Klebsiella pneumoniae and Staphylococcus aureus Infections in Mice: Differences in Uremia and Ammoniagenesis

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Lethal infections by Staphylococcus aureus and Klebsiella pneumoniae were compared for kidney-related effects in mice. K. pneumoniae caused uremia and an increase in blood ammonia that could reach 2.5 times normal. These events did not occur in mice inoculated with S. aureus. Use of germfree animals indicated that most of the increase in ammonia arose from the gut, presumably due to greater availability of urea and ureolysis. Injected ornithine restored blood ammonia to nearly normal levels and extended survival.

In both gram-negative and -positive bacterial infections, the kidney is a site of lodgment of microbial cells and a target for toxin activity. Small amounts of endotoxin reaching the kidney during gram-negative bacterial infections or substances released by host-endotoxin interactions elsewhere can cause lesions reducing renal function and producing a uremic state (3, 8, 9, 20). During infection with Staphylococcus aureus, the kidneys of experimental animals contain alpha-toxin (7) and nuclease (6), and the kidneys are a site of accumulation of injected staphylococcal enterotoxin (11). Renal function in animals infected with S. aureus may decline in parallel with mean arterial function (2), and slow death caused by injection of alpha-toxin is accompanied by severe renal necrosis (1).

Our intent was to compare the outcomes, in terms of kidney-related plasma changes, of the infections caused by S. aureus and Klebsiella pneumoniae. They were found to differ both in uremia and by a striking increase in blood ammonia that followed injection of K. pneumoniae or endotoxin.

MATERIALS AND METHODS

Bacterial cultures and procedures of inoculation. S. aureus strain 14609, a human-derived strain highly virulent for mice (5), and a mouse-derived strain of K. pneumoniae were used. Cultures were kept frozen and transferred before use to Trypticase soy agar slants (Difco) for 24 h at 35 C and then to quiescent culture in tubes of the same medium for 12 h at 35 C, followed by three washings and suspension in physiological saline.

Twenty-five-gram male mice were inoculated intraperitoneally with 1 ml of the suspension containing 10^9 colony-forming units, a treatment followed by death in 4 to 6 h. Alternatively, animals received 0.2 ml of the suspension subcutaneously, which caused death in 24 to 48 h.

Conventional mice were from an inbred strain maintained in the departmental animal colony. Germ free mice were obtained from the Charles River Breeding Laboratories, Inc., Wilmington, Mass.

Assays. Blood and tissue ammonia was determined by the method of McCullough (10), whereas urea was measured by an Autoanalyzer. Arginase was measured by the method of Van Slyke and Archibald (17), and arginine was assayed on a Beckman 120 amino acid analyzer.

To determine gastric emptying, mice were fasted overnight and inoculated intragastrically with 0.25 ml of nearly saturated phenol red delivered via a 1.5-inch (about 3.8 cm), 21-gauge blunt-tipped hypodermic needle. Stomachs from mice so treated were removed immediately or 60 min later, brought to a volume of 2.0 ml with water, and homogenized in a Teflon tissue grinder. The homogenate was centrifuged at 1,000 x g for 10 min, an 0.1 ml of the supernatant fluid was removed, mixed with 0.2 ml of 1.5 N trichloroacetic acid, and recentrifuged at 2,000 x g. The supernatant fluid was mixed with 0.3 ml of 0.1 N NaOH and 3.9 ml of ethanol. Dye absorbance was measured at 560 nm and the percentage of residual dye in the stomach at 60 min was calculated.

Staphylococcal nuclease was measured as described previously (6).

For endotoxin assay, samples, either undiluted or diluted with pyrogen-free water, were mixed 1:1 with 0.1 ml of Limulus lysate in pyrogen-free glass tubes and incubated 60 min at 37 C. Gelation was determined by turning the tube on its side. An endotoxin standard (Difco), suitably diluted, was used to determine endotoxin concentration at the end point.

To remove inhibitors that interfere with the endotoxin assay, homogenates were subjected to pH adjustments (15). Tissue samples were homogenized in pyrogen-free water (1:1, wt/vol).

Limulus amebocyte lysate was prepared by the method of Reinhold and Fine (15). Female Limulus
polyphemus were collected locally and maintained at 18 to 20°C in tanks of running seawater. Blood from the ventral cardiac sinus was placed in a fresh, cold solution of 0.125% N ethylmaleimide in 3% saline adjusted to pH 7.4 with tris(hydroxymethyl)aminomethane buffer. The amebocytes were collected by centrifugation and lysed, and the potency of the lysate was determined. Lysate was frozen for long-term storage or kept at 4°C and periodically retitrated against the standard.

RESULTS

Levels of staphylococcal nuclease in mice infected with S. aureus. Table 1 shows the distribution of staphylococci and their nuclease in organs of mice sacrificed shortly before animals in control observation groups commenced dying. The hepatic and renal levels of nuclease were similar to those reported previously (6), but were somewhat higher. Although bacterial counts were greater in the liver, nuclease activity was higher in the kidney; these ratios were consistently observed in repeated experiments.

Levels of endotoxin in mice infected with K. pneumoniae. Over 99% of the measured endotoxin in infected animals and in those injected with endotoxin was in the liver, as expected (Table 1). Viable counts of Klebsiella, however, were considerably higher in the kidney.

For the two bacterial species, then, the distribution of bacteria and bacterial products differed. However, the presence of staphylococcal nuclease activity in itself demonstrates that growth of the pathogen took place, since we have previously shown (6) that a killed-cell injection does not yield measurable activity. The growth of K. pneumoniae, as evidenced by endotoxin synthesis, also took place, for whereas the injection of killed cells produced pulmonary and cardiac levels of endotoxin comparable to those produced by viable cells, the endotoxin level in the liver was more than 1,200-fold greater with viable cells.

Levels of ammonia and urea in mice infected with S. aureus. Blood urea levels were normal in mice infected with Staphylococcus (Table 2). Similarly, in the absence of convulsions, blood and brain levels of ammonia were not altered in animals infected with S. aureus. In mice undergoing the terminal convulsive period, however, brain ammonia levels were increased.

Levels of ammonia and urea in mice infected with K. pneumoniae. Klebsiella injection resulted in uremia (Table 2). Furthermore, levels of brain and peripheral blood ammonia were elevated in mice infected with this organism.

The time course of ammoniagenesis was observed in animals receiving injections of Klebsiella causing death in either 4 or 24 h. Before any deaths, blood ammonia had increased by 50 to 150% in animals injected with bacteria above controls receiving saline (Fig. 1). Animals surviving longer than the mean time in the shorter-term survival experiments (240 min) had blood levels higher than animals sampled during the interval when most deaths were occurring. Similar survivors in the longer-term experiments (beyond 24 h) had blood ammonia.

Table 1. Distribution of colony-forming units (CFU), nuclease, and endotoxin in the organs of mice injected with Staphylococcus aureus or Klebsiella pneumoniae, killed cells of K. pneumoniae, or endotoxin

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Injection</th>
<th>S. aureus</th>
<th>K. pneumoniae</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Viable count (10⁷ CFU/g)</td>
<td>Nuclease activity (μg of DNA released/μl)</td>
<td>Viable count (10⁷ CFU/g)</td>
</tr>
<tr>
<td>Liver</td>
<td>5.7</td>
<td>52.3 ± 0.7²</td>
<td>38</td>
</tr>
<tr>
<td>Kidney</td>
<td>2.9</td>
<td>72.7 ± 17</td>
<td>150</td>
</tr>
<tr>
<td>Heart</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td>15</td>
<td>25</td>
<td>50</td>
</tr>
<tr>
<td>Brain</td>
<td>0.12</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

* Tissues from five animals that had been sacrificed shortly before deaths began in an observation group (200 min) were pooled.

Each mouse received an intraperitoneal injection of 10 mg of endotoxin (Salmonella typhosa). Animals for analysis were sacrificed at 6 h; deaths began to occur in injected animals in 10 h.

Determined from the ratio (nanograms of endotoxin standard at end point)/(gram of tissue at end point).

Mean and standard deviation of assay. The liver and kidney values are significantly different at P ≤ 0.05.
levels comparable to those of the controls.

When animals were injected with 10 mg of endotoxin, changes in blood ammonia occurred corresponding closely to those observed in the shorter-term experiments with *Klebsiella*.

**Sources of ammonia in *Klebsiella*-treated animals.** Ammonia in the *Klebsiella*- or endotoxin-treated animals did not arise from a failure of the host's urea cycle since serum and liver arginase activity in treated animals was not significantly different from that in controls, nor did liver arginine levels differ. Only in the longer-term experiments was serum arginase elevated in animals surviving after most deaths had occurred.

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**Table 2. Ammonia and urea levels in mice moribund with either *Klebsiella pneumoniae* or *Staphylococcus aureus***

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Urea (mg/100 ml)</th>
<th>Blood ammonia (µg/100 ml)</th>
<th>Brain ammonia (µg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Before convulsion</td>
</tr>
<tr>
<td>Saline</td>
<td>25.1 ± 1.5b</td>
<td>105.8 ± 22.6</td>
<td>13.5 ± 3.7</td>
</tr>
<tr>
<td><em>K. pneumoniae</em></td>
<td>53.2 ± 8.8c</td>
<td>169.6 ± 22.6</td>
<td>18.5 ± 5.1c</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>23.7 ± 2.6</td>
<td>106.0 ± 18.7</td>
<td>15.8 ± 4.3</td>
</tr>
</tbody>
</table>

* Inferred mice go through a terminal convulsion period 2 to 3 min before death.
* Sample mean and standard deviation of groups of mice containing at least 20 animals.
* Sample mean differs significantly (*P* ≤ 0.05) from the control.

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**Table 3. Effect of arginine or ornithine treatment on survival times and blood ammonia levels of mice infected with *Klebsiella pneumoniae***

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Survival time (min)</th>
<th>Blood ammonia (µg/100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>225.6 ± 17.9b</td>
<td>223.3 ± 75.7</td>
</tr>
<tr>
<td>A</td>
<td>254.8 ± 21.9</td>
<td>261.8 ± 134.2</td>
</tr>
<tr>
<td>B</td>
<td>329.8 ± 44.9c</td>
<td>116.2 ± 93.6c</td>
</tr>
<tr>
<td>Ornithine</td>
<td>275.4 ± 15.0b</td>
<td>210.0 ± 88.4</td>
</tr>
</tbody>
</table>

* Saline group A was compared with arginine and saline group B was compared with ornithine.
* Sample mean and standard deviation of groups containing not less than 10 mice. Values were obtained shortly before death.
* One hundred milligrams per mouse administered intraperitoneally in 1.0 ml of physiological saline.
* Significantly different from saline-treated group (*P* ≤ 0.05).

Evidence for an active urea cycle in the host was also obtained when *Klebsiella*-challenged animals received injections of arginine or ornithine immediately after bacterial inoculation (Table 3). Ornithine reduced ammonia concentration to nearly normal levels, significantly extending survival. Arginine did not lower the ammonia level, as expected, since it already contained a urea moiety. It did, however, extend survival times, but to a lesser degree than did ornithine.

Another likely source of the ammonia was the gastrointestinal tract since intestinal bleeding or necrosis would place ammoniogenic substrate in the tract. Histological examination, however, showed no difference between the tracts of control and treated animals. The effects of catecholamines is inferred from the intestinal constriction and inhibition of gastric emptying (Table 4), but blocking these effects with phentolamine did not lower blood ammonia levels.

Cecal pH was 0.4 units more alkaline in affected animals, and increased intestinal alkalinity facilitates diffusion of ammonia into the blood (13). If the uremia in *Klebsiella*-treated...
animals resulted in more urea entering the intestine, then this material would serve as an ammonia-ergic substrate for the intestinal microflora, cause the observed alkaline shift, and produce the observed blood levels of ammonia.

Germfree animals were injected with *K. pneumoniae* in the manner producing shorter survival. These animals died in about the same length of time as conventional animals but showed no significant elevation in blood ammonia, although the average ammonia level seemed somewhat higher than in control animals receiving saline (Table 5). The *Klebsiella*-treated germfree animals did, however, show uremia with a 37% increase in blood urea above control animals.

Germfree animals maintained under conventional laboratory conditions for 2 weeks had a normal gut morphology indicative of bacterial colonization. These mice exhibited typical elevations of ammonia in the blood (195.0 ± 50 μg/100 ml) when challenged with *K. pneumoniae*.

**DISCUSSION**

Although *S. aureus* cells were demonstrable in the kidneys of injected animals and the concurrent presence of nuclease was good presumptive evidence for the presence of the bacterium’s other extracellular enzymes and toxins, no uremia was demonstrable in the shorter-survival treatment. Mukherjee et al. (12) did note excess formation of urea in *S. aureus*-injected mice, apparently due to protein degradation. The difference may be due to differences in bacterial strains used.

*K. pneumoniae* cells and endotoxin were also demonstrable in the kidneys of injected ani-

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**Table 4. Gastric emptying in mice injected with Staphylococcus aureus or Klebsiella pneumoniae**

<table>
<thead>
<tr>
<th>Injection</th>
<th>Percent marker retention&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>40.5 ± 4.9&lt;sup&gt;g&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>73.1 ± 4.6&lt;sup&gt;g&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>K. pneumoniae</em></td>
<td>100.2 ± 8.3&lt;sup&gt;g&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>K. pneumoniae</em> plus 100 μg of phentolamine</td>
<td>69.5 ± 15.2&lt;sup&gt;a, d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mice received phenol red solution 90 min postinfection. Stomachs were sampled 60 min later.
<sup>b</sup> Sample mean and standard deviation of groups containing at least nine animals.
<sup>c</sup> Sample differs significantly from saline group (*P* ≤ 0.05).
<sup>d</sup> The sample is significantly different from the *K. pneumoniae* group (*P* ≤ 0.05). Phentolamine was administered 20 min after the bacterial inoculation.

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**Table 5. Blood ammonia levels in conventional and germfree mice injected with Klebsiella pneumoniae**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Conventional (μg of NH₃⁺/100 ml)</th>
<th>Germfree (μg of NH₃⁺/100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>105.8 ± 22.6&lt;sup&gt;g&lt;/sup&gt;</td>
<td>106.5 ± 19.5</td>
</tr>
<tr>
<td><em>K. pneumoniae</em></td>
<td>169.6 ± 22.6&lt;sup&gt;g&lt;/sup&gt;</td>
<td>121.7 ± 17.6</td>
</tr>
</tbody>
</table>

<sup>a</sup> Sample mean plus standard deviation of groups containing not less than 20 animals. Analysis made shortly before deaths began in an observation group.
<sup>b</sup> Significantly different from saline-treated animals (*P* ≤ 0.05).

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The bulk of the ammonia appears to arise from the action of the intestinal microflora upon increased ammoniagenic substrate, most probably urea, as shown by the experiments with germfree mice. Ureolytic activity of *K. pneumoniae* itself may also contribute to the increase in blood ammonia. Based on the results obtained with germfree mice, however, this possibility appears unlikely. Although increased blood urea was available to the injected *Klebsiella* as a result of uremia, the increase in blood ammonia in germfree animals was not statistically significant, thus indicating that this contribution must be small. Furthermore, endotoxin by itself was capable of producing comparable increases in blood ammonia.

The toxicity of ammonia, particularly to the brain (4), is well recognized in certain noninfectious diseases (16, 18, 19), but its toxic contribution in bacterial disease has not been studied. It seems to be a factor in the lethal events occurring in the shorter-term survival experiments, since ornithine both lowered the level of blood ammonia in these experiments and extended the survival time. The contribution of ammonia...
to toxicity in the longer-term survival experiments seems to be less direct: its rate of increase is slower and the blood concentration of ammonia is about 30% less in animals at the outset of the interval in which most animals die than it is at the outset of the same interval in the shorter-term experiments.

LITERATURE CITED


