Effects of a perfluorochemical emulsion on the fate of circulating Pseudomonas aeruginosa

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Brain, Joseph D., Ramon M. Molina, Malcolm M. DeCamp, and Angeline E. Warner. Effects of a perfluorochemical emulsion on the fate of circulating Pseudomonas aeruginosa. Am. J. Physiol. 276 (Lung Cell. Mol. Physiol. 20): L1037–L1045, 1999.—Because mononuclear phagocytes take up perfluorochemical emulsions (PFCE), we examined how prior treatment with PFCE affects the fate of circulating bacteria. Rats were preinjected with three daily intravenous injections of PFCE (2.0 ml/100 g) containing 12.5% (vol/vol) of a 4:1 mixture of F-dimethyl adamantane and F-trimethylbicyclo-nonane, 2.5% (wt/vol) Pluronic F-68 as the emulsifying agent, and 3% (wt/vol) hydroxyethyl starch as the oncotic agent. Pseudomonas aeruginosa or Staphylococcus aureus were injected 4 h after the third PFCE injection. PFCE pretreatment decreased the rate and extent of vascular clearance of P. aeruginosa, with decreased uptake by the liver. Importantly, there were significant decreases in killing of P. aeruginosa in the liver, lungs, spleen, and kidneys of PFCE animals. PFCE did not alter the clearance of S. aureus from the circulation. However, hepatic uptake was reduced, with concomitant increases in lung and kidney uptake. Ultrastructure of Kupffer cells revealed PFCE inclusions and extensive vacuolization. These experiments demonstrate that the clearance kinetics and organ distribution of circulating P. aeruginosa and their subsequent killing are altered by PFCE. Diminished hepatic phagocyte function leads to a decrease in vascular clearance of circulating bacteria, increased uptake in other reticuloendothelial organs, and decreased bactericidal activity versus P. aeruginosa.

There are indications that there can be depression in their phagocytic function.

We have previously demonstrated significant changes in the ultrastructure of rat Kupffer cells after intravenous injection of PFCE (33). These hepatic macrophages contained abundant vacuoles, many apparently filled with particles of PFCE. Associated with these morphological changes was a reduction in the motion of particle-containing phagosomes and phagolysosomes (1, 33) as detected with magnetometric methods (4, 11). We also observed decreased rates of clearance of inert insoluble $^{57}$Co$_3$O$_4$ particles from the blood and altered organ distribution of these particles in PFCE-injected rats (6). We found that PFCE administration decreased liver uptake, with compensatory increases of cobalt oxide particle uptake in the lungs and spleen.

The primary goal of this study was to examine the effects of PFCE on the function of the components of the MPS with access to the circulating blood in regard to the fate of circulating bacteria. Our goal was to evaluate the clearance kinetics, organ distribution, and intracellular bacterial killing in control rats and in those given PFCE.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (mean weight of 243 ± 8 g) were purchased from Charles River Laboratories (Wilmington, MA). All animals were allowed to acclimate for 1 wk after arrival and were given free access to food and water. One group of rats was preinjected with three daily tail vein injections of PFCE at 2 ml/100 g body wt. This protocol was chosen on the basis of a calculated blood volume addition of ~25%. Age- and weight-matched controls were similarly injected with physiological saline. The lungs and other major organs were grossly and histologically free of pneumonia and parasites postmortem.

Blood substitute (PFCE). The PFCE was prepared by sonicating the components in a CO$_2$ atmosphere to prevent fluoride ion production (12). The emulsion contained 12.5% (vol/vol) of a 4:1 mixture of F-dimethyl adamantane and F-trimethylbicyclo-nonane (Suntech, Marcus Hook, PA). Pluronic F-68 (BASF Wyandotte Chemical, Wyandotte, MI) was used as the emulsifying agent at a final concentration of 2.5% (wt/vol). Hydroxyethyl starch (3%, wt/vol, in the final emulsion) served as the oncotic agent. The aqueous phase of the PFCE was a modified Ringer bicarbonate solution. Osmolarity of the PFCE was 290 ± 2 mosmol/l, and the pH was adjusted to 7.45. The emulsion was filtered through a 0.45-µm cellulose acetate membrane filter (Millipore, Bedford, MA) and was stored at 4°C until needed. Perfluorochemical particle diameters in the emulsion ranged from 0.05 to 0.4 µm, with an average of ~0.2 µm as estimated by electron microscopy. Filtration of the emulsion through cellulose acetate filter removed any perfluorochemical particles larger than

Blood substitutes have been developed for various reasons. Patients who are Jehovah's Witnesses refuse to accept human blood because of their religious beliefs. In some emergencies, adequate supplies of human blood may be lacking. Finally, there are persistent concerns about viral contamination (e.g., hepatitis and human immunodeficiency virus-1) of human blood products. For almost two decades, perfluorochemical emulsions (PFCE) have been studied as alternatives to blood substitutes.
0.45 μm. The endotoxin contamination of the preparation was indirectly evaluated by a pyrogen test, and all preparations were certified to be pyrogen free.

Bacteria. *Pseudomonas aeruginosa* (strain P220; courtesy Dr. J. James Pennington) growing exponentially in trypticase soy broth were washed two times in sterile saline and resuspended at an optical density (620 nm) of 1.2 in sterile saline. Serial aureus (courtesy of Dr. Richard Rose) were similarly grown and washed and suspended in sterile saline.

When radioactive bacteria were used, weighed samples of bacteria, were lysed. Next, 1-ml aliquots of each were plated in trypticase soy agar using standard pour-plate techniques.

Analysis of bacterial content. Rats used for this analysis were briefly anesthetized with vaporized halothane and were injected intravenously with the bacterial suspension. At 1 and 4 h postinjection, the rats were humanely killed. The thorax and abdomen were aseptically opened; next, the liver, spleen, kidneys, and right lungs were removed. The left lung was fixed for examination by light and electron microscopy. The liver, spleen, kidneys, and right lung were weighed, and one to four randomly chosen samples (0.5–1.0 g) were selected from the pool of blocks for each animal; thin sections were cut from these on a Sorvall MT 6000 ultramicrotome using a diamond knife, picked up on 200-mesh uncoated grids, and then stained with uranyl acetate and lead citrate. Electron micrographs were taken on a Philips 300 electron microscope. Sections 0.5–1.0 μm in thickness were cut with glass knives and stained with toluidine blue for light microscopic examination.

Statistical analyses. Student's t-test was used for comparison of means. Statistical significance was assumed at *P* < 0.05.

**RESULTS**

Kinetcs of vascular clearance of injected bacteria. The rates of disappearance of *P. aeruginosa* and *S. aureus* from the blood during the first hour after their intravenous injection are shown in Fig. 1. The rate of clearance of *P. aeruginosa* was considerably slowed by PFCE pretreatment (Fig. 1A). At all time points between 10 and 60 min, there was a significant difference between the amount of bacteria in the blood when the PFCE group was compared with the saline group (*P* < 0.05). One hour after injection of bacteria, nearly 100 times more bacteria remained in the blood of PFCE-treated rats than in control rats. In contrast, no significant difference in the clearance kinetics of *S. aureus* between the two groups of animals was observed (Fig. 1B). We also utilized a radioactive label to follow *S. aureus* clearance (data not shown). We found that the clearance half-time for the iron label appeared longer in PFCE-treated rats than in the corresponding controls (2.16 ± 0.23 vs. 1.67 ± 0.34 min, although *P* > 0.05). At times after 15 min, there was more radioactivity measured in the blood than would be predicted by the colony-forming unit counts. This may reflect a more rapid liberation of 59Fe from the ingested bacteria in the control rats.

Organ distribution of recovered bacteria. The distribution of recovered bacteria throughout the body is sum-
For both bacteria used, the hepatic uptake was significantly diminished by PFCE. For *P. aeruginosa* (Fig. 2A), the liver content decreased from 87.96 ± 5.48 to 68.96 ± 5.19% of the recovered dose. For *S. aureus* (Fig. 2B), the liver uptake decreased from 93.79 ± 1.2 to 81.74 ± 3.86%. Also consistent with Fig. 1A, the animals injected with *Pseudomonas* had significantly more viable organisms in the blood at the time of death. In the animals given *S. aureus*, the reduction in hepatic uptake was accompanied by significant increases in lung and kidney uptake. We observed alterations in the distribution of the 59Fe label of the *S. aureus* that were consistent with Fig. 2B. A significant decrease was seen in hepatic content, and significant increases were also seen in the pulmonary and renal contribution (data not shown). The percent of injected dose of *P. aeruginosa* recovered 1 h after intravenous injection was significantly higher in PFCE-injected rats than in saline control rats. For *P. aeruginosa*, 47.0 ± 14.0% of the injected dose was recovered in the PFCE-injected rats compared with only 14.4 ± 4.7% in saline controls. For *S. aureus*, the values were 35.4 ± 17.7 and 52.3 ± 14.4%, respectively.

**Bacterial Killing.** When we examined the bactericidal activity of the major organs that retained the injected *P. aeruginosa*, we observed a dramatic change induced by pretreatment with the artificial blood. PFCE caused a significant decrease in the microbicidal activity of all four of the organs studied (*P < 0.05*). The changes in the number of viable *P. aeruginosa* between 1 and 4 h (the times of death for the two cohorts) are shown in Fig. 3A. In the liver, spleen, lungs, and kidneys, the saline control animals were able to kill between 77 and 94% of the retained bacteria. In marked contrast, bacterial killing was significantly decreased in PFCE-
injected rats. Killing in the spleen was reduced from 79 to 16% during the 3-h observation period. The bactericidal activity in the liver, lungs, and kidneys was impaired to an even greater extent. In all of these three organs, significant growth of *P. aeruginosa* was observed. The number of colony-forming units increased by 58% in the liver, 136% in the lung, and 49% in the kidneys. However, no significant change in the killing of *S. aureus* was observed in PFCE-injected rats. As shown in Fig. 3B, both PFCE-treated and control animals eliminated the majority of ingested *S. aureus* in all of the organs examined.

### Table 1. Effects of PFCE on the liver and spleen

<table>
<thead>
<tr>
<th></th>
<th>PFCE</th>
<th>Saline</th>
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<tbody>
<tr>
<td>Body weight, g</td>
<td>238.61 ± 11.81</td>
<td>247.12 ± 12.01</td>
</tr>
<tr>
<td>Liver weight, g</td>
<td>13.46 ± 0.68</td>
<td>12.20 ± 0.58</td>
</tr>
<tr>
<td>Spleen weight, g</td>
<td>1.26 ± 0.08</td>
<td>0.64 ± 0.02*</td>
</tr>
<tr>
<td>Change in body weight, % of initial wt</td>
<td>2.99 ± 0.62</td>
<td>6.15 ± 0.67*</td>
</tr>
<tr>
<td>Liver, % of body wt</td>
<td>5.67 ± 0.13</td>
<td>4.97 ± 0.11*</td>
</tr>
<tr>
<td>Spleen, % of body wt</td>
<td>0.54 ± 0.03</td>
<td>0.27 ± 0.01*</td>
</tr>
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Values are means ± SE. PFCE, perfluorochemical emulsions. *P < 0.001, PFCE vs. saline.

Morphological changes caused by PFCE. The three daily intravenous injections of PFCE caused profound morphological changes that were evident at postmortem examination. The body weights of PFCE-injected rats were not different from those of the saline control. However, the increase in body weight over the 3-day period of the PFCE-injected rats was significantly lower than that in saline-injected controls (2.99 vs. 6.15% weight increase). The liver and spleen weights from the PFCE-injected animals expressed as percent of the whole body weight were higher than in saline controls (Table 1). The liver-to-body weight ratio was 13% higher in PFCE than in control animals, and the spleen-to-body weight ratio doubled. Also evident was the pallor of the organs from the PFCE-injected rats compared with that in the saline controls (Fig. 4).

Microscopic examination also revealed morphological changes in the liver induced by PFCE. As shown in Fig. 5, A and B, the liver was dramatically altered. Although no gross necrosis or inflammation was evident, the most striking change was the large number of foamy-appearing cells. Both Kupffer cells and hepatocytes contained large numbers of vacuoles, which probably represent ingested PFCE. Electron microscopy revealed ultrastructural changes consistent with these observations. Accumulation of PFCE within Kupffer cells was frequently observed. Hepatocytes and endothelial cells appeared relatively normal but had occasional clusters of PFCE-containing vacuoles. Kupffer cells, in contrast, were often distorted by large complex vacuoles of PFCE (Fig. 5B). As shown in Fig. 5B, the
hepatocytes appear normal except for occasional clusters of PFCE-containing vacuoles. The overall appearance of the pulmonary parenchyma was relatively normal (Fig. 5C). There was no evidence for an inflammatory response within alveolar spaces. Nevertheless, cells filled with intracellular PFCE particles were frequently seen. At the light microscopy level, it was difficult to distinguish intravascular monocytes from connective tissue macrophages. Occasionally, even alveolar macrophages with similar vacuoles were seen. Electron microscopy revealed the presence of PFCE inclusions within endothelial cells and margined monocytes (Fig. 5D).

Cellular uptake of bacteria. Ultrastructural examination of the lungs and liver revealed that phagocytic cells were primarily responsible for removing bacteria from the circulating blood. The characteristic appearance of *P. aeruginosa* in the liver and lungs 1 h after injection is shown in Fig. 6, A and B. Hepatic macrophages containing PFCE vacuoles were capable of ingesting *P. aeruginosa* (Fig. 6A). A quantitative assessment of the site of *P. aeruginosa* uptake in the liver from PFCE-treated animals revealed that 55% were within Kupffer cells, 2% were in polymorphonuclear cells (PMN), and 43% were in the capillary lumen either free in plasma or adherent to sinusoidal cells.
In the lungs, *P. aeruginosa* were seen far less frequently as is consistent with Fig. 2A. However, free bacteria were occasionally seen in the blood, an observation consistent with Fig. 1A, showing increased persistence of *P. aeruginosa* in the circulating blood. When ingested bacteria were seen, they were always seen within marginated phagocytic cells, usually monocytes. For example, Fig. 6B shows a large marginated mononuclear cell containing a bacterium.

The appearance of *S. aureus* in the liver and lungs 1 h postinjection is shown in Fig. 7. The findings are similar to those seen with *P. aeruginosa*. Intracellular bacteria were confined to phagocytic cells. In the liver, 85% of localized bacteria were found within Kupffer cells, with the remaining 15% found within PMN (Fig. 7A). Many of these cells also contained small and large vacuoles characteristic of PFCE-injected animals. In the lungs, we also observed participation of both PMN as well as circulating monocytes in bacterial localization (Fig. 7B). Many PMN were seen containing ingested bacteria. Generally, PMN had fewer PFCE vacuoles than did the monocytes.

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**Fig. 6.** Uptake of *P. aeruginosa* by phagocytic cells in liver and lungs after bacterial injection in PFCE-injected rats. A: in the liver, *Pseudomonas* (arrow) were found mainly within Kupffer cell. PFCE can be seen as distinct vesicles in phagolysosomes (P) 1 h after injection (magnification, ×6,500). B: in the lungs, 30 min after injection, *Pseudomonas* were seldom found in sections. Bacterial cells (arrows) free within the pulmonary capillary lumen and engulfed by a marginated mononuclear cell (M) can be seen (magnification, ×6,300).

**Fig. 7.** Uptake of *S. aureus* in liver and lungs 1 h after bacterial injection in PFCE-injected rats. The most typical cellular sites of uptake for each organ are shown. A: typical PFCE-laden Kupffer cell (K) can be seen with a bacterial cell (arrow) within a PFCE-containing phagolysosome (magnification, ×6,500). B: neutrophil (N) with engulfed bacterial cells (arrow) within a pulmonary capillary. There are numerous PFCE inclusions (arrowheads) in an adjacent endothelial cell (E) and in a circulating white blood cell (W) (magnification, ×6,500).
DISCUSSION

Side effects of PFCE. Our data show that injection of the PFCE in the blood may have a major impact on phagocytic cells, which have access to the circulating blood. This is consistent with several previous studies in vitro of commercially available PFCE (7, 17, 19). Our data clearly demonstrate that both circulating white blood cells as well as resident macrophages in such organs as the lungs and liver soon contain significant amounts of PFCE vacuoles. Particularly in mononuclear phagocytes, this results in the appearance of large vacuoles and compromised organelle motion. After injections of magnetic iron oxide particles and PFCE into rats, Weinstock et al. (33) used noninvasive magnetometric methods to study the effects of PFCE on phagocytic cells in the liver. The authors reported that magnetic relaxation, a measure of the spontaneous motion of phagosomes within cells, decreased in the livers of animals that received PFCE compared with those that received saline. Weinstock et al. also noted that the majority of iron particles and PFCE particles were within Kupffer cells. A similar decrease in phagosomal motion in Kupffer cells after PFCE and other lipid emulsion administration was reported later (1, 21).

These morphological and magnetometric changes induced by PFCE administration were also accompanied by functional changes. We have reported that PFCE are associated with decreased hepatic and increased pulmonary uptake of injected cobalt oxide particles (6). In the present study, P. aeruginosa were cleared more slowly from the blood, so that 100 times more viable bacteria were still present in the blood at 1 h compared with those in saline control animals. The organ distribution of retained viable organisms was also significantly altered. The hepatic contribution to vascular clearance was significantly decreased, resulting in the persistence of P. aeruginosa in the circulation. Because bactericidal activity of the liver was significantly decreased, that also increased the bacterial burden of other reticuloendothelial organs, including the lungs. Importantly, there was significant bac- terial growth in the lungs, kidneys, and liver of PFCE-injected rats during a 3-h period.

Surprisingly, in spite of these major changes in the way P. aeruginosa, a gram-negative bacteria, was handled, the kinetics of S. aureus (gram-positive) clearance and killing was essentially unchanged. This may reflect different mechanisms of bactericidal activity, such as differences in the relative role of PMN versus mononuclear cells. It could also reflect the slightly lower dose of S. aureus that was used in this study (7 × 10⁷ CFU/100 g vs. 20 × 10⁷ CFU/100 g). Perhaps there could be an impairment in the clearance and killing of S. aureus at higher injected doses of either PFCE or bacteria. Nevertheless, PFCE treatment with this protocol did alter the distribution of cleared S. aureus away from the major reticuloendothelial organ, the liver, shifting bacterial uptake to other organs such as the kidneys, spleen, and lungs.

What are some of the possible mechanisms that explain how PFCE administration changes the clearance, organ distribution, and killing of intravenously injected P. aeruginosa? There are at least four possible mechanisms. First, the phagocytic cells are clearly filled with perfluorochemical, and thus they may be “overloaded.” Previous phagocytic activity devoted to PFCE ingestion may have reduced the available number of bacterial receptors on the plasma membrane. In addition, the presence of PFCE may be a mechanical obstruction to organelle motion, ingestion, and killing. Intracellular killing involves careful orchestration of organelle motion. For example, phagosomes must be formed from plasma membrane, brought deep inside the cell; next, phagosome-lysosome fusion must take place. Second, these functional changes may reflect damage to mononuclear cells. Many have large vac- uoles with no visible internal structure. Rather than being simply coalesced ingested PFCE droplets, these large vacuoles may also reflect cellular damage. Third, there may be fibronectin depletion (23). This protein is important for reticuloendothelial system activity. Because Kupffer cells take up abundant PFCE droplets, the circulating fibronectin levels may be reduced. Fourth, hepatic bacterial clearance is also related to hepatic blood flow. Mechanical obstruction as well as cytokine responses might be involved. It is possible that blood flow to the liver is reduced because of the enlarged PFCE-loaded Kupffer cells, leading to the observed enlargement of the liver after multiple PFCE injections. Likewise, PFCE may cause injury and swelling of the endothelium. This probable increased vascular resistance in the liver might contribute to the longer rates of clearance and altered organ distribution.

Mechanisms and importance of pulmonary uptake. As noted earlier, reduced hepatic uptake was accompanied by compensatory increased uptake in other organs such as spleen, kidneys, and lungs. The dose of bacteria to which the lungs are subjected is an important determinant of lung injury. Previous work in this laboratory (5, 28–30, 32) has emphasized the importance of pulmonary intravascular macrophages (PIMs). Present in ruminants, as well as in other animals such as pigs, horses, and cats, these resident cells in lung capillaries avidly phagocytize both test particles such as gold colloid and iron oxide (27, 28) as well as more relevant materials such as P. aeruginosa (32). Could such cells be responsible for the increased pulmonary uptake observed here? In a comparative study of 13 animals (5), pulmonary uptake of tracer particles is minimal in normal rats compared with that in PIM-containing species such as sheep, calves, pigs, goats, and cats. Those data are consistent with the values obtained for control rats seen in this study. Moreover, extensive electron microscopic studies failed to show any “classic” PIMs in rats. Mature macrophages adherent to the underlying endothelium with characteristic junctional complexes were not observed in rats (27, 34). These junctional complexes involve the plasma membranes of the macrophage and the underlying endothelial cell coming within 10 nm of each other; electron-
dense material can also be seen enveloping both plasma membranes. No such structures were seen in either the control rat lungs or those treated with PFCE.

However, there are circumstances, like PFCE administration, when margination of leukocytes in lung capillaries may increase. There may be even circumstances in which PIMs can develop in non-PIM species (9, 31). Yet, little evidence for it was seen in this study. Instead, the bacteria were always contained in what appeared to be marginated mononuclear cells (probably circulating monocytes) as well as in PMN. These cells not only contained the bacteria but were also loaded with PFCE droplets in varying amounts. We do not know whether PFCE increased the likelihood of these cells marginating in the lung. Increases in cell size or stiffness could contribute. Alternatively, Kupffer cell failure might have led to increased ingestion of bacteria by circulating white blood cells. Next, even normal numbers of marginated white blood cells could give rise to the increased lung burdens of bacteria we observed. Labeling of white blood cells with quantitative measurements of the marginated pool in the lungs and other organs would be necessary to distinguish among these various possibilities.

Liver-lung relationships. Our data add weight to the hypothesis that diminished hepatic function can lead to increased pulmonary pathogen burden and may thus predispose to respiratory failure. This possibility has been raised in a slightly different context by a number of investigators such as Saba (23), who emphasized the possibility that excessive Kupffer cell activity would lead to fibronectin depletion and thus reduced phagocytic performance by Kupffer cells. In turn, this would lead to increased persistence of various pathogenic materials in the blood and thus increase the risk of pulmonary uptake and ultimately lung injury and adult respiratory distress syndrome. Our data do suggest that Kupffer cell function can modulate pulmonary burdens. The data presented in this paper provide a compelling example. We observed reduced hepatic uptake with concomitant increases elsewhere. There is little evidence to suggest that normal humans have significant pulmonary uptake of circulating test particles or pathogens. However, there are numerous reports suggesting that, with certain liver abnormalities, there is significant pulmonary localization of technetium sulfur colloid during liver scintigraphy (15, 16, 24). The data presented here also suggest that liver failure, especially of the Kupffer cells, could lead to increased pulmonary pathogen burden and thus enhance the probability of pulmonary infection and injury.

We showed that multiple PFCE intravenous administrations result in reduced capacity of the reticuloendothelial system to eliminate injected gram-negative bacteria from the blood. Uptake of bacteria by the liver is diminished in PFCE-injected rats, with compensatory increases in uptake by the lungs, kidneys, and spleen. In the liver, the principal cells responsible for uptake of injected bacteria are the Kupffer cells. In the lungs, marginated monocytes and PMN ingest the bacteria. Exposure to PFCE produces major changes in the ability of phagocytic cells to kill bacteria they have ingested. In fact, we observed P. aeruginosa proliferation in liver, kidney, and especially in the lungs of PFCE-injected rats. Our evidence suggests that the use of this perfluorochemical preparation as a blood substitute may alter host defenses and may place the patient at increased risk when bacterial infection is present.

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