Deoxynivalenol and Satratoxin G Potentiate Proinflammatory Cytokine and Macrophage Inhibitory Protein 2 Induction by *Listeria* and *Salmonella* in the Macrophage

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**ABSTRACT**

Health risks from microbial pathogens and toxins encountered in food and the environment continue to be of worldwide concern. The purpose of this research was to test the hypothesis that trichothecene mycotoxins amplify inflammatory responses to foodborne bacterial pathogens. We assessed the capacity of deoxynivalenol (DON) and satratoxin G (SG) to potentiate chemokine and proinflammatory cytokine production in RAW 264.7 murine macrophages induced by *Listeria monocytogenes* and *Salmonella* Typhimurium. When macrophage cultures were incubated with killed irradiated suspensions of the pathogens for 24 h, the minimum *Listeria* concentrations for induction of macrophage inhibitory protein 2 (MIP-2), interleukin-1β (IL-1β), IL-6, and tumor necrosis factor α (TNF-α) were 0.01, 0.01, 1.0, and 1.0 μg/ml (\(P < 0.05\)) and the minimum *Salmonella* concentrations were 0.01, 0.01, 0.1, and 0.1 μg/ml, respectively (\(P < 0.05\)). Induction of all four mediators by both pathogen was potentiated by DON (at 100 and 250 ng/ml); observed responses were significantly higher than predicted additive responses (\(P < 0.05\)). SG (at 2 and 5 ng/ml) also significantly amplified induction of IL-1β and TNF-α (\(P < 0.05\)) by both *Listeria* and *Salmonella*. These results indicate that DON encountered in *Fusarium*-contaminated food and SG from *Stachybotrys*-contaminated indoor environments could magnify innate inflammatory responses to foodborne bacterial pathogens.

Invading microbial pathogens induce both innate and acquired immune responses in the mammalian host. Macrophages serve as professional phagocytes and key effectors of the innate and adaptive immune responses during bacterial infections. These cells have the unique capacity to both recognize conserved pathogen-associated molecular patterns (PAMPs) and trigger inflammatory responses via activation of the toll-like receptor (TLR) family. Endotoxin, a well-studied PAMP, is a biologically active component of the gram-negative bacterial cell wall that exists as complexes of lipopolysaccharide (LPS), and the protein is a prototypical TLR agonist. LPS is a potent immunostimulatory molecule that initiates both rapid changes in macrophage signaling pathways and adaptive changes in macrophage gene expression. Upon binding endotoxin, the CD14 receptor acts by transferring the endotoxin and other bacterial ligands from the circulating LPS-binding protein to the TLR4 receptor, which together with the MD-2 molecule activate innate host defense mechanisms. In addition to TLR4, nine other mammalian TLRs have been identified. Macrophages respond to LPS by producing many mediators, such as proinflammatory cytokines, chemokines, bioactive lipids, and reactive oxygen species. Low-dose LPS exposure stimulates both mononuclear phagocyte function and other host responses that result in removal of invading bacteria. However, moderate LPS exposure often elicits tissue injury mediated by activated neutrophils and intravascular coagulation. High LPS concentrations initiate a chain of inflammatory events that culminate in cell death, frank injury to tissues, and functional failure of various organs. Although modest inflammatory responses may be insufficient to cause overt injury in unstressed individuals, such responses may be amplified in individuals coexposed to chemical stressors, and the net result is frank tissue injury. Xenobiotic chemicals influence the magnitude of responses to LPS in liver, kidney, respiratory tract, and lymphoid tissue. The trichothecenes are a family of more than 180 sesquiterpenoid metabolites that are produced by *Fusarium*, *Stachybotrys*, and other fungi and can adversely impact human health. These toxins bind to eukaryotic ribosomes, inhibit translation, and induce intracellular signaling cascades that evoke gene expression and cell death. Trichothecenes can be both immunostimulatory and immunosuppressive, depending on the dose, exposure frequency, and timing of the functional immune assay. Deoxynivalenol (DON) is a trichothecene that is produced by *Fusarium graminearum* and *Fusarium culmorum* and frequently found in grain-based agricultural products derived from corn, wheat, and barley. Satratoxin G (SG) and other macrocyclic trichothecenes produced by *Stachybotrys chartarum* have been associated with illnesses caused by contaminated indoor air. We and others have previously reported that trichothecenes can interact with LPS, and the immune system is a primary target.
Because of the capacity of trichothecenes to amplify responses to LPS, it is important to determine how these toxins affect inflammatory gene responses to bacterial cells that contain multiple PAMPs and are thus capable of activating different TLRs (8, 14, 16). Of particular interest are foodborne pathogens, and Listeria monocytogenes and Salmonella are among those of most concern. L. monocytogenes, a gram-positive pathogen, is the etiologic agent of listeriosis, a serious infection caused by eating food contaminated with the bacterium. Listeriosis most often affects pregnant women, newborns, and adults with weakened immune systems (16). The gram-negative Salmonella serovars, which are causative agents of gastroenteritis and enteric fevers, are responsible for millions of human illnesses worldwide each year (8). In the present study, a murine macrophage cell culture model was used to test the hypothesis that trichothecene mycotoxins amplify inflammatory cytokine responses to foodborne bacterial pathogens. Killed irradiated murine macrophages were employed to eliminate confounding variables of pathogen growth in macrophage culture. The results suggest that DON and SG potentiate proinflammatory cytokine and chemokine responses to both Listeria and Salmonella in the macrophage.

**MATERIALS AND METHODS**

**Reagents.** DON, cell culture reagents, and all other chemicals were purchased from Sigma Chemical Company (St. Louis, Mo.) unless otherwise stated. Microbiological media were obtained from Becton Dickinson (Sparks, Md.). Satratoxin G was a gift from Dr. Bruce Jarvis (University of Maryland, College Park).

**Bacteria.** Salmonella Typhimurium strain DT 104 (G01071) and L. monocytogenes Scott A (J1-225) were supplied by Dr. Elliot Ryser (Michigan State University, East Lansing), who obtained these from the Centers for Disease Control and Prevention (Atlanta, Ga.) and the Cornell University ILSI North America Listeria Collection (Ithaca, N.Y.), respectively. L. monocytogenes and Salmonella Typhimurium were grown in 100 ml of brain heart infusion broth (Difco, Becton Dickinson) at 20 and 37°C, respectively, until mid-log phase, aliquoted in 25-ml volumes, and centrifuged. Cultures were resuspended in 25 ml of phosphate-buffered saline (PBS) and washed twice more times. Cells were resuspended in 2.5 ml of PBS and immediately stored at −80°C. Frozen cultures were killed by gamma radiation (1 Mrad) at the Huron Irradiation Facility (University of Michigan, Ann Arbor). After irradiation, the absence of viable cells was confirmed by lack of growth on nutrient agar. Gram staining was also used to verify the retention of normal cell morphology. Irradiated aliquots were also assessed for dry weight, which was used for determining concentrations added to macrophage cultures.

**Cell culture.** The murine macrophage cell line RAW 264.7 (American Type Culture Collection, Rockville, Md.) used in this study was maintained in modified Dulbecco modified Eagle medium (DMEM) containing 10% (vol/vol) heat-inactivated fetal bovine serum and 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco BRL, Gaithersburg, Md.) at 37°C in a 6% CO₂ incubator. For concentration response studies, RAW 264.7 cells (2.5 × 10⁵) were cultured for 4 h in flat-bottom 24-well tissue culture plates (Fisher Scientific Co., Cornings, N.J.) containing 1 ml of modified DMEM. Supernatant was removed, and fresh medium containing irradiated Listeria or Salmonella (0.01, 0.1, 1, 10, and 100 µg/ml) was added to adherent cells. After 24 h, supernatants were collected, centrifuged at 450 × g for 5 min at 4°C, and stored at −20°C until used for an enzyme-linked immunosorbent assay (ELISA). In toxin coexposure experiments, RAW 264.7 cells were cotreated with DON (100 and 250 ng/ml), SG (2 and 5 ng/ml), L. monocytogenes (1 µg/ml), Salmonella Typhimurium (1 µg/ml), or combinations of mycotoxins and bacteria for 24 h, after which the supernatant fraction was collected. Fresh modified DMEM was used as the negative control (vehicle). Each treatment was performed in triplicate, and experiments were conducted at least four times. The highest concentrations employed for either toxin reflected those required to inhibit proliferation by 50% (IC₅₀) in previous studies. The next lower concentration that was used represented 40% of the IC₅₀. These DON and SG concentrations are consistent with those found previously to modulate gene responses in leukocyte cell cultures (2–4, 26).

**Cytokine and chemokine assays.** Macrophage inhibitory protein 2 (MIP-2), interleukin-1β (IL-1β), IL-6, and tumor necrosis factor α (TNF-α) in cell supernatant fraction were quantified by ELISA with commercial kits (BD-Pharmingen, San Diego, Calif.) as described previously (3, 26).

**Statistics.** Data were analyzed with Sigma Stat for Windows (Jandel Scientific, San Rafael, Calif.). For determination of interactive effects, values from vehicle-treated cultures were subtracted from those for microbe, toxin, or toxin plus microbe groups. Microbe and toxin replicates were then randomly combined to calculate a predicted mean additive response with variance. This calculated value was compared with that for observed cotreated samples with the Mann-Whitney rank sum test. Data sets were considered significantly different at P < 0.05.

**RESULTS**

The minimum concentrations of killed irradiated Listeria required for inducing chemokine (MIP-2) and cytokine (IL-1β, IL-6, and TNF-α) production were initially assessed. Listeria at 1 µg/ml significantly induced the production of MIP-2, IL-6, and TNF-α compared with induction by vehicle alone, whereas lower concentrations of Listeria (0.01 and 0.1 µg/ml) were ineffective (Fig. 1). The minimum concentration for IL-1β induction was 0.1 µg/ml. For MIP-2, the response to 1 µg/ml was identical to responses at higher concentrations, whereas IL-1β, IL-6, and TNF-α increased in a concentration-dependent fashion from 1 to 100 µg/ml.

The minimum concentrations of killed irradiated Salmonella needed for chemokine and cytokine induction were also determined. Salmonella at 0.1 µg/ml or higher induced MIP-2 and IL-1β, whereas minimum concentrations for TNF-α and IL-6 induction were 0.1 and 1.0 µg/ml, respectively (Fig. 2). Again, IL-1β, IL-6, and TNF-α were induced by Salmonella in a concentration-dependent fashion, whereas MIP-2 was not. Based on these findings, Listeria and Salmonella concentrations of 1 µg/ml were considered to be the minimum needed for induction of all four mediators and were therefore used in cotreatment studies.

The effects of cotreatment of Listeria (1 µg/ml) with either SG (2 and 5 ng/ml) or DON (100 and 250 ng/ml)
were assessed relative to chemokine and cytokine production (Fig. 3). For MIP-2, responses to *Listeria* alone or the combination of *Listeria* and SG (2 and 5 ng/ml) did not differ but were higher than those for either toxin alone (Fig. 3A). *Listeria* with either DON concentration induced more MIP-2 than did *Listeria* or DON alone.

Both DON and SG modulated *Listeria*-induced IL-1β, IL-6, and TNF-α responses. SG and DON at either concentration potentiated IL-1β production when cotreated with *Listeria* (Fig. 3B). SG inhibited IL-6, whereas DON upregulated induction of this cytokine by *Listeria* (Fig. 3C). Both SG and DON enhanced *Listeria*-induced TNF-α production (Fig. 3D).

Effects of coinoculation to *Salmonella* (1 μg/ml) with DON or SG were also assessed (Fig. 4). MIP-2 responses to *Salmonella* alone or cotreatment with 2 ng/ml SG did not differ, whereas 5 ng/ml SG suppressed the response. Cotreatment of *Salmonella* with either DON concentration induced MIP-2 responses (Fig. 4A). Induction of IL-1β by *Salmonella* was higher when cells were cotreated with either concentration of SG or DON (Fig. 4B). Cotreatment of *Salmonella* and 250 ng/ml of DON induced IL-6 production compared with treatment with *Salmonella* alone, whereas 100 ng/ml DON or SG (2 and 5 ng/ml) did not potentiate the response (Fig. 4C). Both SG and DON potentiated *Salmonella*-induced TNF-α (Fig. 4D).

FIGURE 1. Dose-response effects of killed irradiated *Listeria monocytogenes* on chemokine (MIP-2) and cytokine (IL-1β, IL-6, and TNF-α) production in RAW 264.7 cells. Cultures were incubated for 24 h with killed irradiated suspensions of *Listeria*, and supernatants were analyzed by ELISA. Bars without the same letter differ significantly (P < 0.05).

FIGURE 2. Dose-response effects of killed irradiated *Salmonella Typhimurium* on chemokine (MIP-2) and cytokine (IL-1β, IL-6, and TNF-α) production in RAW 264.7 cells. Cultures were incubated for 24 h with killed irradiated suspensions of *Salmonella*, and supernatants were analyzed by ELISA. Bars without the same letter differ significantly (P < 0.05).
FIGURE 3. Effects of SG or DON on chemokine (MIP-2) and cytokine (IL-1β, IL-6, and TNF-α) production by RAW 264.7 cells induced by killed irradiated L. monocytogenes. Cultures were incubated with 1 µg/ml killed irradiated Listeria with or without mycotoxins for 24 h, and supernatants were analyzed by ELISA. ND, none detected; VH, saline vehicle. Bars marked with letters a and b indicate significantly different responses (P < 0.05) from those of vehicle- or Salmonella-treated cultures not treated with toxin, respectively.

FIGURE 4. Effects of SG or DON on chemokine (MIP-2) and cytokine (IL-1β, IL-6, and TNF-α) production in RAW 264.7 cells induced by killed irradiated Salmonella Typhimurium. Cultures were incubated with 1 µg/ml killed irradiated Salmonella for 24 h, and supernatants were analyzed by ELISA. ND, none detected; VH, saline vehicle. Bars marked with letters a and b indicate significantly different responses (P < 0.05) from those of vehicle- or Listeria-treated cultures not treated with toxin, respectively.

DISCUSSION

A critical research question in food safety relates to why certain individuals that encounter food pathogens are more susceptible to infection or are more likely to have more severe disease with complications. Inflammation is a critical component of infections with Listeria and Salmonella. Although such innate immune responses can be inherently beneficial, exaggerated inflammatory responses underlie many chronic diseases, some of which are sequelae to bacterial infections. DON and SG can be commonly encountered in food and the environment, respectively (18). The observations reported here are important because they suggest that these trichothecenes can magnify inflammatory gene responses to foodborne pathogens. The three key observations made in this study were that (i) trichothecenes amplified responses to both gram-negative and gram-positive bacterial cells, (ii) the degree of potentiation followed...
a rank order of IL-1β > TNF-α > IL-6 > MIP-2, and (iii) DON at equitoxic concentrations was more effective at potentiating IL-6 and MIP-2 than was SG.

Increases in mediator responses in cotreatment groups might have simply resulted from the additive effects of pathogen and toxin. Table 1 is a comparison of predicted additive MIP-2, IL-1β, IL-6, and TNF-α responses (pathogen alone plus toxin alone) and observed responses (co-treatment of pathogen and toxin). Predicted additive and observed MIP-2 responses were not different for cells coexposed to either *Listeria* or *Salmonella* and SG (2 and 5 ng/ml). However, cotreatment with DON at 250 ng/ml potentiated increased MIP-2 responses to both pathogens. Observed IL-1β responses in cells cotreated with the pathogens and mycotoxins were markedly greater than the predicted additive responses. Observed IL-6 responses were not higher than predicted responses in cells cotreated with SG but were higher in cells that were cotreated with DON at either concentration. Cotreatment of the cells with either pathogen plus DON markedly induced TNF-α, compared with the predicted additive response, whereas SG amplified the effects of *Listeria* but not those of *Salmonella*. Thus, in most cases, potentiation did not simply reflect additive responses.

The approach utilized in this study should enable further exploration of how bacteria might interact with xenobiotic agents such as the trichothecenes to modulate immune function and potentially injure the immune system. The bacterial suspensions employed here were used to mimic the multiple PAMPs that are presented by microbes during the course of an infection. Radiation was used to kill the pathogens to eliminate complications of bacterial growth during culturing. Radiation enabled killing without obvious disruption of cell structure such as might occur during heating. Critical PAMPs that are likely to evoke inflammatory gene responses in macrophages are the lipoteichoic acid–containing cell wall of *Listeria* (21) and the LPS–gram-negative cell wall of *Salmonella* (19). Other potential PAMPs might include DNA, RNA, and flagella (14).

The capacity of these mycotoxins to upregulate pathogen-induced IL-1β, TNF-α, and IL-6 could contribute to inflammatory sequelae associated with these microbes (8, 16); sepsis (15) is particularly important. When the relative responses were compared among cytokines and chemokines, elevation of IL-1β was most robust in cotreated cultures. This finding is important because IL-1β is a critical proinflammatory cytokine for innate immune responses (5) and can modulate immune and inflammatory responses (9) and contribute to apoptosis in vivo (10, 13, 20, 22). IL-1β also plays an essential role in inducing corticosterone and subsequent leukocyte apoptosis in mice exposed to LPS and DON (10, 11).

The results presented here suggest that trichothecene mycotoxins can amplify innate responses to bacterial pathogens in the macrophage. These findings are consistent with those of previous in vivo studies of DON and LPS (10, 11, 27, 28). Tai and Pestka (23, 25) determined that the trichothecene T-2 toxin lowered the LD₅₀ for *Salmonella* Typhimurium by five orders of magnitude. Increased inflamma-

### Table 1. Comparison of predicted additive and observed responses of RAW macrophages to irradiated *Listeria* or *Salmonella* co-cultured with either SG or DON and relative to the induction of proinflammatory cytokines by DON (ng/ml)

<table>
<thead>
<tr>
<th>Microbe</th>
<th>TNF-α (ng/ml)</th>
<th>IL-6 (ng/ml)</th>
<th>MIP-2 (ng/ml)</th>
<th>SG (ng/ml)</th>
<th>DON (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Listeria</em></td>
<td>0.7 ± 0.3</td>
<td>1.1 ± 0.04</td>
<td>0.7 ± 0.3</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td><em>Salmonella</em></td>
<td>1.1 ± 0.04</td>
<td>1.7 ± 0.4</td>
<td>1.1 ± 0.04</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td><em>Listeria</em>+SG</td>
<td>1.1 ± 0.04</td>
<td>1.5 ± 0.15</td>
<td>0.7 ± 0.3</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td><em>Salmonella</em>+SG</td>
<td>1.1 ± 0.04</td>
<td>1.5 ± 0.15</td>
<td>0.7 ± 0.3</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td><em>Listeria</em>+DON</td>
<td>1.1 ± 0.04</td>
<td>1.5 ± 0.15</td>
<td>0.7 ± 0.3</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td><em>Salmonella</em>+DON</td>
<td>1.1 ± 0.04</td>
<td>1.5 ± 0.15</td>
<td>0.7 ± 0.3</td>
<td>0.2</td>
<td>0.2</td>
</tr>
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* SG, satratoxin G; DON, deoxynivalenol; MIP, macrophage inflammatory protein; IL, Interleukin; TNF, tumor necrosis factor.

* Actual response is significantly different from predictive response (*P < 0.05*).
tory mediators during bacteremia might have mediated this response. Further study is needed to determine the potential for trichothecenes and other toxic chemicals to amplify responses to LPS and other PAMPs.

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REFERENCES