; Zuckerman et al., 2004). This fact encouraged us to speculate that the inocula of *V. vulnificus* might also translocate from the intestine to liver in a liver disordered mouse model. Here, to address this hypothesis, *V. vulnificus* was administered orogastrically to hepatic disordered mice. The purpose of this study is the development of a mouse model that mimics liver diseases.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions**

*Vibrio vulnificus* K1 was isolated from the blood of septicemic patients at Kurashiki Central Hospital in Japan. Challenge bacteria were prepared as follows. K1 was routinely grown in marine broth (Difco, Detroit, MI, USA) at 37°C for 12 h. The bacterial concentration was adjusted to an optical density of 1.0 at 600 nm, and then 1 mL of adjusted bacterial culture was added to 200 mL of fresh marine broth. This was then further grown for 3 h at 37°C with agitation to obtain logarithmic growth-phase bacteria. Bacteria were harvested by centrifugation at 2500 × g for 10 min and suspended in phosphate-buffered saline (PBS). The desired bacterial concentration was adjusted by measuring the optical density at 600 nm and checked by plating serial dilutions of the samples on thiosulfate–citrate–bile salt sucrose (TCBS) agar (Nissui Pharmaceutical Co., Ltd, Tokyo, Japan) and counting the bacterial colony-forming unit (CFU) after incubation.

**Animal experiments**

All experiments involving mice were carried out in accordance with the Kitasato University guidelines for animal treatment (approval no. 09–022). Male 6-week-old Balb/c mice were obtained from Charles River Laboratories, Japan, Inc. (Yokohama, Japan). The mice were housed in plastic cages in a group and were maintained on a standard laboratory diet (rat chow MF, Oriental Yeast Co., Ltd, Tokyo, Japan) and tap water under a 12 h light and dark cycle. Ambient temperature during the study was maintained at about 21°C. For experiments with bile duct ligation (BDL), mice were anesthetized. After midline laparotomy, the bile duct was double-ligated and transected between the ligatures. The sham operation was performed similarly without BDL. Serum chemistries, liver histology, translocation of the intestinal commensal bacteria and iron content were carried out at 3 weeks after the BDL or sham operation without infection of *V. vulnificus*.

**Orogastric challenge of *V. vulnificus***

Nine sham mice and eleven BDL mice were generated. Three weeks after the BDL or sham operation, these mice were orogastrically infected with 300 μL of the desired concentrations of K1 strain. These mice were carefully monitored, and the mouse showing the potentially lethal signs, such as low body temperature and lethargy, was euthanized by intraperitoneal injection of sodium pentobarbital at an overdose immediately, due to ethical considerations, and was counted as a dead mouse. The intestine, liver and spleen were removed and then weighed in sterile weigh boats, and placed in separate sterile tissue grinders containing nine volumes of cold sterile PBS. Each tissue was homogenized, serially diluted in sterile PBS and plated in duplicate onto TCBS agar (Nissui Pharmaceutical Co., Ltd, Tokyo, Japan). These plates were incubated at 37°C for 24 h. The number of bacterial CFU was normalized per gram of tissue (CFU g⁻¹).

**Serum chemistries**

Three weeks after BDL, serum was obtained for biochemical and histological analysis. Serum albumin (ALB), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), γ-glutamyl transpeptidase (γ-GTP) and total bilirubin (T-Bil) were measured with a chemical analyzer AU400 (Olympus Corporation, Tokyo, Japan).

**Serum iron content**

After 3 weeks of the sham and BDL operation, or at 3 h after the iron dextran via intramuscular injection (5 mg kg⁻¹), 100 μL of sera were obtained from the sham (n = 4), BDL (n = 3) and iron dextran-treated mice (n = 3). These sera were dried and dissolved with 1 mL of nitric acid in a glass test tube. After rehydrating, these samples were dissolved with 10% nitric acid, and total iron levels in serum were determined using an atomic absorption spectrophotometer (Shimadzu AA-630-01).

**Liver histology**

Liver samples taken 3 weeks after BDL were fixed with 10% (vol/vol) buffer formalin solution (pH 7.2), dehydrated in graded alcohol, embedded in paraffin and cut into 3 μm sections. After hematoxylin and eosin and Azan–Mallory staining, the sections were examined under a microscope.

**Analysis of bacterial translocation**

The ileum and liver were weighed and homogenized. Ileum and liver homogenates were serially diluted 10⁴-fold in sterile PBS. Each homogenate was inoculated onto fresh blood agar plates (containing 5% horse blood) or Gifu anaerobic medium (GAM) agar plates (Nissui pharmaceutical Co., Ltd, Tokyo, Japan), and the plates were incubated under aerobic or anaerobic condition at 37°C for 24 h. The number of CFU was normalized per gram of tissue (CFU g⁻¹).

**Statistical analysis**

The data from biochemistry markers for liver injury were expressed by box plot. To compare the differences in the data between the sham group and the BDL group, we used
Figure 1. Liver biochemistry markers and liver histology in the Sham and BDL-operated mice. (a) Serums were obtained from the Sham (n = 11) or the BDL-operated mice (n = 12) 3 weeks after the operation, and then following liver biochemistry markers were tested. AST; asparate aminotransferase, ALT; alanine transaminase, ALP2; alkaline phosphatases-2, γ-GTP; γ-glutamyltransferase, ALB; albumin, D-B; direct bilirubin, T-B; total bilirubin. The boxplot displays the median, upper and lower quantiles. The whiskers indicate the 10th and 90th percentiles. *P < 0.05, **P < 0.001, significantly different from the Sham-operated control (Mann–Whitney’s U test). (b) Azan–Mallory staining of the liver from the Sham and the BDL-operated mice. Collagen fibers surrounding the proliferating bile duct in the BDL-operated mice were increased significantly compared with the Sham-operated mice. Bar = 100 μm.
Evaluation of the bacterial translocation in the Sham and BDL-operated mice. Intestinal commensal bacteria were detected from both the intestine and the liver in V. vulnificus uninfected sham and BDL mice by using blood agar and GAM agar. Both blood agar and GAM agar were incubated under aerobic and anaerobic conditions. Higher numbers of bacteria were detected only in the liver in the BDL mice compared with the sham mice. Data are represented as means ± S.D. ∗P < 0.05, ∗∗P < 0.001, significantly different from Sham-operated control (Mann–Whitney's U test).

Mann–Whitney’s U-test, and a P-value less than 0.05 was considered statistically significant. The other results are recorded as mean ± SD. For data with a normal distribution, differences between the treatment and control groups were compared with Tukey’s test. Pearson’s chi-square analysis was used to evaluate the significance of difference in mortality rate or isolation of V. vulnificus.

RESULTS

Evaluation of the BDL operation on liver by plasma chemistry and histological analysis

In order to assess the effect of BDL on the mouse liver, plasma chemistry and liver fibrosis were evaluated 3 weeks after the BDL operation. As shown in Fig. 1a, the levels of AST (P < 0.01), ALT (P < 0.01), ALP2 (P < 0.01), γ-GTP (P < 0.005), DB (P < 0.01) and TB (P < 0.01) in serum were significantly higher in the BDL-operated mice than in the sham mice, while ALB levels were unaffected. In the histological analysis, Azan–Mallory staining demonstrated that severe fibrosis was observed only in the liver of BDL mice (Fig. 1b). These results suggested that the BDL operation caused acute hepatitis.

Higher numbers of bacteria were detected from the liver in BDL mice than sham mice

Many previous studies reported that translocation of intestinal bacteria from intestine to liver occurred in liver disorders due to a breakdown in intestinal barrier function (Campillo et al., 1999; Ersöz et al., 1999; Pascual et al., 2003; Zuckerman et al., 2004). We hypothesized that V. vulnificus could also translocate from intestine to livers and cause septicemia in mice with disordered liver function. We investigated whether our BDL operation in mice caused bacterial translocation. Although there was no significant difference in the number of intestinal commensal bacteria in intestinal samples between the sham and BDL mice, a much larger number of bacteria was detected in the livers of BDL mice than in sham mice on both agars and culture conditions in this study (Fig. 2). On the other hand, when V. vulnificus was administered to these mice groups, there were no significant differences in the detection rate (Table 1) or the number of V. vulnificus in the intestine, liver and spleen between the sham and BDL mice (Fig. 3).

Table 1. Detection rates of V. vulnificus from the intestine, liver and spleen in the Sham and BDL-operated mice.

<table>
<thead>
<tr>
<th></th>
<th>Sham (n=9)</th>
<th>BDL (n=11)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intestine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V. vulnificus (+)</td>
<td>7</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>V. vulnificus (-)</td>
<td>2</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V. vulnificus (+)</td>
<td>5</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>V. vulnificus (-)</td>
<td>4</td>
<td>4</td>
<td>p=1.000</td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V. vulnificus (+)</td>
<td>4</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>V. vulnificus (-)</td>
<td>5</td>
<td>7</td>
<td>p=1.000</td>
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</tbody>
</table>

No significant differences were observed at any level of challenge bacteria in the Sham and BDL-operated mice. The chi-square test was used for statistical analysis. P-value was represented by Yates’ correction.
The BDL operation induced a decrease of serum iron concentration in mice

Iron-overloaded mouse injected with iron dextran is usually used as a hepatic disordered model in the many previous studies of *V. vulnificus* (Stelma et al., 1992; Paranjiye et al., 1998; Lee et al., 2007). However, the iron concentration of these mice was not measured so far. To explore the reason why the BDL mouse had not suitable model for orogastric infection of *V. vulnificus* in this study, we measured and compared the levels of serum iron concentration between the sham, BDL and iron-overloaded mice. The serum iron concentration in the sham mice was 42.6 ± 18.0 ppm, and that of the BDL mice was 12.7 ± 4.3 ppm (Fig. 4). On the other hand, the serum iron concentration of iron-overloaded mice was extremely high (498.3 ± 53.4 ppm) compared with that of the sham and BDL mice (Fig. 4). Thus, the level of serum iron concentration was not elevated by the BDL operation in mice.

The mortality by *V. vulnificus* was not enhanced in the BDL mice compared with the sham mice

To evaluate the effects of the BDL operation on mortality rates by *V. vulnificus* infection, the sham and BDL mice were orogastrically challenged with the indicated numbers of *V. vulnificus* (Table 2). As shown in Table 2, there were no differences in mortality rate in this study between the sham and BDL mice.

There was no significant difference in detection rate of *V. vulnificus* from organs between the Sham and BDL mice

In the intestine, *V. vulnificus* was detected in seven out of nine sham mice and eight of eleven BDL mice. In the liver, *V. vulnificus* was detected in five out of nine sham mice and seven out of eleven BDL mice, and in the spleen in four out of nine sham mice and four out of eleven BDL mice (Table 1). Thus, there was...
Concentration of serum iron in the Sham and BDL-operated mice. Iron concentrations in the serum of the BDL-operated (n = 5), Sham-operated (n = 5) and iron-overloaded (n = 3) mice were determined by atomic absorption spectrophotometer. Data are represented as means ±S.D. *P < 0.05, **P < 0.001, significantly different from Sham-operated control (Mann–Whitney’s U test).

Table 2. Mortality rates in the Sham and BDL-operated mice after orogastric administration of V. vulnificus.

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>BDL</th>
<th>P-value</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Number administered (CFU/ml)</td>
<td>Survival</td>
<td>Dead</td>
</tr>
<tr>
<td>10⁶</td>
<td>1</td>
<td>2</td>
<td>p=0.165</td>
</tr>
<tr>
<td>10⁵</td>
<td>1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>10⁴</td>
<td>3</td>
<td>0</td>
<td></td>
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</table>

No significant differences were observed at any level of V. vulnificus administration between the Sham and BDL-operated mice. Mouse showing the potentially lethal clinical signs, such as low body temperature and lethargy, was euthanized and counted as a dead mouse. The chi-square test was used for statistical analysis.

No significant difference in the detection rates of V. vulnificus in any organs between the sham and BDL mouse. Furthermore, as shown in Fig. 3, similar amounts of V. vulnificus were detected in the intestine, liver and spleen both in the sham vs BDL mice (in intestine; 6.46 ± 2.01 vs 6.23 ± 2.06, liver; 4.82 ± 1.36 vs 4.57 ± 0.99, spleen; 3.99 ± 0.95 vs 4.73 ± 0.71 log10 CFU g⁻¹, respectively).

Detection rates of V. vulnificus from the liver and spleen in the surviving mice were significantly lower than that in the dead mice

Next, we tried a reanalysis of our data by comparing the surviving and dead mice groups. As shown in Table 3, the number of dead mice with V. vulnificus in the liver and spleen was significantly higher than that of the surviving mice, but no differences were observed in the intestine. Notably, V. vulnificus was not detected in the spleen of any mouse in the surviving group (Table 3). Thus, it was clearly indicated that mice were able to survive if they could eliminate V. vulnificus from the spleen and/or liver.

The numbers of V. vulnificus in the intestine and spleen of dead mice were significantly higher than that of the surviving mice

We compared the amount of V. vulnificus detected in the intestine, liver or spleen in the surviving and dead mice. The amounts of V. vulnificus detected from the intestine and spleen in the dead mice were significantly higher than those in the surviving mice, though no differences were seen in the liver (Fig. 5).

DISCUSSION

Vibrio vulnificus infects individuals who are suffering from underlying diseases such as liver diseases, immunocompromising diseases or diabetes (Hlady and Klontz 1996; Linkous and Oliver 1999; Strom and Paranjpye 2000). Among these, V. vulnificus infection is strongly associated with liver diseases (Park, Shon and Joh 1991). Several factors have been proposed to
explain why morbidity is increased in the presence of liver disease. The shunting of portal blood containing *V. vulnificus* infection around a diseased liver may lead to septicemia. Opsonized activity of polymorphonuclear leukocytes and Kupffer’s cells was reduced in patients with chronic liver diseases and this may lead to a decreased clearance of bacteria from the portal circulation (Koenig, Mueller and Rose 1991). However, the exact risk factors for *V. vulnificus* infection in liver disease patients have not yet been demonstrated using an animal model. Therefore, we tried to define the exact risk factors for *V. vulnificus* infection in liver disease patients using orogastric infection in a hepatic disordered mouse model.

It is thought that the challenge inoculums of *V. vulnificus* to mice via orogastric administration gain access from intestine to liver via the portal vein or bile duct, and then diffuse into general circulation. Because it has been known that bacterial translocation from intestine to liver occurs in liver disease patients (Campillo et al., 1999), we thought that the translocation of *V. vulnificus* from intestine to liver might be also detected in a liver disordered mouse model. In this study, the effects on *V. vulnificus* infection were investigated in a liver disordered mouse that was generated by BDL. The levels of T-Bil and D-Bil in the serum of BDL mice were increased compared with those of the sham mice, indicating that obstructive jaundice was caused by the BDL operation. The increased levels of AST, ALT, ALP2, γ-GTP, D-B and T-B indicated that hepatitis was induced in the BDL mice, whereas the levels of ALB were not affected (Fig. 1a).

In addition, liver fibrosis was observed in the BDL mice (Fig. 1b).
These data indicated that while hepatocellular damages were caused by the BDL operation, protein synthesis in the liver was maintained, as shown by the fact that levels of ALB were unaffected in the BDL group. We concluded that our BDL operation caused acute hepatitis in mice. There have been many reports that BDL operation caused translocation of intestinal bacteria to liver in mice (Deitch et al., 1990; Berg 1992). Similarly, our study detected a much higher number of bacteria in the liver of BDL mice than in the liver of sham mice. Although it seems that bacterial translocation was specifically increased in BDL mice, the BDL operation had no significant influence on the mortality rates or the number of V. vulnificus in any organ after orogastric administration. In addition, the intestinal commensal bacteria were also detected in the livers of sham mice (Fig. 2). From these data, we speculated that the intestinal commensal bacteria in the liver or gallbladder, which had existed before the BDL operation, were trapped by the BDL operation and then proliferated in the liver. Concerning another critical determinant factor for proliferation of V. vulnificus, it has been known that elevation of iron concentration in blood becomes a risk factor for V. vulnificus infection both in human and mouse (Wright, Simpson and Oliver 1981; Koenig, Mueller and Rose 1991). In our study, the serum iron levels were significantly lower in the BDL mice than in the iron-overloaded mice which were typically used as the infection model with V. vulnificus (Stelma et al., 1992; Paranjiyye et al., 1998; Lee et al., 2007) (Fig. 4). This lower level of serum iron in the BDL mice did not reflect the condition of liver disease patients. Thus, we concluded that the BDL mice were not a suitable model for the analysis of oral infection with V. vulnificus in liver disease patients.

Next, the BDL and sham mice were divided either into the surviving or dead group, and the data were reanalyzed. The number of mice with V. vulnificus in the liver and spleen was higher in the dead group than in the surviving group. This finding strongly indicated that the host defense mechanisms in the liver and/or spleen were critical for the elimination of V. vulnificus. In fact, Koenig, Mueller and Rose (1991) reported that opsonized activity of Kupffer’s cells was reduced in patients with chronic liver diseases, and suggested that this may lead to decreased clearance of bacteria from the portal circulation. In addition, we found that the number of V. vulnificus in the intestine and spleen was significantly higher in the dead than in the surviving group, but levels in the liver were unaffected (Fig. 5). These data indicated that if V. vulnificus was able to grow in the intestine and spleen, the mice would die, while the amount of V. vulnificus in the liver was not an indicator of fatal outcome in V. vulnificus infected mice. We could confirm again that host defense in the liver and/or spleen plays a critical role in the clearance of V. vulnificus. It was also revealed that intestinal proliferation of V. vulnificus was needed for sepsis to develop after oral administration of this bacterium.

Taken together, the acute hepatitis induced by BDL operation did not generate a critical risk factor for orogastric infection with V. vulnificus in this murine model. However, we found that the elimination of V. vulnificus in the liver and/or spleen plays a critical role in survival of the host. These findings should lead to new insights into why hepatic disorders are a risk factor for orogastric infection with V. vulnificus.

**FUNDING**

This work was partly supported by a Grant from the School of Veterinary Medicine and Animal Sciences, Kitasato University.

**Conflict of interest statement.** None declared.

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