Impairment of Host Resistance to *Listeria monocytogenes* Infection in Liver of db/db and ob/ob Mice

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Leptin is a small peptide hormone secreted primarily by the adipocyte. Leptin-deficient ob/ob mice (1,2) and receptor-deficient db/db mice (3) were characterized initially as diabetes-obesity syndromes (4). Moreover, these animals exhibit a severe dysregulation of reproductive and hormonal traits (5) as well as a disturbance of hematopoietic and immune functions (6).

Lord et al. (7) first reported that leptin increases T-helper (Th1) cytokine production and suppresses Th2 cytokine production. Implication of leptin in inflammatory responses, including experimental autoimmune encephalomyelitis (8) and experimental arthritis (9), was demonstrated. On the other hand, a recent study showed that neutrophils express leptin receptors and that leptin enhances oxidative species production by neutrophils (10). Moreover, ob/ob mice exhibit impaired host resistance to intratracheal gram-negative *Klebsiella pneumoniae* infection due to impaired alveolar macrophage phagocytosis and neutrophil complement-mediated phagocytosis (11,12).

Diabetes is often identified as an independent risk factor for infections (13,14). We were interested in investigating the role of leptin and its correlation to host resistance to *Listeria monocytogenes* infection. *L. monocytogenes* is an intracellular-growing bacterium that is ordinarily non-pathogenic to healthy people; however, it is important as an opportunistic pathogen. The individuals at highest risk are pregnant women and their fetuses, new born infants, debilitated elderly people, and immunocompromized hosts including patients with diabetes (15). Host resistance to *L. monocytogenes* is controlled by cell-mediated immunity and regulated endogenous cytokines. This pathogen promotes the induction of the Th1 response, and interferon (IFN)-γ plays a critical role in anti-listerial resistance (16–18). Activated macrophages are the major effector cells in anti-listerial resistance (19), while neutrophils play a critical role in the resistance, especially early in infection (20). Chemokines are essential for accumulation of neutrophils and macrophages at the infectious foci (21). It was reported that mice lacking CCR2, a receptor for monocyte chemoattractant protein-1 (MCP-1) (CCL2), are highly susceptible to *L. monocytogenes* infection (22). Similarly, anti-listerial resistance is reportedly decreased in mice lacking CXCR3, a receptor for macrophage inflammatory protein-2 (MIP-2) (CXCL8) and KC (23). MCP-1 is a potent monocyte activator that has been associated with monocyctic infiltration in several inflammatory diseases, and MIP-2 and KC are powerful chemoattractants for neutrophils (24).

In the present study, we investigated host resistance to *L. monocytogenes* infection in db/db and ob/ob mice. We showed that these mutant mice were highly susceptible to...
this pathogen, and the elimination of bacteria from liver from these mice was inhibited. We demonstrate that leptin is required for host resistance to L. monocytogenes infection and that hyperglycemia caused by leptin deficiency is involved in the inefficient elimination of bacteria from the liver. Moreover, a defect of MCP-1 expression in liver may be involved in the attenuated host resistance in these mutant mice.

**RESEARCH DESIGN AND METHODS**

Female C57BL/Ks-db/db mice and their lean heterozygote littermates (db/m) were purchased from Clea Japan (Tokyo, Japan). Female C57BL/6j-ob/ob mice and their lean heterozygote littermates (ob/+) were obtained from The Jackson Laboratories (Bar Harbor, ME). Mice were maintained under specific pathogen-free condition at the Institute for Animal Experiment, Hirosaki University School of Medicine. All mice were allowed free access to water and food and were studied at 13–15 weeks of age. This study was carried out in accordance with the Guidelines for Animal Experimentation of Hirosaki University.

**Bacterial infection.** L. monocytogenes 1b 1684 was prepared as previously described (25). The concentration of washed cells was adjusted spectrophotometrically at 550 nm, and cells were stored at −80°C until use. Mice were infected intravenously with 0.2 ml of a solution containing 5 × 10⁸ or 5 × 10⁹ colony-forming units (CFU) of viable L. monocytogenes cells in 0.1 mol/l PBS (pH 7.4). The numbers of viable L. monocytogenes in the liver and spleen of infected animals were established by plating serial 10-fold dilutions of organ homogenates in RPMI-1640 medium (Nissui, Tokyo, Japan) containing 1% CHAPS (3-[3-cholamidopropyl]-dimethylammonio)-1-propanesulfonate) (Wako, Osaka, Japan) on tryptic soy agar (BD Biosciences, Sparks, MD). Colonies were routinely counted 18–24 h later.

**Histology and immunohistochemistry.** The liver tissues from db/db, db/m, ob/+, and ob/ob mice were fixed with 10% phosphate-buffered formalin and embedded in paraffin. Four-micrometer-thick sections were prepared, deparaffinized, and stained with hematoxylin and eosin for routine histopathological examination. For immunohistochemistry, deparaffinized sections were immunostained using the avidin-biotin-peroxidase complex (ABC) method with a Vectastain ABC kit (Vector, Burlingame, CA). They were then incubated with rat anti-mouse neutrophil antibody (diluted 1:100; Serotec Product, Oxford, U.K.), rabbit anti-human T-cell antibody CD3 (diluted 1:10; DakoCytomation, Carpinteria, CA), or rat anti-mouse macrophage antibody F4/80 (diluted 1:100; Serotec Product) overnight at 4°C. Diaminobenzidine was used as the chromogen. The immunolabeled sections were counterstained with hematoxylin. For immunostaining with CD3, the sections were pretreated for 30 min at 37°C with proteinase K (Gibco, Gaithersburg, MD) at a concentration of 10 mg/ml. For immunostaining with anti-neutrophil antibody, the sections were heated in 0.01 mol/l sodium citrate buffer (pH 6.0) by microwave for 15 min. Quantitative analysis of inflammatory cell density was performed by counting the number of neutrophils, T-cells, and macrophages using the specific antibodies described above. A blinded observer counted the inflammatory cells in the liver in 10 random high-power (>200×) fields of each immunohistochemically stained section.

**Real-time quantitative RT-PCR.** Total RNA was isolated from pieces of spleen and liver (0.05 g each) using a guanidium thiocyanate-phenol-chloroform single-step method (26). First-strand cDNAs were synthesized by reverse transcription of 1 μg total RNA using random primers (Takara, Shiga, Japan) and reverse transcriptase Moloney murine leukemia virus (Invitrogen, CA). The following primers were used for MCP-1, forward 5’-AGTGAAGGCTCTCTTCTCC-3’ and reverse 5’-TTCTTCTCTGGGGTGTCAGACACG-3’; for KC, forward 5’-GGATCTAATCTGACCACTCACCC-3’ and reverse 5’-CACCCTTACTAGCACTGGTGTGGA-3’; for IL-10, forward 5’-GGTTTCAGCGACTCACCC-3’ and reverse 5’-GACCATTGACCGTCCGCA-3’; for IFN-γ, forward 5’-CTGCACTTACTGAGGCGGAC-3’ and reverse 5’-GACCTGACCAACAGCCATCGT-3’; and for GAPDH, forward 5’-GAGGAAACGCAATACCTGACCAC-3’ and reverse 5’-AGTGGAGGCAACCTGACCC-3’. These primers and the corresponding probes were as follows: MCP-1, forward 5’-CTCTTCTTCCTC-3’ and reverse 5’-CGATCTCTTCCTC-3’; KC, forward 5’-GGCAACACAGCAAGCAGGAC-3’ and reverse 5’-GCCAGACGAAAAGACACGAC-3’; IL-10, forward 5’-GGAACCTGAGGGAAGGCGGAAC-3’ and reverse 5’-GGATGAGAGAAGCTGAGAACA-3’; IFN-γ, forward 5’-GCAGTCTACCCAGTCTCTCTC-3’ and reverse 5’-GGATCTAATCTGACCACTCACCC-3’. The predicted sizes of amplified products for MCP-1, KC, IL-10, and GAPDH were 151, 127, 137, and 107 bp, respectively. SYBER Green Supermix (Bio-Rad) was used as a PCR solution. PCR was run following the protocol: initial activation of TaqDNA polymerase for 5 min at 94°C, 1 min at 94°C for denaturing, 2 min at 60°C for annealing, and 3 min at 72°C for elongation, and 40 PCR cycles were performed. All experiments were run in duplicate to control for PCR efficiency. All parameters to detect primer-dimer conformation and nonspecific amplification were set and determined in relation to the amplification plot of GAPDH. The detection threshold is set to the log linear range of the amplification curve and kept constant (0.05) for all data analysis.
mice (Fig. 3A). Consistent with previous studies (28), severe fatty degeneration was observed in the ob/ob mice but not in the ob/? mice (Fig. 2C and D). Mild hydropic degeneration of hepatocytes, swelling of Kupffer cells, and formation of abscesses were observed in both the ob/ob and the ob/? mice 48 h after infection. Hydropic degeneration and abscess formation were more severe in the ob/ob than in the ob/? mice. On the other hand, mononuclear cell infiltration was much more prominent in the ob/? than in the ob/ob mice (Fig. 2C and D). Quantitative analysis revealed that the majority of inflammatory cells in the ob/? mice were T-cells and that the numbers of T-cells and macrophages were significantly higher in the ob/? than in the ob/ob mice (Fig. 3B).

Expression of chemokine mRNA is reduced in the liver of db/db and ob/ob mice. Chemokines including MCP-1, KC, and MIP-2 play an important role in the recruitment of phagocytes (22,23). We examined whether the impaired bacterial clearance exhibited by db/db and ob/ob mice is associated with the ability of chemokine expression. db/db and db/m mice were infected with $5 \times 10^5$ CFU of L. monocytogenes, and the expression of MCP-1, KC, and MIP-2 mRNA in the liver at various time points was determined by real-time quantitative PCR. The MCP-1 mRNA expression in the liver of db/m mice was significantly upregulated 42 h after infection compared with that in the db/db mice (Fig. 4A) (P < 0.05). Similarly, the KC mRNA expression in the liver of db/m mice was significantly higher than that in the db/db mice 24 h and 42 h after infection (Fig. 4B) (P < 0.05). However, the levels of MIP-2 mRNA in the liver of db/m mice were comparable with those in db/db mice during L. monocytogenes infection (Fig. 4C). In contrast, the expression of MCP-1, KC, and MIP-2 mRNA was equally increased in the spleen of db/db and db/m mice by L. monocytogenes infection (data not shown). We also assessed the chemokine mRNA expression in the liver of ob/ob and ob/? mice 42 h and 48 h after infection. At 48 h, the MCP-1 mRNA expression in the liver of ob/? mice was significantly upregulated compared with that in the ob/ob mice (Fig. 5B) (P < 0.05), while the levels of KC and MIP-2 mRNA in the liver of ob/? mice were comparable with those in ob/ob mice (Fig. 5C and D). Similar results were also obtained 42 h after infection (data not shown).

Host resistance to L. monocytogenes infection and chemokine mRNA expression is improved by leptin
replacement in ob/ob mice. We examined whether the impaired bacterial clearance and the expression of chemokine mRNA exhibited might be improved by injections of leptin. When ob/ob mice were injected with recombinant murine leptin at 12-h intervals for 10 days, the blood glucose level, body weight, and liver weight were reduced to the approximate values of ob/? mice (Table 1). The ob/ob mice were infected with 5 × 10⁴ CFU of L. monocytogenes on the following day after the last injection of leptin or PBS, and the bacterial numbers in the liver were determined 48 h later (Fig. 5A). The growth of bacterial cells in the liver of leptin-treated ob/ob mice was significantly inhibited compared with that of the PBS-treated ob/ob mice. We next assessed histology of the liver of leptin-treated mice 48 h after infection. Fatty and hydropic degeneration of hepatocytes were much milder in the leptin-treated ob/ob mice than in the PBS-treated ob/ob mice. The number of abscesses was smaller in the leptin-treated ob/ob mice than in the PBS-treated ob/ob mice (Fig. 6A and B). We also determined the expression of MCP-1, KC, and MIP-2 mRNA in the liver by real-time quantitative PCR 48 h after infection. The MCP-1 mRNA expression in the leptin-treated ob/ob mice was improved to the level of ob/? mice (Fig. 5B). In contrast, the levels of KC and MIP-2 mRNA were not affected by the leptin treatment in the ob/ob mice (Fig. 5C and D). These results suggest that the absence of leptin contributes to decreases in anti-listerial resistance and MCP-1 mRNA expression in ob/ob mice.

Host resistance to L. monocytogenes infection and chemokine mRNA expression are improved in db/db mice by insulin treatment. We next examined whether the impaired bacterial clearance exhibited might be associated with hyperglycemia. When db/db mice were injected with insulin for 14 days, the blood glucose levels were reduced to the approximate values of db/m mice (Table 1). Moreover, their body and liver weight were slightly decreased by insulin treatment. The db/db mice were infected with 5 × 10⁴ CFU of L. monocytogenes on the following day after the last injection of insulin, and the bacterial numbers in the liver were determined 48 h later (Fig. 7A). The growth of bacterial cells in the liver of insulin-treated db/db mice was significantly decreased compared with that of the PBS-treated db/db mice (P <
0.05). We next assessed histology of the liver of insulin-treated mice 48 h after infection. Hydropic degeneration and abscess formation were milder in the insulin-treated \( \text{db/db} \) mice than in the PBS-treated \( \text{db/db} \) mice (Fig. 6C and D). We also determined the expression of MCP-1, KC, and MIP-2 mRNA in the liver by real-time quantitative PCR 48 h after infection. The expression of both MCP-1 and KC mRNA in the insulin-treated \( \text{db/db} \) mice was improved to the levels of \( \text{db/m} \) mice (Fig. 7B and C). In contrast, the level of MIP-2 mRNA expression was not improved by insulin treatment in the \( \text{db/db} \) mice (Fig. 7D). These results suggest that hyperglycemia is involved in the decrease of anti-listerial resistance and the expression of MCP-1 and KC in \( \text{db/db} \) mice.

**DISCUSSION**

Our study showed that leptin is a critical factor in host resistance to infection with an intracellular-growing bacterium, \( \text{L. monocytogenes} \), as well as an extracellular-grow-
ing bacterium, *K. pneumoniae* (11,12). In this study, *db/db* and *ob/ob* mice were succumbed by sublethal *L. monocytogenes* infection (Fig. 1A and B). Death from listeriosis is caused by multiple organ failure, including liver damage (29). Indeed, the bacterial growth in the liver of *db/db* and *ob/ob* mice was significantly enhanced compared with that in their heterozygote littermates (Fig. 1D and E). These results suggested that leptin deficiency attenuates the defense against *L. monocytogenes* infection in liver.

In this study, bacterial growth was quite different between the spleen and the liver of *db/db* and *ob/ob* mice (Fig. 1C–E). Activated macrophages are the major effector cells in host resistance to *L. monocytogenes* infection (19), while neutrophils are also critical in the defense (20). Previous studies suggested that defense mechanisms to *L. monocytogenes* infection are different between spleen and liver (19,20,30); >60% of *L. monocytogenes* cells injected intravenously are recovered in the liver and multiply in hepatocytes. Massive infiltration of neutrophils occurs in the liver soon after. Explosive bacterial growth is revealed in the liver but not in the spleen of neutrophil-depleted mice early in infection. However, there have been little precise studies on the difference of defense mechanisms against *L. monocytogenes* infection between liver and spleen. In our histological observations, abscess formation occurred in the liver of *db/db* mice but not in the liver of *db/m* mice (Fig. 2A and B). Moreover, inflammatory cell infiltration including macrophages and neutrophils was more prominent in the *db/m* than in the *db/db* mice (Fig. 3A). Similarly, hydropic degeneration and abscess formation were more severe in the *ob/ob* than in the *ob/* mice, and infiltration of inflammatory cells including macrophages and neutrophils was much more prominent in the *ob/* than in the *ob/ob* mice (Fig. 2C and D). From these results, it is possible that dysregulation of bacterial clearance in the liver of *db/db* and *ob/ob* mice is caused by reducing infiltration of inflammatory cells.

Chemokines are essential for accumulation of neutrophils and macrophages at the infectious foci (21). MCP-1 attracts and activates monocytes, and MIP-2 and KC are powerful chemoattractant for neutrophils (24). Mice lacking CCR2, a receptor for MCP-1, are highly susceptible to *L. monocytogenes* infection (22), although excessively produced MCP-1 results in higher sensitivity to the infection (31). Similarly, anti-listerial resistance is reportedly decreased in mice lacking CXCR3, a receptor for MIP-2 and KC (23). Implication of these chemokines in host resistance has also been reported in other bacterial infections such as *Mycobacterium tuberculosis* (32), *Staphylococcus aureus* (33), and *Klebsiella pneumoniae* (34). In this study, the induction of MCP-1 and KC mRNA expression, but not MIP-2 mRNA expression, by *L. monocytogenes*

### Table 1

Effect of treatment with leptin or insulin on blood glucose concentration, body weight, and liver weight

<table>
<thead>
<tr>
<th>Mice</th>
<th>Treatment</th>
<th>Blood glucose (mmol/l)</th>
<th>Body weight (g)</th>
<th>Liver weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>ob/</em></td>
<td>None</td>
<td>8.6 ± 1.4</td>
<td>30 ± 2</td>
<td>1.48 ± 0.07</td>
</tr>
<tr>
<td><em>ob/ob</em></td>
<td>PBS</td>
<td>11.1 ± 8.7</td>
<td>58 ± 6</td>
<td>3.99 ± 0.52</td>
</tr>
<tr>
<td><em>ob/ob</em></td>
<td>Leptin</td>
<td>6.3 ± 3.8</td>
<td>48 ± 2</td>
<td>2.15 ± 0.26</td>
</tr>
<tr>
<td><em>db/m</em></td>
<td>None</td>
<td>6.4 ± 1.3</td>
<td>26 ± 2</td>
<td>1.16 ± 0.02</td>
</tr>
<tr>
<td><em>db/db</em></td>
<td>PBS</td>
<td>&gt;33.3</td>
<td>51 ± 5</td>
<td>3.12 ± 0.30</td>
</tr>
<tr>
<td><em>db/db</em></td>
<td>Insulin</td>
<td>10.7 ± 1.5</td>
<td>47 ± 2</td>
<td>2.55 ± 0.33</td>
</tr>
</tbody>
</table>

Data are means ± SD. *ob/ob* mice were injected with leptin twice daily with for 10 days, and *db/db* mice were injected with 50 units insulin or PBS for 14 days before infection with 5 × 10^8 CFU of *L. monocytogenes*, respectively. Each result was obtained from mice 48 h after infection.

*FIG. 6. Histology of liver from leptin-treated *ob/ob* and insulin-treated *db/db* mice during *L. monocytogenes* infection. *ob/ob* mice were injected twice daily with murine recombinant leptin or PBS for 10 days, and *db/db* mice were injected with insulin or PBS for 14 days before infection with 5 × 10^8 CFU of *L. monocytogenes*, respectively. Liver sections from leptin-treated *ob/ob* mice (*A*), control *ob/ob* mice (*B*), insulin-treated *db/db* mice (*C*), and control *db/db* mice (*D*) were obtained 48 h after infection with 5 × 10^8 CFU of *L. monocytogenes*. Abscess formation (arrows) and hydroptic degeneration are much milder in the leptin-treated *ob/ob* mice (*A*) than in the control *ob/ob* mice (*B*), and they are less prominent in the insulin-treated *db/db* mice (*C*) than in the control *db/db* mice (*D*). Magnification: ×400.*
genes" infection was suppressed in the liver of db/db mice (Fig. 4). In contrast, only MCP-1 mRNA expression was attenuated in ob/ob mice (Fig. 5). These results suggest that the decrease of MCP-1 might be involved in the attenuated anti-listerial resistance in the liver of db/db and ob/ob mice.

In this study, the weight loss and the decrease of the blood glucose levels in ob/ob mice were observed when leptin was administrated in the same scheduled doses as that reported previously (12) (Table 1). The elimination of bacteria from the liver was significantly enhanced by leptin replacement (Fig. 4A). The degree of fatty and hydropic degeneration of hepatocytes and the number of abscesses were reduced by leptin replacement (Fig. 6A and B). Moreover, the MCP-1 mRNA expression in leptin-treated ob/ob mice was improved to the level of ob/ob mice (Fig. 5B). These results suggest that leptin is able to restore anti-listerial resistance and MCP-1 production in leptin-deficient mice. Herein, the essential role of leptin in host resistance to L. monocytogenes infection was demonstrated by leptin-deficient mice and leptin replacement.

Patients suffering from diabetes are recognized as a risk group for opportunistic infections including L. monocytogenes (13–15). The available literature suggests two patterns of susceptibility to infections in the diabetic host. First, certain types of pulmonary infections may occur with an increased frequency in diabetic patients (35). Second, although certain pulmonary infections do not occur with increased frequency, they may be associated with increased morbidity and mortality in diabetic patients (36). In this study, hyperglycemia was improved by insulin treatment in db/db mice (Table 1). In parallel, the induction of MCP-1 and KC mRNA expression in the liver of insulin-treated db/db mice reversed to the levels of db/m mice (Fig. 7). These results suggested that hyperglycemia suppressed the expression of MCP-1 and KC mRNA in the liver of L. monocytogenes–infected db/db mice. On the other hand, the elimination of L. monocytogenes cells from the liver was recovered in insulin-treated db/db mice, but the improvement of resistance did not reach the levels of db/m mice (Fig. 7A). These results suggest that hyperglycemia is an important factor for the high susceptibility to L. monocytogenes infection. However, it is possible that other mechanisms mediated by leptin may be involved in the resistance of db/db mice.

In conclusion, db/db and ob/ob mice were highly susceptible to L. monocytogenes, and suppressed clearance of bacteria was especially revealed in the liver. It is possible that the decreased expression of MCP-1 is involved in attenuated anti-listerial resistance. Leptin is required for host resistance to L. monocytogenes infection, and hyperglycemia is included in leptin-mediated host defense. The present findings may provide a novel approach to elucidate a mechanism of high susceptibility to infections in diabetes.

REFERENCES