Systemic Inflammatory Response Associated with Augmentation and Activation of Leukocytes in Candida/Indomethacin Administered Mice

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We have previously shown that the combination of β-glucan and nonsteroidal anti-inflammatory drugs (NSAIDs) was lethal to mice. In this study, we examined the effect of Candida on this treatment and found that Candida showed similar lethal toxicity when used in combination with indomethacin. It was also confirmed that LPS preparations from various origins and by various procedures did not show lethality. Candida/indomethacin induced lethality was found to be associated with increased number of leukocytes in various organs and activation of these cells. These findings strongly suggest that pathogenic fungi augment the side effects of non-steroidal anti-inflammatory drugs.

Key words Candida; β-glucan; indomethacin; NSAIDs; side effect; lethal toxicity

Candida spp. is a medically important fungus which induces disseminated candidiasis and candidemia in hospitalized immuno-compromised patients. The cell wall of Candida is mainly composed of two polysaccharides, mannan and β-glucan. The β-glucan moiety is composed mainly of β-(1→3)-β-glucan and β-(1→6)-β-glucan moieties. At least part of the β-glucan is basically insoluble in aqueous neutral as well as alkaline solution. In contrast, appearance of an aqueous β-glucan in the blood specifically occurred with deep-seated fungal infections.1–3 We have demonstrated that intravenously administered Candida spp. was distributed in the liver and the spleen, and the β-(1→3)-β-glucan moiety was deposited in these organs for quite a long period of time.4–7 Degradation and excretion of other fungal components, such as proteins, nucleic acids, lipids, and carbohydrates, easily proceeded within a couple of days as shown by metabolic labeling methodologies.7) Deposition and slow metabolism of the β-(1→3)-β-glucan would be due to the absence of β-glucan hydrolase in the host. The only mechanism to degrade β-glucan which can be postulated is oxidative degradation by products of phagocytes: O₂⁻, H₂O₂, and hypochlorous acid (HOCl).

Indomethacin is a nonsteroidal anti-inflammatory drug (NSAID) that suppresses prostaglandin (PG) synthesis by inhibiting cyclooxygenases I and II.8,9) Individuals treated with such a drug are relieved of pain and inflammation. Several studies have also shown that indomethacin is an immunomodulator that augments macrophage, T cell, and natural killer (NK) cell activities and regulates production of cytokines including tumor necrosis factor (TNF)-α, interleukin (IL)-1, and interferon (IFN)-γ.10–13) Despite the advantages of NSAIDs, several adverse reactions are known such as damage to the gastroduodenal mucosa and liver dysfunction.14–17)

During previous screenings to identify both the useful and detrimental activities of β-glucans, we found that pretreatment of mice with β-glucan significantly increased mortality by subsequent indomethacin administration.18,19) The lethal toxicity was induced by pretreatment or simultaneous treatment of mice with β-glucan, and by single as well as multiple dosing. Since IFN-γ, IL-6 and CSF concentrations in sera of β-glucan/indomethacin treated mice were significantly elevated, the lethality in mice was likely due to mal-adjustment of the cytokine network. Additionally, other NSAIDs, such as aspirin, diclofenac, and sulindac induced similar toxicity, but nabumetone did not. These findings strongly suggest that certain underlying immunological as well as inflammatory events modify or enhance the side effects of β-glucan and/or NSAIDs, and are individually controlled in each NSAID. Precise characterization of this phenomenon and its mechanism are very important to reduce the lethal adverse effects of these medicines.

In this study, we investigated whether this phenomenon is caused by the medically important fungus, Candida, the major cell wall component of which is β-glucan. We found that in addition to β-glucans, whole cell preparation of Candida also enhanced the lethal toxicity. In addition, inflammatory mediators and function of leukocytes in Candida/indomethacin administered mice were studied.

MATERIALS AND METHODS

Animals ICR male mice between 5 and 8 weeks of age were purchased from Japan SLC, Inc., Shizuoka and were maintained under specific pathogen-free conditions. Breeding and handling of all animals in this experiment were approved by the Committee on the Animal Experiments in the School of Pharmacy, Tokyo University of Pharmacy and Life Sciences.

Reagents Lipopolysaccharides (LPS) (from Escherichia coli Serotype O111:B4, phenol extract; from E. coli Serotype O111:B4 TCA extract; from E. coli Serotype O127:B4 Butanol extract; from Salmonella minnesota phenol extract; from Serratia marcescens phenol extract; from Pseudomonas aeruginosa Serotype 10 (Habs) phenol extract; and from Salmonella minnesota Re595 (Re mutant), and dimethylsulfoxide (DMSO) were purchased from Sigma Chemical Co. Candida albicans IFO1385 defatted whole cells (CA) were prepared as described previously.20) Indomethacin (Wako Pure Chemical Industries, Ltd., Osaka) was suspended with 2% polyoxyethylene (20) sorbitan monooolate in 0.5% sodium carboxymethyl cellulose (CMC).

Administration Schedule of Microbial Products and In-
domethacin Each candidate material suspended or dissolved in physiologic saline was intravenously or intraperitoneally administered to ICR mice every other day a total of three times (days −5, −3, −1). Indomethacin (5 mg/kg) was orally administered daily from day 0 for 14 d. Survival of these mice was monitored.

Measurement of \( \text{H}_2\text{O}_2 \) Production by Peritoneal Macrophages Production of hydrogen peroxide was assayed continuously at 37°C with the micro-assay using the scoopletin technique. Briefly, the cultures were washed 3 times with 50 \( \mu l \) of Krebs-Ringer phosphate buffer supplemented with 5.5 mm glucose (KRPG). The assay mixture consisted of 30 mm scoopletin, 1 mm Na\(_2\)S, 1 purpurogallin unit/ml horse radish peroxidase, in the presence or absence of phorbol myristate acetate (PMA) in KRPG was added in each well. Immediately after the addition of the assay mixture the plate was placed in a filter fluorometer (microplate reader MTP-32, Corona Electric Co., Ltd., Tokyo) and the fluorescence was recorded with an excitation wavelength of 365 nm and an emission wavelength of 450 nm. After incubation for 30, 60, 90, or 120 min at 37°C, the fluorescence in each well was again recorded using the microplate reader. The amount of hydrogen peroxide produced by macrophages was calculated from the fluorescence reduction using hydrogen peroxide solution as standard.

Cell Preparation Leukocytes were harvested from various mouse tissues. Hepatic lymphocytes were isolated by Histopaque (1.077) density gradient. Briefly, after sacrificing mice, the liver was removed and cut into small pieces, digested with an enzyme mixture (20 mg/mouse collagenase (Wako), 2 mg/mouse deoxyribonuclease (Sigma), 0.5 mg/mouse hyaluronidase (Sigma) in 10% FCS RPMI) for 3 h. After washing with phosphate buffered saline, leukocytes were prepared by Histopaque density gradient. Leukocyte fraction was adhered onto a dish and the non-adherent population was collected. Splenocytes were obtained by pressing the spleen through 200-gauge steel mesh. Erythrocytes in the spleen were disrupted by ACK-lysis buffer (8.29 g/l NH\(_4\)Cl, 1 g/l KHCO\(_3\), 37.2 mg/l EDTA/2Na). Peritoneal exudate cells (PEC) were collected from the peritoneal cavity by washing twice with sodium nitrite in the range of 0—100 \( \mu M \).

ELISA IL-6: A 96-well plate (Sumitomo Bakelite Co., Tokyo) was coated with rat anti-mouse IL-6 monoclonal antibody (mAb) (Pharmingen, CA, U.S.A.) in 0.1 M NaHCO\(_3\) (pH 8.2) by incubation at 4°C overnight. The plate was washed with phosphate buffered saline (PBS) containing 0.05% Tween 20 (Wako Pure Chemical Industries) (PBST) and blocked with 0.5% bovine serum albumin (Sigma) (BPPBST) at 37°C for 40 min. After washing, the plate was incubated with recombinant mouse IL-6 (Pharmingen, CA, U.S.A.) or 50 \( \mu l \) of samples at 37°C for 40 min. The plate was washed with PBST and blocked with PBST at room temperature for 10 min, then treated with biotinylated rat anti-IL-6 mAb (Pharmingen, CA, U.S.A.). Next, the plate was treated with peroxidase-conjugated streptavidin (ZyMED Laboratories Inc.) and developed with a TMB substrate system (KPL Inc., MD, U.S.A.). Color development was stopped with 1 nM phosphoric acid and the optical density at 450 nm was measured.

IFN-\( \gamma \): a 96-well plate (Sumitomo Bakelite Co., Tokyo) was coated with rat anti-mouse IFN-\( \gamma \) monoclonal antibody (mAb) (Pharmingen, CA, U.S.A.) in 0.1 M NaHCO\(_3\) (pH 8.2) by incubation at 4°C overnight. The plate was washed with PBS containing 0.05% Tween 20 (Wako Pure Chemical Industries) (PBST) and blocked with 0.5% bovine serum albumin (Sigma) (BPPBST) at 37°C for 40 min. After washing, the plate was incubated with recombinant mouse IFN-\( \gamma \) (Pharmingen, CA, U.S.A.) or 50 \( \mu l \) of samples at 37°C for 40 min. The plate was then washed with PBST and treated with biotinylated rat anti-IFN-\( \gamma \) mAb (Pharmingen, CA, U.S.A.), further treated with peroxidase conjugated streptavidin (ZyMED Laboratories Inc.) and developed with a TMB substrate system (KPL Inc., MD, U.S.A.). Color development was stopped with 1 nM phosphoric acid and the optical density at 450 nm was measured.

Statistics Statistical analysis of survival was done by the Kaplan-Meier method with Logrank test followed by a statistics program, StatView for Windows version 5.0. All other \textit{in vivo} and \textit{in vitro} experiments were examined in duplicate or triplicate and repeated twice. The findings are expressed as mean±standard deviation (S.D.). The significance of differences between means was measured with the Students \( t \)-test.

RESULTS

Effect of Combination Treatment of \textit{Candida} and Indomethacin on Mortality of Mice To examine the lethality induced by a combination of \textit{Candida} and indomethacin in mice, defatted whole cell preparation of \textit{Candida} was intraperitoneally or intravenously administered to mice every other day for three times, and then indomethacin was orally administered for 2 weeks. Rectal temperature, blood glucose level and mortality were monitored. None of the mice died by administration of \textit{Candida} without indomethacin treatment (data not shown). As shown in Table 1, both i.p. and i.v. treatment enhanced mortality and i.v. treatment was more toxic. In the most toxic group, \textit{C. albicans} (500 \( \mu g/mouse, \) i.v.), the average life span was 7.7±3.6 d and the mortality rate was 93%. Rectal temperature and blood glucose concentration of each mouse are shown in Fig. 1, and both levels were significantly lowered near death. These facts strongly suggest that \textit{Candida} also enhanced mortality in combination with indomethacin.

Since LPS often contaminate various preparations and are well-characterized microbial products showing strong immunopotentiating activity, their contribution to the lethality was examined. Various concentrations of LPS preparation

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from *E. coli* O111 phenol extract (250, 50, 10, 2, 0.4 mg/mouse) were prepared and the same protocol as shown above was followed. None of the doses of LPS preparation enhanced mortality. In particular, none of the mice died by 50 or 10 mg/mouse of LPS administration (Table 2). To examine the LPS-associated membrane components involved in this response, other methods of extracting LPS such as butanol and TCA were used, but toxicity did not occur. Lethality was not enhanced even by s.c. administration of LPS (Table 2). LPS preparations from various organisms, such as *Salmonella minnesota*, *Serratia marcescens*, *Pseudomonas aeruginosa* and from rough mutants such as *Salmonella minnesota* Re595 also showed no effect (Table 2). These facts strongly suggest that fungal components are responsible for the toxicity and no contaminated lipopolysaccharide is important in inducing lethality.

**Accumulation of Leukocytes in Various Organs in *Candida*/indomethacin Administered Mice**

In the previous study,18,19 we showed that immune/inflammatory parameters of β-glucan/indomethacin administered mice are uncontrolled. To make a comparison, leukocyte numbers in organs and cytokine concentrations were examined in mice that were administered *Candida* three times and indomethacin twice. Figure 2 shows leukocyte counts in the blood and spleen weights after indomethacin treatment and these values were significantly increased in the *Candida*/indomethacin group. Figure 3 shows leukocyte numbers in lung, peritoneal cavity, and spleen, and the numbers in *Candida*/indomethacin administered mice were significantly increased. Figure 4 shows organ weight of liver and the weight was significantly increased. These facts strongly suggest that leukocyte numbers in various organs were increased in *Candida*/indomethacin administered mice. In the preliminary experiment, leukocyte population was examined by flow cytometer, the ratio of neutrophils was significantly increased in various organs and activation markers of these cells were also increased (data not shown), suggesting the enhancement of damage in these organs.

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**Table 1. Effect of *C. albicans* on Mortality of Indomethacin-Administered Mice**

<table>
<thead>
<tr>
<th>Dose×3 (μg/mouse)</th>
<th>Route</th>
<th>Number of mice</th>
<th>Life span (d, mean±S.D.)</th>
<th>p vs. control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (IND)</td>
<td>—</td>
<td>70</td>
<td>12.9±2.3</td>
<td>—</td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>500 i.v.</td>
<td>15</td>
<td>7.7±3.6, &lt;0.0001</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>100 i.v.</td>
<td>10</td>
<td>9.2±3.5, &lt;0.0001</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>500 i.p.</td>
<td>5</td>
<td>6.8±2.4, &lt;0.0001</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>100 i.p.</td>
<td>5</td>
<td>12.4±2.3</td>
<td>—</td>
</tr>
</tbody>
</table>

*C. albicans* (500, 100 μg/mouse) was suspended in 99% EtOH overnight. After drying, the cells were suspended in saline. These materials (500, 100 μg/200 μl) were i.v. administered 3 times on days −5, −3, −1. Indomethacin (5 mg/kg) was then p.o. administered once a day from day 0 to day 14 and mortality was monitored.

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from *E. coli* O111 phenol extract (250, 50, 10, 2, 0.4 μg/mouse) were prepared and the same protocol as shown above was followed. None of the doses of LPS preparation enhanced mortality. In particular, none of the mice died by 50 or 10 μg/mouse of LPS administration (Table 2). To examine the LPS-associated membrane components involved in this response, other methods of extracting LPS such as butanol and TCA were used, but toxicity did not occur. Lethality was not enhanced even by s.c. administration of LPS (Table 2). LPS preparations from various organisms, such as *Salmonella minnesota*, *Serratia marcescens*, *Pseudomonas aeruginosa* and from rough mutants such as *Salmonella minnesota* Re595 also showed no effect (Table 2). These facts strongly suggest that fungal components are responsible for the toxicity and no contaminated lipopolysaccharide is important in inducing lethality.

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**Activation of Leukocytes in *Candida*/indomethacin Administered Mice**

Activation of leukocytes in relation to inflammation/immunotoxicity was examined by several parameters. Figure 5 shows inflammatory cytokine synthesis in sera, and the *Candida*/indomethacin administered group showed enhanced production of IFN-γ and IL-6. Figure 6 shows hydrogen peroxide synthesis of peritoneal exudate cells and leukocytes in liver, and leukocytes of both organs in *Candida*/indomethacin administered mice showed enhanced production both in the presence and absence of PMA stimu-
translation. Figure 7 shows nitric oxide synthesis of spleen cells and *Candida*/indomethacin administered mice also enhanced its production. All of these data strongly suggest that the inflammatory response of leukocytes was augmented in various organs in *Candida*/indomethacin administered mice.

### Table 2. Effect of LPS on Mortality of Indomethacin-Administered Mice

<table>
<thead>
<tr>
<th>Number of mice</th>
<th>Life span (d, mean±S.D.)</th>
<th>p vs. control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sum</td>
<td>Dead</td>
<td>Sum</td>
</tr>
<tr>
<td>Control (IND)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td><em>E. coli</em> O111: B4 phenol extract</td>
<td>500</td>
<td>i.v.</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>i.v.</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>i.v.</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>i.v.</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>i.v.</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>i.v.</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>s.c.</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>s.c.</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>s.c.</td>
</tr>
<tr>
<td><em>E. coli</em> O111: B4 TCA extract</td>
<td>50</td>
<td>i.v.</td>
</tr>
<tr>
<td><em>E. coli</em> O127: B8 butanol extract</td>
<td>50</td>
<td>i.v.</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> Serotype 10 phenol extract</td>
<td>250</td>
<td>i.v.</td>
</tr>
<tr>
<td>Serratia marcescens phenol extract</td>
<td>50</td>
<td>i.v.</td>
</tr>
<tr>
<td><em>Salmonella minnesota</em> phenol extract</td>
<td>50</td>
<td>i.v.</td>
</tr>
<tr>
<td><em>Salmonella minnesota</em> Re 595 phenol-chloroform-petroleum ether extract</td>
<td>250</td>
<td>i.v.</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>i.v.</td>
</tr>
</tbody>
</table>

*Various doses of LPS (500, 250, 50, 10, 2, 1, 0.4 μg/200 μl) were i.v. or s.c. administered 3 times on days −5, −3, −1. IND (5 mg/mouse) was p.o. administered once a day from day 0 to day 14 and mortality was monitored.*

Fig. 2. Kinetics of WBC and Spleen Weight in *Candida*/Indomethacin-Administered Mice

*Candida* (CA; 500 μg/mouse) was i.v. administered 3 times on days −5, −3, −1. Indomethacin (IND) was p.o. administered on day 0 (0 h) and day 1 (24 h). Each time (0, 6, 12, 24, 32 and 48) mice were killed and spleen weight and number of white blood cells (WBC) were measured. Results are shown as mean±S.D. The significance was evaluated by Student's t-test against the indomethacin-group. *p<0.05, **p<0.01, ***p<0.001.

Fig. 3. Effect of *Candida*/Indomethacin Administration on Leukocyte Number in Bronchoalveolar Lavage, Peritoneal Cavity and Spleen

*Candida* (CA; 500 μg/mouse) was i.v. administered 3 times on days −5, −3, −1. Indomethacin (IND; 5 mg/kg) was p.o. administered once a day on day 0 and day 1. Leukocytes were counted on day 2. Results are shown as mean±S.D. The significance was evaluated by Student’s t-test against the indomethacin-group. *p<0.05, **p<0.01.
DISCUSSION

We have previously shown that a combination of β-glucan and NSAIDs induced lethality in mice.\textsuperscript{18,19} In this study, we examined the effect of Candida on this treatment and found that it showed similar toxicity in combined use with indomethacin. This was also confirmed by the fact that LPS preparations from various origins and by various procedures did not show lethality. These facts suggested the independence and the specificity of the toxicity from the biological activity of LPS. Candida/indomethacin induced lethality was found to be associated with increased number of leukocytes in various organs and activation of these cells. In addition to the findings shown here, in a preliminary investigation we found a reduced expression of L-selectin in leukocytes, significant granuloma formation in liver, and increase of GOT and GPT in sera (data not shown). These findings strongly
suggest that Candida/indomethacin induced lethality is a systemic inflammatory response syndrome similar to endotoxin shock.

Prostaglandins and leukotrienes are potent eicosanoid lipid mediators derived from arachidonic acid released from the host cell-membrane by phospholipases (PLA2). These lipid mediators are involved in numerous homeostatic biological functions and inflammation, and act through specific G protein-coupled receptors; many of these receptors have been recently cloned. Prostaglandins are formed by most cells in our bodies and act as autocrine and paracrine lipid mediators. They are not stored but are synthesized when cells are activated by mechanical trauma or by specific cytokine, growth factor, and other stimuli. In contrast, leukotrienes are made predominantly by inflammatory cells, such as polymorphonuclear leukocytes, macrophages, and mast cells. Cellular activation by immune complexes, bacterial peptides, and other stimuli elicit a sequence of events that include PLA2, 5-lipoxygenase (5-LO) translocations to the nuclear envelope to initiate biosynthesis. Several studies have shown that indomethacin is an immunomodulator that augments macrophage, T cell, and natural killer (NK) cell activities and regulates production of cytokines including TNF-α, IL-1, and IFN-γ.\(^{10-13}\) These immunomodulatory actions would be strongly related to the shift of balance of actions among prostaglandins and leukotrienes due to the inhibition of prostaglandin synthesis. Similarly, β-glucan with other NSAIDs, such as aspirin, diclofenac, and sulindac also inhibited prostaglandin synthesis. Similarly,\(^{14}\) prostaglandins and leukotrienes due to the inhibition of cyclooxygenase inhibitors, including indomethacin, dramatically reduced the viability of the yeast and the production of prostaglandins, suggesting that a cyclooxygenase like enzyme may be responsible for fungal prostaglandin production. In addition, a PGE series lipid was purified from C. albicans and was biologically active on both fungal and mammalian cells. The discovery that pathogenic fungi produce and respond to immunomodulatory eicosanoids reveals a virulence mechanism that has potentially great implications for understanding the mechanisms of chronic fungal infection. Additionally, it has been reported that a major component of the cell wall mannan components of C. albicans produced pyrogenic responses which were completely inhibited by indomethacin.\(^{26}\) These facts suggested the strong relationship between prostaglandins and fungal infection. In contrast to these quoted data, the results shown in the present study were paradoxical and strongly suggested the induction of systemic inflammatory response with maladjustment of the cytokine network shown in this and earlier studies. The overall mechanism of Candida/indomethacin-mediated lethality is not yet clearly understood, however, the data in the present study indicate an additional interaction of pathogenic fungi and prostaglandin which may be considered in therapeutic strategies.

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REFERENCES


