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Differential Antibiotic-Induced Endotoxin Release in Severe Melioidosis

A. J. H. Simpson,1,3 S. M. Opal,4 B. J. Angus,1,3 J. M. Prins,5 J. E. Palardy,4 N. A. Parejo,4 W. Chaowagul,2 and N. J. White1,3

Severe melioidosis is a life-threatening systemic bacterial infection caused by *Burkholderia pseudomallei*. A prospective, randomized treatment trial was conducted in northeast Thailand to compare ceftazidime (a penicillin-binding protein [PBP]-3–specific agent that causes release of large amounts of endotoxin in vitro) and imipenem (a PBP-2–specific agent that kills *B. pseudomallei* more rapidly but releases low amounts of endotoxin) in severe melioidosis over a 6-h time course after the first dose of antibiotic. Despite similar clinical, microbiological, endotoxin, and cytokine measures at study entry, ceftazidime-treated patients (n = 34) had significantly greater systemic endotoxin (*P* < .001) than patients treated with imipenem (n = 34) after the first dose of antibiotic. No overall difference in mortality was observed (35% in both groups [95% confidence interval, 20%–50%]). Differential antibiotic-induced endotoxin release is demonstrable in severe melioidosis. These differences in endotoxin release did not appear to have a significant impact on survival in this group of patients.
attempted to analyze antibiotic-induced endotoxin release. Some previous investigations have indicated that antibiotic-induced endotoxin release occurs and has measurable clinical consequences [14, 15, 17, 18], whereas others have not observed this effect [12, 16, 19, 20].

The current study represents the first large clinical trial to test the hypothesis that differential antibiotic-induced endotoxicin release occurs and has clinical consequences in a specific infection. This study was designed to examine the impact of the initial dose of antimicrobial therapy with either a PBP-2-specific or a PBP-3-specific \( \beta \)-lactam agent in a single-organism infection in a relatively homogeneous population and at a single study site.

**Patients and Methods**

**Clinical study.** Patients were randomized prospectively to receive either ceftazidime at 100 mg/kg/day (usual dose, 2 g every 8 h) or imipenem at 50 mg/kg/day (usual dose, 1 g every 8 h) alone to treat suspected acute severe melioidosis. This trial was part of a recently published [21] clinical study conducted at Sappasitprason Hospital, Ubon Ratchathani, northeast Thailand, and followed a protocol similar to those of previously reported treatment trials [5, 7]. Patients were allocated to either treatment by envelope randomization; envelopes were opened only after enrollment in the trial. Dose adjustments were made in cases of renal impairment, but the first dose was always 2 g of ceftazidime or 1 g of imipenem. Investigators were not blinded as to drug therapy.

After enrollment, all patients were seen at least daily by one of the study team until the patients were discharged. Patient details were recorded on a standard case report form. The current study of endotoxin release was conducted from July 1996 through November 1997. Subjects in the endotoxin-release study were from a convenience sample collected over the last 2 years of a large treatment trial [21]. Clinicians obtained the full series of blood samples for endotoxin and cytokine analysis when sufficient time was available to collect all necessary samples. Patients who had received prior empiric therapy with \( \beta \)-lactam agents possessing activity against *B. pseudomallei*, such as amoxicillin-clavulanate, ceftriaxone, or cefotaxime, were excluded from this study. Only those patients in whom systemic infection with *B. pseudomallei* (n = 68) was confirmed by culture were included in the analysis. *B. pseudomallei* was isolated and identified by standard methods [21]. All clinical isolates were tested for susceptibility to both ceftazidime and imipenem by standard laboratory methods [22, 23].

**Endotoxin and cytokine measurements.** Endotoxin determinations were derived from platelet-rich plasma obtained at baseline (before treatment) and then every 30–60 min, up to 6 h after the first dose of either ceftazidime or imipenem. Endotoxin measurements were analyzed by use of a quantitative turbidimetric limulus amebocyte lysate assay (Associates of Cape Cod, Woods Hole, MA). Plasma was collected in heparinized, endotoxin-free glassware (Chromogenix, Molndal, Sweden). Specimens were centrifuged and then frozen at –30°C within 15 min of collection. Plasma was diluted 1:10, passed through a 0.45-mm filter, and heated to 70°C for 5 min to remove membrane-bound endotoxin and any serum inhibitors. The functional limit of detection of the assay was 2 pg/mL endotoxin.

Cytokine concentrations were also measured at the same time points as the endotoxin assays by use of immunoassays for human interleukin (IL)-6, tumor necrosis factor (TNF)–\( \alpha \), and IL-10. Samples were collected into potassium EDTA tubes containing Trasylol (50 \( \mu \)L/mL blood). Specimens were centrifuged and then frozen at –30°C within 15 min of collection. Cytokines were each measured by ELISA. TNF–\( \alpha \) was measured by a commercially available assay for human TNF–\( \alpha \), according to the instructions of the manufacturer (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam). IL-6 was measured by use of anti–human IL-6 monoclonal antibody (Pharmingen, San Diego; clones MQ2-13A1 and MQ2-39C3). IL-10 was measured by use of anti–human IL-10 monoclonal antibody (Pharmingen; clones JES3-9D7 and JES3-12G8). The detection limits were 3 pg/mL (TNF), 5 pg/mL (IL-6), and 4 pg/mL (IL-10). Baseline cytokine assays were done for all patients entered into the larger ceftazidime versus imipenem clinical trial; full results of these cytokine assays will be reported separately. All cytokine and endotoxin assays were done in a blinded fashion, without knowledge of the treatment assignment or clinical outcome of each patient.

**Statistical methods.** Results are presented as the median plus 25%–75% interquartile (IQ) range for numeric data, unless otherwise stated. For categorical variables, proportions were compared by use of the \( \chi^2 \) test with Yates’s correction. Normally distributed continuous data were compared by use of the Student’s \( t \) test. Data not conforming to a normal distribution were compared by use of the Mann-Whitney \( U \) test. The area under the curve (AUC) for endotoxin release for each antimicrobial agent was calculated by use of the generalized Wilcoxon statistic. All analyses were done with the statistical computing package SPSS for Windows, version 7.5 (SPSS, Chicago), and EpiInfo, version 6 (Centers for Disease Control and Prevention, Atlanta).

**Results**

**Clinical characteristics at study onset.** From July 1996 through November 1997, 73 patients were enrolled. Five of these patients were not proven to have melioidosis by culture and were excluded from the subsequent analysis. Demographic and clinical characteristics for the remaining 68 patients who had culture-confirmed systemic infection with *B. pseudomallei* and who were enrolled in this study are presented in table 1. All isolates were susceptible to both ceftazidime and imipenem. Baseline clinical and demographic characteristics of the patients were similar. Although some randomization imbalances were found between the 2 treatment groups at study entry, none of these differences were statistically significant (table 1).

The imipenem-treated group had slightly higher APACHE II scores (\( P = .4 \)). The majority of patients (60 [88%]) had an underlying disease, but there was no difference between the 2 therapy arms. Preexisting diabetes mellitus (DM) occurred in 56% of patients in each arm and renal disease in 21% of each group. Bacteremia at the time of study entry was slightly more
Of the 20 patients who either were in shock at study entry or subsequently went into shock, the mortality rate was 80% (16/20), compared with 17% among those who were not in shock (8/48). Patients with hepatocellular dysfunction at study entry did significantly worse than patients without hepatocellular dysfunction (mortality 20 [50%] of 40 patients vs. 4 [14%] of 28; P = .006). The presence of acute renal failure (P = .69) or advanced age (>64 years; P = .59) did not worsen mortality. APACHE II scores were significantly higher in nonsurvivors than in survivors (median, 22 [17–27] vs. 12 [8–16]; P < .001).

A total of 60% of patients had positive blood cultures for B. pseudomallei at the time of study entry. The mortality rate was 49% (20/41) among bacteremic patients, whereas nonbacteremic patients had a significantly lower mortality rate of 15% (4/27; P = .009). There was a trend toward a lower mortality in patients with DM (10 [26%] of 38), compared with those without DM (14/30, 47%), but this was not statistically significant (P = .14). Mortality in this series was slightly higher among patients who had received antibiotics before entry into the study (7 [28%] of 25 vs. 17 [40%] of 43; P = .49), but these 2 groups had similar fever clearance times (P = .68) and durations of hospital stay (P = .97).

**Table 1.** Baseline characteristics of study patients.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Ceftazidime (n = 34)</th>
<th>Imipenem (n = 34)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age in y, mean ± SD</td>
<td>51.7 ± 10.6</td>
<td>52.8 ± 12.8</td>
<td>.70</td>
</tr>
<tr>
<td>Sex, male</td>
<td>59</td>
<td>59</td>
<td>1</td>
</tr>
<tr>
<td>APACHE II score (IQ range)</td>
<td>12 (10–21)</td>
<td>16 (10–20)</td>
<td>.39</td>
</tr>
<tr>
<td>Prior use of other antibiotics</td>
<td>62</td>
<td>65</td>
<td>1</td>
</tr>
<tr>
<td>Preexisting diabetes mellitus</td>
<td>56</td>
<td>56</td>
<td>1</td>
</tr>
<tr>
<td>Bacteremia</td>
<td>65</td>
<td>56</td>
<td>.62</td>
</tr>
<tr>
<td>Presence of shock at study entry</td>
<td>7</td>
<td>16</td>
<td>.43</td>
</tr>
<tr>
<td>Hepatic dysfunction at study entry</td>
<td>59</td>
<td>59</td>
<td>1</td>
</tr>
<tr>
<td>Acute renal failure at study entry</td>
<td>32</td>
<td>32</td>
<td>1</td>
</tr>
<tr>
<td>Site of pulmonary/pleural infection</td>
<td>62</td>
<td>71</td>
<td>.63</td>
</tr>
<tr>
<td>Site of skin/soft tissue infection</td>
<td>44</td>
<td>32</td>
<td>.45</td>
</tr>
<tr>
<td>Site of hepatic/splenic infection</td>
<td>34</td>
<td>35</td>
<td>1</td>
</tr>
<tr>
<td>Other sites of infections (urine, bone, central nervous system)</td>
<td>29</td>
<td>29</td>
<td>1</td>
</tr>
</tbody>
</table>

**NOTE.** Data are %, unless otherwise noted. IQ, interquartile range.

**Endotoxin levels.** Results of plasma endotoxin measurements over the first 6 h of initial treatment with either of the 2 intravenous β-lactam agents are provided in figure 1. Only 3 (4.4%) of 68 patients had plasma endotoxin levels below the limit of detection at baseline, that is, before the first dose of study antibiotic. The median endotoxin concentration in the remaining 65 patients was 47.4 pg/mL (overall range, <2–9540 pg/mL; IQ range, 13.6–172.2 pg/mL). Seven patients (10.3%) had baseline levels >500 pg/mL, and 5 (7.4%) had levels >1000 pg/mL. Baseline results are shown in table 2. Baseline plasma endotoxin concentrations were similar in the 2 treatment arms (P = .12) and were similar in survivors and nonsurviving patients (P = .77; table 3). There were no differences in endotoxin levels between patients who were septicemic compared with those who were not (P = .88), and in these septicemic patients endotoxin levels were similar regardless of outcome (P = .86).

There were no correlations between baseline endotoxin concentrations and various clinical and laboratory parameters overall, including APACHE II scores or plasma lactate concentration (table 4). However, among septicemic patients, there was an inverse correlation with aspartate aminotransferase (r = −.31; P = .046).

Peak plasma endotoxin levels after the first dose were significantly higher in the ceftazidime-treated patients (table 5; P = .008). The quantitative AUC for circulating endotoxin was also significantly higher in the ceftazidime-treated patients than in the imipenem-treated patients (P < .001). Although this difference in AUC was most apparent in those patients who had not received any prior antimicrobial agents (P < .001), the same finding was observed in those patients who received other antimicrobial agents in addition to either imipenem or ceftazidime (P = .05). Peak endotoxin concentrations were correlated with serum alkaline phosphatase concentrations only (r = .33; P = .046).

![Figure 1. Plasma endotoxin levels over the first 6 h after the initial dose of imipenem (n = 34) or ceftazidime (n = 34) in patients with acute septicemic melioidosis. Results are presented as median values with 25%–75% interquartile ranges.](image)
Table 2. Baseline cytokine and endotoxin levels as predictors of fatal outcome.

<table>
<thead>
<tr>
<th>Substance, pg/mL</th>
<th>Survivors (n = 44)</th>
<th>Nonsurvivors (n = 24)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>0 (0–3.7)</td>
<td>14.4 (5.2–5.4)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>IL-6</td>
<td>116.5 (45.4–245.2)</td>
<td>825.2 (228.8–2829.0)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>IL-10</td>
<td>18.9 (9.1–41.1)</td>
<td>191.9 (65.6–526.6)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Endotoxin</td>
<td>58.4 (13.8–170.3)</td>
<td>34 (10.8–146)</td>
<td>.77</td>
</tr>
</tbody>
</table>

NOTE. Data are median (interquartile range). TNF, tumor necrosis factor; IL, interleukin.

Cytokine levels. Baseline TNF-α, IL-6, and IL-10 levels are shown in table 3. As reported elsewhere in a different series, elevated baseline cytokine levels were strongly predictive of a lethal outcome in this group of patients [24] (table 2). There were strong positive correlations between levels of all 3 cytokines at baseline (IL-6 vs. IL-10, Spearman rank-correlation coefficient: r = .58; P < .001; IL-6 vs. TNF: r = .40; P = .001; IL-10 vs. TNF: r = .64, P < .001). There were also strong positive correlations between peak levels of all 3 cytokines (IL-6 vs. IL-10: r = .72, P < .001; IL-6 vs. TNF, r = .50, P = .002; IL-10 vs. TNF, r = .68, P < .001). None of the 3 cytokines (baseline concentrations) showed any correlation with baseline endotoxin levels (TNF: r = .07, P = .59; IL-6: r = .1, P = .92; IL-10: r = .04, P = .75), nor did peak levels of the 3 cytokines show any correlation with peak endotoxin levels (TNF: r = .02, P = .93; IL-6: r = .23, P = .18; IL-10: r = .10, P = .58). Despite the clear differences in endotoxin release between the ceftazidime- and imipenem-treated groups, the mortality rate during the course of hospitalization for melioidosis was similar in both treatment groups (35%). In patients who died, the time to death after study enrollment was similar for the 2 arms: in the ceftazidime group, the median was 4 days (IQR range, 1–8 days); and in the imipenem group, the median was 2 days (IQR range, 1–11 days; P = .46). In surviving patients, the median duration of fever after starting ceftazidime therapy (288 h; IQR range, 93–648) was similar to that for the imipenem-treated group (240 h; IQR range, 66–396; P = .52). Four patients were never febrile (3 in the imipenem arm), and 5 patients were still febrile when discharged (4 in the ceftazidime arm). Total duration of hospitalization in surviving patients in the ceftazidime-treated group (median, 17 days; IQR range, 14–23 days) was similar to that for imipenem patients (median, 17 days; IQR range, 14–21 days; P = .98).

Discussion

The current standard therapy for severe melioidosis is high-dose intravenous ceftazidime, followed by maintenance therapy for extended periods with either amoxicillin-clavulanate or the 4-drug combination of doxycycline, chloramphenicol, and trimethoprim/sulfamethoxazole [5, 7, 25]. Maintenance regimens lasting several months after successful acute treatment are required to prevent relapse, which is common in short-course treatment [26].

Table 3. Baseline cytokine and endotoxin levels by drug treatment.

<table>
<thead>
<tr>
<th>Substance, pg/mL</th>
<th>Ceftazidime</th>
<th>Imipenem</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>0 (0–19.7)</td>
<td>3.7 (0–14.3)</td>
<td>.59</td>
</tr>
<tr>
<td>IL-6</td>
<td>211.6 (71.6–730.1)</td>
<td>223.9 (99.2–1021.6)</td>
<td>.33</td>
</tr>
<tr>
<td>IL-10</td>
<td>39.5 (12.6–143.7)</td>
<td>38.1 (14.0–131.5)</td>
<td>.62</td>
</tr>
<tr>
<td>Endotoxin</td>
<td>65.5 (28.4–130.8)</td>
<td>28.2 (5.8–146)</td>
<td>.12</td>
</tr>
</tbody>
</table>

NOTE. Data are median (interquartile range). TNF, tumor necrosis factor; IL, interleukin.

Despite dramatic reductions in mortality in severe melioidosis since the introduction of ceftazidime, acute mortality remains high [5, 7]. For this reason, alternative treatment strategies have been sought, including the use of carbapenems such as imipenem. A large clinical trial to prospectively evaluate the relative efficacy of imipenem compared with ceftazidime in the treatment of severe melioidosis provided an opportunity to investigate the incidence and effects of differential antibiotic-induced endotoxin release in this disease.

Melioidosis generally occurs as a community-acquired infection in a homogeneous, ambulatory patient population. It represents an ideal infectious-disease process in which to determine the clinical relevance of antibiotic-induced endotoxin release in a single, defined infection [12]. This type of investigation can be performed unencumbered by the myriad clinical, microbiological, and immunological variables found in series of patients with gram-negative bacterial infections of multiple etiology and treated with multiple doses of antimicrobial agents [12, 16, 19, 20]. Melioidosis is now well characterized and is recognized in endemic areas as an important cause of community-acquired, life-threatening illness [1, 3]. Although many patients have underlying conditions such as DM or renal disease [27], these are not usually acutely life threatening in themselves. This greatly simplifies the analysis of the clinical impact of the infection itself.

The heterogeneous patient groups that constitute septic-shock study populations in developed countries typically exhibit a multitude of underlying conditions. Concomitant underlying diseases and comorbidities often dominate the clinical course and mortality rates [12, 16]. Severe melioidosis itself, like primary meningococcemia, is the predominant factor determining outcome [1, 3, 5]. Although many patients with severe melioidosis have DM or renal disease, most would otherwise have survived had the systemic infection not occurred. Finally, the immunodominant LPS structure of B. pseudomallei exists as a single major serotype [28]. This greatly simplifies quantitative measurement of endotoxin release in melioidosis. There is considerable variability inherent in measurements of endotoxin from different bacterial species because of the variety of LPS biochemical structures and the degree of substitution with O-specific side chains [12].

It has been clearly shown for in vitro and animal models that PBP-3–specific inhibitors induce filament formation during the course of killing gram-negative bacilli [9, 10, 14]. These filaments generate large quantities of free endotoxin, which
could have adverse consequences during the treatment of gram-negative infections [13, 29]. In contrast, imipenem and other penicillin-binding protein type 2–specific agents induce spheroplast formation and rapid cell lysis in gram-negative bacilli, with release of minimal amounts of endotoxin [9, 13, 18].

Since the beginning of the antimicrobial era, it has been speculated that initial treatment of gram-negative infections with antimicrobial agents may paradoxically injure the host, because large quantities of bacterial endotoxin may be released during bacterial killing [17, 19]. This observation was first made in the late 1940s [30] and has been the subject of much debate and controversy over the last 4 decades [19]. It has been difficult to verify the clinical relevance of antibiotic-induced endotoxin release, although it has been studied in considerable detail in gram-negative bacilli, with release of minimal amounts of endotoxin [9, 13, 18].

The results of the current study show that significant differences in systemic endotoxin release occur in severe melioidosis after initial antimicrobial therapy with a PBP-2–specific agent (imipenem) versus a PBP-3–specific agent (ceftazidime). However, the excess endotoxin release induced by PBP-3–specific agents did not result in a significant worsening in mortality when compared with the case of PBP-2–specific agents in this group of patients. The mortality rate was 12 (35%) of 34 in the ceftazidime-treated group and 12 (35%) of 34 in the imipenem-treated group. The rather small sample size generates wide confidence intervals (CIs) with respect to potential differences in mortality rates (95% CI, 20%–50%). Thus, it is apparent that differential antibiotic-induced endotoxin release alone is not sufficient to explain excess mortality in patients with severe melioidosis. Numerous other determinants of survival, independent of antibiotic-induced endotoxin release, affect the outcome in patients with systemic bacterial infections [12, 19, 30, 31].

Combination therapy with aminoglycosides and bacteriostatic antimicrobial agents affects the amount of endotoxin release associated with β-lactam antimicrobial agents [10, 14, 19]. The fact that the majority of patients in this study had received other antimicrobial agents before the first dose of either imipenem or ceftazidime may also have affected the results and minimized the differences in mortality rates between the 2 groups. Endotoxin was detectable in most patients at significant concentrations before starting treatment. In this small clinical study, modest increases in circulating endotoxin associated with PBP-3–specific agents may not have been sufficient to cause statistically significant differences in outcome.

It is possible that *B. pseudomallei* endotoxin is simply less potent in vivo than LPSs from other bacterial species—there is both animal and in vitro evidence to this effect [32]. This is important if antiendotoxin strategies are shown to be of benefit in other forms of sepsis, such as meningococcal infection, and are considered possible candidates for future therapeutic trials in melioidosis [33]. It is also possible that, because most patients had detectable endotoxin levels at study entry, the subsequent incremental increase in endotoxin concentrations associated with ceftazidime was insufficient to significantly worsen the outcome [12]. However, baseline endotoxin levels were similar in survivors and nonsurvivors and were not related to the presence of bacteremia. Baseline levels in this study did not correlate with other surrogate markers of outcome, such as plasma lactate concentration or APACHE II score, or with plasma cytokine concentrations. This also suggests that endotoxin release is of relatively minor importance in melioidosis.

In summary, this study demonstrates that PBP-3–specific β-lactam agents induce high levels of free endotoxin in acute, severe melioidosis when compared with PBP-2–specific agents. This excess endotoxin release was not associated with delayed resolution of fever or longer hospitalization and did not affect overall mortality. Endotoxin from *B. pseudomallei* is probably not of major pathophysiologic importance in melioidosis. Whether the lack of clinical consequences of differential antibiotic-induced endotoxin release observed in this study can be

<table>
<thead>
<tr>
<th>Substance, pg/mL</th>
<th>Ceftazidime</th>
<th>Imipenem</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>10 (0–37.6)</td>
<td>13.7 (3.1–30.9)</td>
<td>.62</td>
</tr>
<tr>
<td>IL-6</td>
<td>291.9 (139.0–1366.0)</td>
<td>594.8 (70.8–4305.0)</td>
<td>.36</td>
</tr>
<tr>
<td>IL-10</td>
<td>36.9 (10.8–301.6)</td>
<td>39.6 (12.8–348.1)</td>
<td>.74</td>
</tr>
<tr>
<td>Endotoxin</td>
<td>725.8 (231.8–2477.2)</td>
<td>146 (54.2–893.0)</td>
<td>.008</td>
</tr>
</tbody>
</table>

**NOTE.** Data are median (interquartile range). TNF, tumor necrosis factor; IL, interleukin.
ized to other microbial causes of gram-negative sepsis remains to be determined.

Acknowledgments

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