Hepatic Clearance of Salmonella typhimurium in Silica-Treated Mice

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Scanning electron microscopy demonstrates that crystalline silica destroys liver Kupffer cells but has no other obvious deleterious effects on the liver. Silica-treated livers still retain the ability to trap large numbers of bacteria perfused through the portal vein even though the rate of clearance is well below normal. In vivo, silica treatment decreases the rate of bacterial clearance from the blood, alters the in vivo organ distribution of cleared bacteria, and decreases the mean lethal dose of Salmonella typhimurium over 100-fold. Cumulatively, the data indicate that silica treatment enhances susceptibility to gram-negative infection, probably by destruction of macrophages.

Crystalline silica is a specific macrophage toxin (1, 2, 3, 9, 14, 17). It enhances susceptibility of experimental animals to certain viral (16, 22, 29) and parasitic (15) infections as well as to Mycobacterium tuberculosis (7, 26, 27). Silica also potentiates growth of M. tuberculosis in macrophage culture (4). The effect of silica treatment in gram-negative infections has not been determined.

This study evaluates the effect of crystalline silica on both in vitro and in vivo parameters of host defenses against gram-negative infection. In vitro analysis includes an evaluation of the ability of the perfused liver to trap Salmonella typhimurium in a single pass. Our hypothesis is that destruction of macrophages should reduce trapping but not eliminate it, since a previous report from our laboratory (18) clearly demonstrates extensive trapping of bacteria in liver sinusoids but outside Kupffer cells. A second objective includes determining the biological consequences of macrophage destruction on the clearance and killing of S. typhimurium in vivo and its implication for host survival against gram-negative disease.

MATERIALS AND METHODS

Animals. Spartan (HA/ICR) female mice (Haslett, Mich.) weighing 18 to 25 g were used in all experiments. Animals were kept under standard laboratory conditions. Water and Purina laboratory chow were available ad libitum.

Bacteria. S. typhimurium strain SR-11 was used in all investigations. Cultures (18 to 24 h) grown in brain heart infusion broth were centrifuged at 8,000 × g for 15 min. The bacteria were suspended in either M-199 (GIBCO, Grand Island, N.Y.) or sterile saline. All injections of bacteria were by the intravenous route.

Silica. Three types of crystalline silica were evaluated. Dörenturp silica, particle size 5 μm or less (DQ12) (21), was kindly supplied by Gustavo Cudkowicz, Department of Pathology, State University of New York at Buffalo, N.Y. Crystalline silica no. 03741 was obtained from Spex Industries, Metuchen, N.J., and particle sizes of 5 μm or less were prepared by the Cummings sedimentation technique (10). A third silica preparation, 5-μm Min-U-Sil silica (Whittaker, Clark and Daniels, South Plainsfield, N.J.), was also tested. All preparations were autoclaved in powder form and then suspended in sterile saline. Before injection, the suspensions were exposed to ultrasonic vibration in a Bronsonic ultrasonic cleaner (no. B220, Branson Instruments Co., Sketgon, Conn.) to suspend the silica evenly. Various doses were injected over a 3-day period (Table 1), and mice were used for experiments on day 4, unless otherwise noted. All injections of silica were by the intravenous route.

Carbon clearance tests performed by the methods of Biozzi et al. (6) were used to determine the extent of reticuloendothelial system blockade by various silica preparations. Five milligrams of colloidal carbon (Pelikan carbon suspension C11/143/1, Gunther Wagner, Hanover, Germany) was injected into mice. They were bled from the retro-orbital plexus at 2 and 15 min. Blood (0.05 ml) was lyzed in 4.0 ml of 0.1% Na2CO3. The concentration of carbon was determined photometrically in an Hitachi Perkin-Elmer spectrophotometer (Coleman 111), with tungsten light at a wavelength of 650 nm. The phagocytic index, K, was determined by the equation \[ K = \frac{\log C_1 - \log C_2}{t_2 - t_1} \], where \( C_1 \) and \( C_2 \) represent the blood colloid concentration at time 1 \( (t_1) \) and time 2 \( (t_2) \), respectively. The clearance of carbon from the blood was also expressed as biological half-life \( (t_1) \) according to the formula \( t_1 = 0.693/K \). In normal mice, the phagocytic index was 0.056, which is equivalent to a biological half-life of 5.38 min. (Table
Table 1. Effect of various silica preparations on carbon clearance in mice*

<table>
<thead>
<tr>
<th>Type of silica</th>
<th>Phagocytic index</th>
<th>Biological half-life (min)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.056</td>
<td>5.38</td>
<td>0.001</td>
</tr>
<tr>
<td>Dörenturp</td>
<td>0.020</td>
<td>15.15</td>
<td>0.001</td>
</tr>
<tr>
<td>Spec</td>
<td>0.050</td>
<td>6.02</td>
<td>NS*</td>
</tr>
<tr>
<td>Min-U-Sil (5 μm)</td>
<td>0.068</td>
<td>4.43</td>
<td>NS*</td>
</tr>
</tbody>
</table>

* Average value from at least eight separate experimental determinations.

1) In mice receiving a total of 10 mg of Dörenturp silica (DQ12) over 3 days, the phagocytic index (K) was significantly decreased, to 0.020, thereby increasing the t1/2 to 15.15 min. No other silica preparation depressed carbon clearance, and, hence, only DQ12 silica was used in succeeding experiments.

Mouse liver perfusion. Mouse liver perfusion was performed according to the method of Moon et al. (18).

Scanning electron microscopy of mouse liver. After liver perfusion, 5 to 10 ml of glutaraldehyde solution (2.5% glutaraldehyde in 0.2 M phosphate buffer at pH 7.4) was infused. Fixed livers were excised, cut into small blocks, and dehydrated in sequential 15-min steps with 30, 50, 70, 90, and 100% ethanol. The blocks were allowed to stand overnight at 4°C in a fresh change of 100% ethanol. The dehydrated blocks were cryofractured by immersing them in liquid nitrogen for 1 min. The tissue was fractured with a precooled single-edge razor blade held by locking forceps. The fractured tissue was placed in metal baskets under liquid nitrogen and transferred to the critical-point dryer. The tissue was dried in an Omar SPC 900/EX critical-point dryer with CO2 as the carrier gas. The dried specimens were mounted on stubs with double-stick Scotch tape, and the stub edge was painted with television Tube Koat (G. C. Electronics) to prevent charging. The specimens were coated with gold (200 to 300 A) by the EMS-41 Minicoater (Film Vac. Inc., Englewood, N.J.) and viewed in an AMR-900 scanning electron microscope. Micrographs were made with Kodak positive/negative (P/N) film.

Distribution and survival of S. typhimurium in mice. Mice injected intravenously with 1.0 x 10^9 S. typhimurium were killed after 15 or 30 min. The liver and spleen were removed and homogenized separately in 9 ml of sterile deionized water with a Teflon and glass homogenizer. The carcass, excluding the stomach, intestinal tract, skin, paws, and tail, was homogenized in 99 ml of deionized water in a Waring blender for 3 min. Quantitative pour plates of the homogenates were prepared.

WBC and differential blood counts. Leukocyte (WBC) counts were determined by bleeding mice from a lateral tail vein. The drawn blood was mixed with 2% acetic acid solution, and hemocytometer counts were made. Differential counts were determined from blood smears stained with Wright stain. Blood counts were done 24 h after each silica injection.

S. typhimurium infection in silica-treated mice. Groups of mice were treated with a total of 10 mg of DQ12 silica. One group served as a control, and the others were injected intravenously with doses of S. typhimurium ranging from 2 x 10^6 to 2 x 10^9. Survival was observed for 2 weeks. The mean lethal dose (LD50) for normal and silica-treated mice was determined by the method of Reed and Muench (20).

Statistics. Where appropriate, data were evaluated by the White rank order method (28).

RESULTS

Scanning electron microscopy of livers from normal and silica-treated mice. Kupffer cells of normal livers reside in the lumen of the sinusoids and possess many membranous cytoplasmic appendages that anchor the body of the cell to the sinusoidal wall (Fig. 1A). Kupffer cells of silica-treated livers were not as spread out as normal Kupffer cells. Many were rounded up (Fig. 1B and D) and engorged with silica (Fig. 1C and E), appearing to have part or all of their outer plasma membrane destroyed. Fewer membrane appendages were apparent (Fig. 1C through E), and attachment to walls of the sinusoids was decreased (Fig. 1B through E). The crystalline-like material seen in many of the Kupffer cells looks similar to Dörenturp silica (Fig. 1F). No detrimental effects of silica on the portal veins, hepatic veins, central veins, sinusoids, and parenchymal cells of the liver were observed.

Trapping of S. typhimurium by perfused livers of normal and silica-treated mice. In vitro normal livers trapped an average of 63.5% of a 1 x 10^10 to 2 x 10^10 dose of S. typhimurium on a single pass, with an average of 42.3% recovered in the effluent (Table 2). Silica-treated livers trapped only 31.3% (P < 0.001) with 65.9% recovered in the effluent (P < 0.05). Scanning electron micrographs of silica-treated livers perfused with bacteria revealed extensive sinusoidal trapping of the organisms (Fig. 2).

Clearance, distribution, and survival of S. typhimurium in vivo in normal and silica-treated mice. Normal and silica-treated mice were injected with 1 x 10^9 to 2 x 10^9 S. typhimurium, and the phagocytic index for clearance of bacteria was determined in a manner similar to carbon clearance analysis. In normal mice the phagocytic index was 0.092 (t1/2 = 32.7), and in silica-treated mice it was 0.058 (t1/2 =
5.20) (Fig. 3). The differences were statistically significant at all time points ($P < 0.001$). This decrease in clearance rate was reflected in an altered distribution of bacteria in vivo (Table 3). In normal mice, after 15 min, 50.5% of the bacteria were recovered as viable cells, with 40.2% recovered from the liver, 7.5% from the carcass, and 2.8% from the spleen. Statistically similar total numbers of bacteria were recovered from silica-treated mice. In this instance, however, the liver had only 12.7% of the bacteria, the spleen had 6.9%, and the carcass...
had 23.5%. After 30 min, 18.5% of the bacteria was recovered from the liver, spleen, and carcass in both normal and silica-treated mice. The altered distribution of viable bacteria was similar to that observed at 15 min. Our failure to recover greater numbers of bacteria in the silica-treated mice after 15 and 30 min, despite extensive damage to liver macrophage and decreased clearance rates, suggested that silica may have enhanced alternate bactericidal capacities of the host simultaneous with Kupffer cell destruction. Polymorphonuclear leukocytes (PMN) are logical candidates for such change. Table 4 shows the effect of silica treatment on total WBC, PMN, and lymphocyte counts during the course of silica injections. Twenty-four hours after the first injection of 3 mg of silica, total WBC counts decreased significantly. Differential counts showed approximately 31% PMN and 69% lymphocytes. Only an occasional monocyte was observed. After the second injection of silica (3 mg), total WBC was elevated above controls, and, by 24 h after the final injection of silica (4 mg), the total WBC count had more than doubled. The percentages of PMN and lymphocytes remained relatively constant throughout the silica treatment. The net effect, however, was over a threefold increase in the absolute numbers of PMN.

**Susceptibility of silica-treated mice to S. typhimurium infection.** Normal and silica-treated mice were injected with $2 \times 10^8$ to $2 \times 10^9$ bacteria, and survival was monitored for 14 days. Representative data are shown in Fig. 4. With $2 \times 10^9$ organisms, 60% of normal mice survived compared with only 20% of silica-treated mice (Fig. 4A). With $2 \times 10^8$ bacteria, 17% of normal mice survived compared with no survivors in the silica-treated group (Fig. 4B). Silica-treated mice also died sooner. By chi-square analysis, the rate of death was significantly different after the fourth observation day at the $2 \times 10^8$ dose. The LD$_{50}$ for normal mice was $3.9 \times 10^4$ organisms, and for silica-treated mice the LD$_{50}$ was $3 \times 10^4$ organisms, indicating at least a 100-fold increase in susceptibility to infection in the silica-treated animals. No deaths occurred in mice given silica alone.

**DISCUSSION**

Moon et al. (18) recently demonstrated that bacterial clearance by the perfused liver is not synonymous with phagocytosis by Kupffer cells. *S. typhimurium* is trapped in the liver sinusoids, giving a "log-jam" appearance. No bacterial killing occurred when blood was omitted from the perfusion media. Fifty-percent killing occurred when blood was added to the perfusion media. No killing occurred when bacteria were incubated with whole blood alone.

The present data suggest that physical factors may be involved in bacterial trapping by the perfused liver. Scanning electron micrographs clearly demonstrate that DQ12 silica caused damage and destruction of Kupffer cells (Fig. 1) but has no other apparent histotoxic effects on the liver. No normal Kupffer cells, as in Fig. 1A, were seen during extensive scanning electron microscopy examination of silica-treated livers. The destruction of Kupffer cells significantly decreased trapping of perfused *S. typhimurium* (Table 2), but, still, 31.3% of the bacteria were trapped. These data indicate that for maximal bacterial trapping, Kupffer cells must be intact and viable. However, livers devoid of most Kupffer cells can still trap large numbers of bacteria in the sinusoids (Fig. 2), reinforcing the concept that bacterial trapping also involves non-Kupffer cell components of the liver. The physical trapping by the liver may involve either surface receptors on endothelial cells or may simply be due to the sinusous nature of the liver sinusoids.

Our results further demonstrate that the perfused liver model is an accurate indicator of bacterial clearance ability of this organ in vivo (18). Perfused, silica-treated livers trapped a statistically lower number of bacteria than normal livers (Table 2). These results directly correlate to the data obtained in vivo on the distribution of viable bacteria after intravenous injection into mice (Table 3). The increase in number of bacteria in the effluent of the perfused liver reflects the increased number of bacteria recovered in the carcass of silica-treated mice. No bacterial killing occurred in perfused livers from either normal or silica-treated mice. At all times, 100% of the *S. typhimurium* was accounted for by total viable cells found in the
Fig. 2. Scanning electron micrographs of silica-treated livers perfused with $1 \times 10^{10}$ to $2 \times 10^{10}$ S. typhimurium. (A) Bacteria trapped in sinusoid (note Kupffer cell lysed by silica (S) and rounded-up Kupffer cell (K)) ($\times 2,600$). (B) Bacteria trapped in sinusoids ($\times 6,500$). (C) Bacteria trapped in sinusoid with rounded-up Kupffer cell at sinusoidal junction (K) ($\times 5,800$).
The recovery of S. typhimurium 15 and 30 min after intravenous injection was essentially the same in normal and silica-treated mice (Table 3). This result was unexpected, since we postulated that reduced clearance (Fig. 3) would enhance the survival time of bacteria in vivo in silica-treated mice. One possible explanation for these results may involve the absolute numbers of PMN in the circulation after silica injection. Table 4 shows that total PMN tripled after silica injection, suggesting that this leukocytosis may explain the similar rate of whole animal bactericidal activity despite the destruction of many Kupffer cells. Still, mice were sensitized to the infection and, in the end, still succumbed to the infection. These data suggest that PMN is not the major defense mechanism against S. typhimurium infections, an interpretation consistent with studies by Collins (8), which say that liver phagocytic cells and blood macrophages play the ultimate role in host phagocytic defense to Salmonella infection.

Crystalline silica has a profound effect on susceptibility of mice to S. typhimurium infection (Fig. 4). The LD₅₀ for normal mice was 3.9 × 10⁴ organisms, and for silica-treated mice the LD₅₀ was 3 × 10⁵ organisms. This difference represents over a 100-fold increase in susceptibility and is the first demonstration of the ability of silica to enhance susceptibility to gram-negative infection.

**ACKNOWLEDGMENTS**

We wish to acknowledge the excellent technical assistance offered by Gary Hooper and the staff of the Electron Optics Laboratory of Michigan State University in the preparation of the scanning electron micrographs and the assistance of Stuart Pankarte in preparation of the final plates.

**Table 4. Total and differential cell counts of mice before and during the course of silica treatment**

<table>
<thead>
<tr>
<th>Silica injections</th>
<th>Total WBC</th>
<th>Differential (%)</th>
<th>PMN</th>
<th>Lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>9,000 ± 1,497⁺</td>
<td>17.8 (1,602)⁺</td>
<td>82.2 (7,398)⁺</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>6,050 ± 2,072</td>
<td>31.2 (1,887)</td>
<td>68.8 (4,163)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>16,075 ± 5,910</td>
<td>14.6 (2,346)</td>
<td>85.4 (13,729)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>98,250 ± 6,853⁺</td>
<td>21.2 (5,555)</td>
<td>78.8 (20,885)</td>
<td></td>
</tr>
</tbody>
</table>

⁺ Cell counts done 24 h after each silica injection.
⁺⁺ Average ± standard deviation of 4 to 6 experimental determinations.
⁺⁺⁺ Approximate numbers of cells calculated from total WBC. Blood monocytes were present as <1% of the WBC and were not included in the differential tabulations.
⁺⁺⁺⁺ Versus control P < 0.001.

**Table 3. Survival of S. typhimurium 15 and 30 min after intravenous injection into normal and silica-treated mice**

<table>
<thead>
<tr>
<th>Area of clearance</th>
<th>Recovery (%) after:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15 min</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
</tr>
<tr>
<td>Liver</td>
<td>40.2 ± 5.8</td>
</tr>
<tr>
<td>Spleen</td>
<td>2.8 ± 1.8</td>
</tr>
<tr>
<td>Carcass</td>
<td>7.5 ± 1.4</td>
</tr>
<tr>
<td>Total</td>
<td>50.5 ± 7.8</td>
</tr>
</tbody>
</table>

⁺⁺⁺⁺ Average value from at least six separate experimental determinations.
⁺⁺⁺⁺⁺⁺ P < 0.001.
⁺⁺⁺⁺⁺⁺⁺⁺ Not statistically significant.
FIG. 4. Susceptibility of normal and silica-treated mice to infection by $2 \times 10^3$ (4A) and $2 \times 10^4$ (4B) S. typhimurium. Normal control (■); 10 mg of silica (●).

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LITERATURE CITED


