CONCISE COMMUNICATION

Viable but Nonculturable *Salmonella* Species Recovery and Systemic Infection in Morphin-treated Mice

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In vivo recovery and pathogenicity of *Salmonella enterica* serovar Oranienburg that became viable but nonculturable (VNC) in food were examined. VNC cells were completely eliminated in normal mice but caused systemic bacteremia and were subsequently lethal to morphin-treated mice. Therefore, morphin-treated mice might be a useful bioassay for VNC *Salmonella* species in implicated food items.

Nontyphoidal *Salmonella* species that usually cause gastroenteritis are important pathogens in foodborne diseases, and foodborne salmonellosis occurs frequently in humans. Isolating *Salmonella* organisms from fecal or blood samples from patients is essential to identify salmonellosis, and isolating *Salmonella* is important in confirming what is the infected food. However, bacterial pathogens easily become viable but nonculturable (VNC) in food by normal bacterial detection techniques. When the causative pathogen can be isolated from humans but not confirmed in the host food, the pathogen is VNC.

A diffuse outbreak caused by dried processed squid contaminated with *S. enterica* serovar Oranienburg (*S. oranienburg*) occurred in 1999 in Japan, and >1500 people developed septicemia and gastroenteritis [1]. When we processed experimentally dried squid contaminated with the causative strain, bacterial cells became VNC and were not culturable on agar plates [1]. Although we speculated that the dried squid was contaminated with numerous VNC *S. oranienburg* [1], we had no means of recovering and determining the pathogenicity of VNC cells in vivo. Here we describe an in vivo protocol for assessing the pathogenicity of VNC *Salmonella* organisms by using morphin-treated mice.

Materials and Methods

**Bacteria and media.** We used 2 *Salmonella* strains in this study: *S. oranienburg* Sa99004, isolated from a patient in an outbreak caused by dried processed squid [1], and *S. enterica* serovar Enteritidis (*Salmonella Enteritidis*) 363 [4]. The liquid culture media were nutrient broth (NB; Difco) lacking NaCl and buffered peptone water (BPW; pH 7.2). The agar plate was DHL agar (Eiken).

Processing of dried squid experimentally infected with *Salmonella* species. In all, 50 mL of 7% NaCl solution containing 5 g of raw squid was inoculated with 6.6 × 10⁷ cells of *S. oranienburg* Sa99004 grown at 37°C for 18 h in NB with shaking and incubated at 4°C for 24 h. The squid was removed, dried at 45°C for 24 h, soaked in 5 mL of BPW at pH 7.2, and incubated at 37°C for 1 h. We showed elsewhere that, in 1 mL of such a suspension, no culturable cells are detected on DHL plates but >90% of the cells are viable under fluorescence microscopy by use of the LIVE/DEAD BacLight Bacterial Viability kit (Molecular Probes). Therefore, we concluded that bacterial cells in 1 mL of the suspension become VNC [1]. We inoculated mice in the present study with the 1-mL suspension and its serial dilutions.

Infection and morphin treatment protocols. Pathogen-free female 25–30-g BALB/c mice (CLEA Japan) were treated with morphine, essentially by the method of Bhaskaran et al. [5]. In brief, 0.1 mL of sterile saline containing morphine sulfate (100 mg/kg of body weight; Wako Chemicals) was subcutaneously injected into mice every 12 h for 14 days. *Salmonella* cells grown in NB at 37°C for 18 h with shaking were intraperitoneally inoculated into mice that had been or had not been given morphine. These mice were observed daily, and mortality was scored for 14 days. During the experiments, morphine was subcutaneously inoculated into mice every 12 h. The survival percentages and mean survival times (MSTs) were calculated. For statistical analysis, we tested differences in survival by Student’s t test. Surviving mice were killed with chloroform 14 days after infection, for collection of liver and spleen and were homogenized in 1 mL of PBS to measure the colony-forming units, as described elsewhere [1].

Results

In our previous study [1], when 6.6 × 10⁷ cells of *S. oranienburg* Sa99004 freshly grown in NB were intraperitoneally in-
oculated into healthy mice, all mice survived with no symptoms for ≥40 days, although organisms were isolated from liver and spleen. Therefore, we speculated that large numbers of strain Sa99004 might be lethal to mice. In this study, we grew 3.6 × 10⁹ cells of *S. oranienburg* Sa99004 in NB that were intraperitoneally inoculated into healthy mice. Simultaneously, 6.6 × 10⁸ or 8.1 × 10⁸ cells of *Salmonella* Enteritidis grown in NB were intraperitoneally infected into mice as control specimens, resulting in 100% mortality with MSTs of 3.6 and 6.4 days, respectively (figure 1). However, all mice inoculated with *S. oranienburg* survived (figure 1). We concluded that *S. oranienburg* induced systemic infection, but its pathogenicity could not be evaluated in healthy mice because the infection did not harm the mice.

Because morphine increases the susceptibility to bacterial pathogens in mice [5, 6], we presumed that morphine-treated mice might be useful to evaluate the pathogenicity of vegetative *S. oranienburg* cells, because the treatment causes systemic immunosuppression, suppressed intestinal vermication, and stagnated *Salmonella* in vivo [5, 6]. Also, we presumed that even if some VNC *Salmonella* organisms retained pathogenicity in animals, they might cause infection and be pathogenic for such immunosuppressed mice.

In total, 25 mice were first treated with morphine, as described in Materials and Methods, and then intraperitoneally inoculated with various numbers of strain Sa99004, grown freshly in NB. This resulted in 100% mortality, with MSTs of 6.4 and 7.8 days in groups of morphine-treated mice inoculated with 6.3 × 10⁸ and 3.2 × 10⁸ cells of strain Sa99004, respectively (figure 2A). Three morphine-treated mice inoculated with 2.8 × 10⁴ and 1 morphine-treated mouse inoculated with 7.1 × 10³ cells of strain Sa99004 died (MSTs of 8.3 and 10 days, respectively), but all morphine-treated mice inoculated with 65 cells of strain Sa99004 survived 14 days (figure 2A), showing that the morphine increased susceptibility to *S. oranienburg* in mice (statistically significant). In addition, *Salmonella* species were recovered from liver and spleen of all surviving mice at 14 days after infection (data not shown). Because systemic infection by all *Salmonella* serotypes usually cannot be reproduced in mice, morphine-treated mice would be a good animal model in which to evaluate and verify *Salmonella* infection.

Because 1 mL of the BPW suspension prepared as described in Materials and Methods contained no cultivable cells, that suspension and its serial dilutions were intraperitoneally inoculated into all groups of mice. When 1 mL of the VNC suspension was intraperitoneally inoculated into healthy mice, all mice survived (figure 2B), and no bacterial cells were isolated from mice (data not shown), suggesting that healthy mice were inadequate for assessing the pathogenicity of VNC *Salmonella* species. However, all morphine-treated mice inoculated with 1 mL or 100 μL of the VNC suspension died with MSTs of 2.6 and 2.8 days, respectively, and 2 morphine-treated mice inoculated with 10 μL of the suspension died with an MST of 4.5 days (figure 2B). All morphine-treated mice inoculated with 1 μL of the suspension or saline survived. *Salmonella* organisms were recovered from tissue samples of all surviving morphine-treated mice (data not shown). Therefore, we conclude that morphine increases susceptibility to VNC cells in mice.

**Discussion**

Because 2.8 × 10⁴ and 7.1 × 10⁴ cells of freshly grown *S. oranienburg* killed 3 and 1 morphine-treated mice, respectively (figure 2A), and 10 μL of the VNS suspension killed 2 morphine-treated mice (figure 2B), the 10-μL suspension was estimated to contain ≥10³ pathogenic cells. Although no culturable cells were found in 1 mL of the BWP suspension, our results show recovery of VNC *Salmonella* species in morphine-treated mice. Because the VNC state makes it difficult to detect *Salmonella* cells in an implicated food, morphine-treated mice may be useful to confirm the causative infected foods.

Because no animal model for assessing microbial pathogenicity

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**Figure 1.** Survival of BALB/c mice infected with *Salmonella* species. Groups of 5 mice were orally inoculated with either *S. enterica* serovar Enteritidis or *S. enterica* serovar Oranienburg. Data in parentheses are survivors/group.
Survival of morphine-treated BALB/c mice against *Salmonella enterica* serovar Oranienburg (*S. oranienburg*) infection (treatment groups and control specimens, 5 mice/group). A, Healthy and morphine-treated mice were orally inoculated with various nos. of *S. oranienburg* (open symbols). $P < .0001$, for healthy mice vs. morphine-treated mice with $6.3 \times 10^5$ and $3.2 \times 10^5$ *S. oranienburg* cells. $P < .05$, for healthy mice vs. morphine-treated mice with $2.8 \times 10^4$ cells. B, Healthy and morphine-treated mice orally inoculated with buffered peptone water (BPW) suspension of viable but nonculturable (VNC) *S. oranienburg* cells or saline. Healthy mice were also intraperitoneally inoculated with 1 mL of the suspension. $P < .0001$, healthy mice with VNC suspension and morphine-treated mice with saline vs. morphine-treated mice with 1 mL and 100 µL of VNC suspension. $P < .15$, healthy mice with VNC suspension and morphine-treated mice with saline vs. morphine-treated mice with 10 µL of VNC suspension. Data in parentheses are survivors/group.

has been established for all pathogens, this method may be available for that purpose. However, because we did not reproduce the pathogenicities for *Shigella* or *Yersinia* species or of enteropathogenic *Escherichia coli* in morphine-treated mice (data not shown), this method cannot be used for all bacterial pathogens.

Because morphine treatment induces the degradation of macrophages in mammalian hosts [5], morphine-treated mice may be a useful bioassay for intracellular pathogens that cause no lethal infection for general breeding mice. For example, there is no animal model system in which to assess pathogenicity of *Listeria monocytogenes*, and therefore immunologic responses have been investigated mainly by intravenous infection in mice (the bacterium is not lethal for mice) [7].

E-cadherin A transgenic mice would be useful as animal models for *L. monocytogenes* infection, because they permit organisms to cross the intestinal barrier [8], but they are difficult to breed and are expensive. We often use mice to evaluate *Brucella* infection, but both virulent and avirulent strains can multiply in mice (authors’ unpublished data), and there is no animal model system in which to reproduce *Brucella* systemic infection. Therefore, we propose that morphine treatment in mice might be a useful bioassay for further investigation and also optimum to evaluate latent pathogenicity in narcotic addicts.

Narcotic abusers have a high risk for human immunodeficiency virus infection and have a high potential risk for secondary infection by bacteria or parasites because of immunosuppression [7]. Although morphine is usually used for patients as an analgesic and for heroin users, our results suggest that such groups might be at increased risk not only for various pathogens but also for VNC cells in foods.

References

1. Asakura H, Makino SI, Takagi T, et al. Passage in mice causes a change in the ability of *Salmonella enterica* serovar Oranienburg to survive NaCl


