Leukocyte antibacterial functions are not impaired by perfluorocarbon exposure in vitro

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Submitted 21 August 2007; accepted in final form 24 April 2008

LIQUID VENTILATION WITH PERFLUOROCARBONS (PFC) has been successfully applied in experimental and human acute lung injury (18, 20, 27, 36). Beside positive effects on gas exchange, lung mechanics, and ventilation/perfusion matching, animals treated with liquid ventilation also exhibited attenuated pulmonary damage with concomitant reduction in pulmonary neutrophil sequestration (19, 21, 32, 39). A clinical trial investigating PFC treatment in trauma patients with acute lung injury demonstrated reduced neutrophil counts and decreased levels of proinflammatory cytokines in bronchoalveolar lavage fluid (7). Although removal of alveolar inflammatory exudate by dense, water-insoluble PFC probably contributes to mitigation of pulmonary hyperinflammation, a direct interference with immune cell function may also be involved. This proposition is supported by recent in vivo studies using aerosolized PFC in a surfactant-washout model that reported PFC to decrease mRNA expression of proinflammatory cytokines and adhesion molecules in lung tissue samples as well as in microdissected alveolar macrophages and pulmonary parenchymal cells (42, 49, 50). Further evidence for cellular anti-inflammatory effects of PFC is provided by in vitro experiments showing a decreased response of activated polymorphonuclear neutrophils (PMN) and monocytic cells in terms of cytokine secretion, chemotaxis, cyclooxygenase-2 (COX-2) expression, and NF-κB activation (5, 25, 37, 46, 51). Liquid ventilation with PFC did not result in improved survival or reduced need for ventilatory support in recent multicenter trials of patients with acute respiratory distress syndrome (ARDS) (17, 24). Nevertheless, intrinsic anti-inflammatory properties of PFC might be an effective mechanism, introducing PFC as a therapeutic strategy in other illnesses aside from established acute lung injury. New forms of application such as inhalation of aerosolized or vaporized PFC, being less invasive than liquid ventilation, may facilitate this (3, 50).

The elimination of pathogenic microorganisms by phagocytic leukocytes constitutes a fundamental part of innate immunity. First stages of this process are leukocyte chemotaxis to sites of local inflammation and attachment of bacterial and fungal microbes to the cell surface of PMN, monocytes, and macrophages. Subsequently, infectious particles are ingested (phagocytosis) and killed intracellularly by oxygen-dependent (respiratory burst) and oxygen-independent mechanisms. Although cellular anti-inflammatory PFC effects such as attenuated phagocyte activation may be beneficial in states of pulmonary hyperinflammation, they also could compromise bacterialic functions and thus increase susceptibility to nosocomial lung infection. Almost all previous studies only reported on short-term application of PFC in the treatment of acute lung injury and ARDS, thus not being able to detect delayed adverse effects in terms of compromised host defense. To date, there is no data regarding an interaction of pure PFC with distinct leukocyte populations in respect of bacterial adherence, phagocytosis activity, and subsequent bacteria-induced intracellular generation of reactive oxygen species (ROS). Therefore, we investigated the influence of PFC on these key functions of human PMN and monocytes in an in vitro model. We hypothesized that the known anti-inflammatory effects of perfluorohexane also affect cellular bacterialic functions, resulting in impaired leukocyte uptake of Escherichia coli and subsequent respiratory burst activity.

MATERIALS AND METHODS

Sample preparation for ultrastructural studies of bacterial phagocytosis. After receiving approval by the local ethics committee and obtaining written informed consent, we collected heparinized venous blood from healthy human volunteers. Morphological studies were...
performed separately on populations of neutrophils (PMN) and peripheral blood mononuclear cells (PBMC). PMN were isolated from whole blood that was incubated for 45 min with an equal volume of dextran 6% (Gentran 70; Baxter, Lessines, Belgium) to allow red blood cell sedimentation, followed by density gradient centrifugation over Biocoll separation solution (density 1.077 g/ml; Biochrom, Berlin, Germany). After being washed in phosphate-buffered saline (PBS), remaining erythrocytes were eliminated by hypotonic lysis for 30 s and PMN were resuspended in DMEM medium (Biochrom) supplemented with 10% fetal calf serum (FCS), yielding a concentration of \( 3 \times 10^6 \) cells/ml. PBMC were isolated from whole blood by standard density gradient centrifugation over Biocoll, washed twice in PBS, and resuspended at \( 3 \times 10^6 \) cells/ml in RPMI 1640 (Biochrom) containing 10% FCS. Both cell preparations resulted in a population purity of >95% as determined with an automated hematology analyzer (Gen-S; Beckman Coulter, Krefeld, Germany). Before and after in vitro cultivation, viability of both PMN and PBMC was assessed by means of trypan blue exclusion.

Aliquots of suspended PMN and PBMC were preexposed to 25% (vol/vol) perfluorhexane (PFH; ABCR, Karlsruhe, Germany) with a purity of 99% for 1 (PFH-1h group) or 4 h (PFH-4h group). PFH [\( CF_3(CF_2)4CF_3 \)] is a clear, liquid, water-immiscible PFC with a purity of 99% for 1 (PFH-1h group) or 4 h (PFH-4h group). PFH means of trypan blue exclusion. In vitro cultivation, viability of both PMN and PBMC was assessed by a trypan blue exclusion. After samples were maintained for 15 min at 37°C, PFH was allowed to settle down spontaneously by gravity, and PFH-free cell suspensions in the upper phase were carefully removed for morphological analysis of bacterial phagocytosis. In contrast to these two test series of PFH preexposure, additional experiments were performed on cell suspensions that were incubated with 25% (vol/vol) PFH for 4 h without subsequent removal of the agent (PFH-4h+ group). Because PFH is a dense and water-immiscible liquid, its presence does not result in a dilutonal. Therefore, control samples consisted of cell suspensions that were preincubated for 1 or 4 h without PFH but treated similarly otherwise.

*E. coli* K12 (strain 5698; DSZM, Braunschweig, Germany) were prepared in nutrient broth and grown at 37°C to the midlogarithmic phase (optical density at 578 nm = 0.4–0.6). After being harvested and washed with PBS, bacteria were opsonized by incubation with autologous serum for 30 min at 37°C. *E. coli* were resuspended in culture medium and added to cell suspensions at a final concentration of \( 1.2 \times 10^9 \) colony-forming units (cfu)/ml, representing a bacteria-to-leukocyte ratio of \( 40:1 \) (see *Quantitative assessment of leukocyte phagocytosis activity*). After samples were maintained for 15 min at either 37°C in a rotating incubator to enable phagocytosis or at 0°C to prevent *E. coli* ingestion, leukocytes were gently washed and subjected to preparation for electron microscopy.

**Electron microscopy.** For scanning electron microscopy, PMN or PBMC were seeded on poly-l-lysine-coated slides for 30 min under standard culture conditions, washed with PBS, and fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) overnight at 4°C. Subsequently, cells were prepared by washing in 0.1 M cacodylate buffer, dehydration in graded series of ethanol, critical point drying with liquid CO2 (E3000; Gala Instruments, Schwalbach, Germany), and sputtering with gold in a Balzers MED010 sputtercoater (70 s, 50 mA). Specimens were studied with a LEO 5430 scanning electron microscopy.

For transmission electron microscopy, cell pellets were resuspended in 100 μl of 2% fibrinogen (Sigma, Deisenhofen, Germany) and incubated with 40 μl of thrombin (100 U/ml; Sigma) until clot formation was reached. The fibrin clots including the cells were fixed in 0.1 M sodium cacodylate buffer (pH 7.4) containing 2.5% glutaraldehyde overnight at 4°C. After clots were postfixed with 1% osmium tetroxide (pH 7.4) in 0.1 M cacodylate buffer for 1 h at 4°C, dehydration was performed with ethanol and ethanol-Epon. Thereafter, clots were Epon-embedded overnight at 4°C, and Epon-filled flat embedding molds were subsequently polymerized for 48 h at 60°C. Ultrathin sections (60–80 nm) were mounted on nickel grids and contrasted with lead citrate and uranyl acetate. Ultrastructural studies were carried out using an EM 906 electron microscope (Carl Zeiss, Oberkochen, Germany). All buffers, fixatives, and embedding materials for electron microscopy were purchased from Serva (Heidelberg, Germany).

**Quantitative assessment of leukocyte phagocytosis activity.** Samples from each subject were studied separately. Aliquots of 100 μl of heparinized whole blood were transferred to plastic tubes and, under gentle agitation, were preexposed for 1 or 4 h to 25% (vol/vol) PFH either with (PFH-1h, PFH-4h) or without (PFH-4h+) subsequent PFH removal. Therefore, all probes had their respective controls without PFH (Con-1h, Con-4h, and Con-4h+, respectively).

A commercial test kit (Phagotest; Orpegen Pharma, Heidelberg, Germany) was used to study quantitative whole blood leukocyte phagocytosis activity. The protocol for quantitative analysis was described previously in a slightly modified form (16). Briefly, samples of 100 μl were incubated with fluorescein isothiocyanate (FITC)-marked, opsonized *E. coli* (bacteria-to-leukocyte ratio of \( 40:1 \); optimal leukocyte recruitment by a ratio of 25–50:1 according to the manufacturer’s instructions) either at 0 or 37°C for 10 min. During this period, labeled *E. coli* were ingested by PMN and monocytes in the 37°C assay, whereas incubation at 0°C prevented this process (negative control). For the measurement of phagocytosis, fluorescence of extracellular bacteria bound to the cell surface membrane was excluded by subsequent quenching with ice-cold trypan blue solution. An additional subset of experiments was performed with nonquenched aliquots to calculate the degree of bacterial adhesion to leukocytes (fluorescence of nonquenched minus that of corresponding quenched samples). Whole blood was lysed and fixed, and probes were washed twice in PBS. To exclude artifacts from dead cells, cell debris, and aggregated bacteria and platelets, we performed DNA staining with propidium iodide (red fluorescence, FL-2). Phagocytosis activity was determined by flow cytometry (FACScan; Becton Dickinson, Heidelberg, Germany) at an excitation wavelength of 488 nm. Before data acquisition, a gate was set in the red fluorescence channel (FL-2) to dot plot to count only events that had at least the DNA content of human diploid cells. Separate analyses were carried out for populations of PMN and monocytes that could be discriminated by their characteristic size-to-granularity ratio in the forward vs. sideward scatter plot. Green fluorescence was measured in channel FL-1, and data from 15,000 cells were processed using WinMDI 2.8 (by J. Trotter, Scripps Research Institute, La Jolla, CA). Depending on whether or not samples were treated with quenching solution, mean fluorescence intensity correlated with the number of phagocytosed or total cell-associated FITC-*E. coli*. To determine the percentage of cells having phagocytosed bacteria (recruitment), we set a marker in the FL-1 histogram in quenched, negative controls (0°C) so that <1% of the cells were positive. In quenched samples incubated at 37°C, the percentage of recruited cells was calculated from the quotient of cell counts above this marker position and the total number of analyzed cells.

To analyze whether putative PFH effects differ depending on bacterial quantity and whether assay sensitivity is adequate, we conducted additional experiments (Con-4h+ and PFH-4h+ groups) using decreased bacteria-to-leukocyte ratios \( (4 \times 10^{9/2}; 4 \times 10^{9/1}) \) and cytochalasin D. This inhibitor of actin polymerization and phagocytosis was added to blood samples 30 min before *E. coli* challenge (bacteria-to-leukocyte ratio \( 4 \times 10^{9/1} \)) in a final concentration of 1 μg/ml.

**E. coli-induced generation of reactive oxygen species.** Generation of intracellular reactive oxygen species (ROS) was analyzed using a Phagoburst test kit (Orpegen). Individual PFH-exposed samples and respective controls were treated as described above in preparation for phagocytosis experiments. Aliquots (100 μl) of heparinized whole blood were incubated with 20 μl of PBS or opsonized *E. coli* (2 \( \times 10^7 \) cfu) for 10 min at 37°C, and formation of leukocyte reactive oxidants was monitored by means of an intracellular oxidation step (10 min,}
37°C). Thereby, the nonfluorescent substrate dihydrorhodamine 123 was taken up by phagocytes and converted to a green fluorescent compound (rhodamine 123). Subsequently, whole blood samples were lysed, fixed, washed twice, and finally stained with propidium iodide. Intracellular dihydrorhodamine 123 oxidation was measured using flow cytometry in FL-1 at a 488-nm excitation wavelength. Data acquisition and gating of leukocyte subpopulations were performed as described above. Mean cellular fluorescence of samples treated with PBS and *E. coli* represented spontaneous ROS production and bacteria-induced respiratory burst activity, respectively. For determining the percentage of cells with respiratory burst activity (recruitment), a marker in the FL-1 histogram was set in PBS-treated probes so that <1% of the cells were positive. In samples incubated with *E. coli*, the percentage of cells with stimulated ROS production was calculated from the quotient of cells above this marker position and the total number of analyzed cells.

**Data analysis.** Data are means ± SD. Results of quantitative assessment of leukocyte phagocytosis activity are expressed as percentages of PFH-treated cells compared with nonexposed controls unless stated otherwise. Parametric methods were used for all analyses, since a normal distribution of data was confirmed with the Kolmogorov-Smirnov test. Differences between PFH-treated cells and respective controls were evaluated with the paired Student’s *t*-test. The statistical association between different PFH treatment groups was detected using one-way analysis of variance (ANOVA) and Tukey’s honestly significant difference post hoc test. Differences in experiments with lower bacteria-to-leukocyte ratios and cytochalasin D were assessed nonparametrically with the Mann-Whitney *U*-test. Calculations were performed using the statistical package SPSS 11.0 (SPSS, Chicago, IL), and significance was accepted at *P* < 0.05.

**RESULTS**

**Blood samples.** Samples had a hemoglobin concentration of 14.0 ± 1.2 g/dl and contained 4.47 ± 0.40 × 10¹²/l erythrocytes, 270 ± 47 × 10⁹/l platelets, and 6.6 ± 1.4 × 10⁹/l leukocytes, 53 ± 8% of which were neutrophils, 37 ± 8% lymphocytes, and 7 ± 2% monocytes (all means ± SD). Before and after in vitro cultivation, viability of both PMN and PBMC was >98% in all experiments without significant differences between groups as assessed by trypan blue exclusion (data not shown).

**Electron microscopy.** In scanning electron microscopy, control monocytes displayed a normal appearance of cell surface (Fig. 1A). Treatment with PFH for 1 h was associated with small membrane-bound particles (Fig. 1B), increasing in number and size after 4 h of incubation, whereas cell morphology was preserved otherwise (Fig. 1, C and D). After challenge with opsonized *E. coli*, monocytes were intensely covered with adherent bacteria, which were to some extent wrapped by the cell membrane (Fig. 1E). When PFH was present before (PFH-1h and PFH-4h) or during (PFH-4h+) *E. coli* incubation, both normally shaped bacteria and particles were observed (Fig. 1, G and H). Similar morphological findings were obtained using isolated neutrophils (not shown).

The cytoplasm of neutrophils exposed to PFH for 1 h contained multiple small circular vacuoles as revealed by transmission electron microscopy (Fig. 2B). The size of intracellular inclusions was strikingly increased after 4 h of incubation (Fig. 2, C and D). Appearance of cell membrane, nucleus, and granula corresponded to that of untreated controls (Fig. 2A). Figure 2E shows early uptake of opsonized *E. coli* 15 min after addition to the neutrophil suspensions. Free extracellular and phagocytosed, unlysed bacteria could be observed in controls and all PFH groups (Fig. 2, E–H). The latter also contained the above-mentioned vacuoles, occasionally together with ingested *E. coli* (e.g., Fig. 2G) but were otherwise similar to untreated cells. Additional ultrastructural studies on PFH-exposed monocytes confirmed these observations (not shown).

**Flow cytometry of *E. coli* adherence and phagocytosis.** After FITC-*E. coli* challenge for 10 min at 37°C, 95–99% of PMN and 69–80% of monocytes were positive for cell-associated bacteria, irrespective of a treatment with or without PFH. Therefore, except for a significant but negligible increase in

![Image](https://example.com/image.png)

Fig. 1. Scanning electron micrographs of isolated peripheral blood mononuclear cells (PBMC) without (A–D) and with *Escherichia coli* exposure (E–H) after treatment without (control) and with 25% (vol/vol) perfluorohexane (PFH) for 1 or 4 h (PFH-1h, PFH-4h, and PFH-4h+) as indicated. See text for detailed description of groups. Images are representative of 3 independent experiments. Arrowheads, membrane-bound PFH particles; arrows, adherent bacteria.

AJP-Lung Cell Mol Physiol • VOL 295 • JULY 2008 • www.ajplung.org
one group (PMN-4h), PFH did not influence the portion of phagocytosing cells (Table 1). Mean fluorescence of adherent and phagocytosed FITC-*E. coli* did not differ between controls and cells pretreated with PFH in both PMN and monocytes (PFH-1h and PFH-4h, Fig. 3, A and B). In the presence of PFH during *E. coli* challenge (PFH-4h+), the amount of cell surface-bound bacteria was moderately decreased in PMN (*P* < 0.05) and tended to be lower in monocytes (*P* = 0.1) compared with respective controls. In contrast, PFH did not alter the corresponding quantity of intracellular FITC-*E. coli* in PMN and even slightly increased respective values in monocytes (PFH-4h+, Fig. 3, A and B, *P* < 0.05). Decreasing bacteria-to-leukocyte ratio from 40:1 to 4:1 and coincubation with the phagocytosis inhibitor cytochalasin D reduced the percentage of phagocytosing leukocytes and fluorescence intensity of ingested *E. coli* in both PMN and monocytes (Table 2 and Fig. 4, A and B). As with the high bacteria-to-leukocyte-ratio, we did not observe impairment of *E. coli* phagocytosis by PFH at these lower ratios.

**Leukocyte generation of ROS.** PFH exposure significantly decreased spontaneous generation of intracellular ROS in both resting PMN and monocytes in all treatment groups compared with respective controls (Fig. 5A, *P* < 0.05). PFH pretreatment of PMN resulted in a negligible increase in cellular recruitment to respiratory burst activity (PFH-1h, PFH-4h), whereas in the PFH-4h+ group the percentage of positive cells was significantly elevated (Table 3, *P* < 0.05). Likewise, more monocytes responded to *E. coli* stimulation in samples preexposed to PFH for 4 h than in controls (*P* < 0.05). Mean cellular rhodamine fluorescence after *E. coli* challenge did not differ significantly between samples with PFH preexposure for 1 h and corresponding controls (Fig. 5B). PFH significantly attenuated respiratory burst in PFH-4h+ (PMN) and PFH-4h groups (monocytes). In addition, stimulated ROS generation tended to be diminished in the corresponding PFH-4h (PMN) and PFH-4h+ subgroups (monocytes) (*P* = 0.063 and *P* = 0.074, respectively).

**DISCUSSION**

Bacterial pneumonia and sepsis are leading causes of acute lung injury, and superimposed pulmonary infection (ventilator-associated pneumonia, or VAP) is a common complication in patients with ARDS, leading to prolonged mechanical ventilation (11). In this respect, epidemiological studies revealed a frequent occurrence of *Pseudomonas* species, *Staphylococcus aureus*, and, especially in pediatric critical care units, gram-negative *Enterobacteriaceae* including *E. coli* (2, 33). Since anti-inflammatory PFC effects might impair leukocyte bactericidal functions, thereby compromising pulmonary host defense of these patients, we evaluated for the first time the in vivo influence of a pure PFC on bacterial adherence, phagocytosis, and subsequent intracellular burst activity in distinct human leukocyte populations.

To date, the mechanism of cellular PFC effects is not known. Some authors favor the theory that the presence of PFC forms a microbarrier on the cell surface that interferes with binding, successive removal of PFH from suspension. PMN, polymorphonuclear neutrophils.

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**Table 1. Percentage of leukocytes positive for ingested *E. coli* as assessed by flow cytometry**

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<tr>
<th>Group</th>
<th>PMN</th>
<th>Monocytes</th>
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<tr>
<td></td>
<td>Con</td>
<td>PFH</td>
</tr>
<tr>
<td>1 h</td>
<td>95.3±3.9</td>
<td>94.6±2.6</td>
</tr>
<tr>
<td>4 h</td>
<td>94.7±4.4</td>
<td>96.3±4.3</td>
</tr>
<tr>
<td>4 h+</td>
<td>91.6±4.3</td>
<td>89.1±3.3</td>
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Data are means ± SE of 7 independent experiments, expressed as percentages of phagocytosing cells exposed to *Escherichia coli* that were left untreated (Con) or pretreated with perfluorohexane (PFH) for 1 or 4 h (PFH-1h and PFH-4h). PFH-4h+, group exposed to 4 h of PFH incubation with no subsequently removal of PFH from suspension. PMN, polymorphonuclear neutrophils.
signal transduction, or transmembrane migration of inflammatory stimuli or soluble mediators (1, 38, 47). However, there is clear evidence of a time-dependent PFC diffusion into lipid bilayers that has been shown to alter cellular responses to activation and stress (31, 52). Accordingly, the groups in our experiments were set to discriminate between effects due to PFH uptake into leukocyte membranes at different time points (PFH-1h, PFH-4h) and a putative cellular “microcoating” in the presence of PFH that may alter leukocyte-bacteria contact (PFH-4h+). Since PFC have been shown to differ in their interaction with distinct leukocyte populations (30), both neutrophils and monocytes were evaluated separately.

### Table 2. Percentage of leukocytes with phagocytosed FITC-E. coli as assessed by flow cytometry, with application of different bacteria-to-leukocyte ratios or cytochalasin D

<table>
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<tr>
<th>Ratio</th>
<th>PMN</th>
<th>Mono</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Con-4h+</td>
<td>Con-4h+</td>
</tr>
<tr>
<td>4 × 10^1:1</td>
<td>92.8±7.1</td>
<td>79.9±9.2</td>
</tr>
<tr>
<td>4 × 10^1/2:1</td>
<td>70.7±8.6</td>
<td>57.6±21.8</td>
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<tr>
<td>4 × 10^2:1</td>
<td>42.8±6.1</td>
<td>31.2±18.7</td>
</tr>
<tr>
<td>4 × 10^3:1</td>
<td>84.7±13.4</td>
<td>62.2±28.6</td>
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<td>4 × 10^3:1</td>
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Data are means ± SD of 4 independent experiments, expressed as percentages of phagocytosing cells with different bacteria-to-leukocyte ratios or 1 μg/ml cytochalasin D (cyt D).
In previous studies, a time-dependent cellular uptake of PFC was shown (43), with an exposure time of 5 min not sufficient to allow PFC phagocytosis (12). We morphologically demonstrated adhesion of PFH particles to the surface and subsequent intracellular ingestion after incubation for 1 and 4 h in both neutrophils and monocytes. The size of cytoplasmic vacuoles increased with incubation time, most likely due to a confluence of phagocytosed PFH particles. In contrast, electron microscopy analysis by other groups revealed only small intracellular vacuoles of perfluorooctylbromide and perfluorodecalin after similar incubation times (30). This is most likely due to differences in physicochemical properties of PFC such as vapor pressure and surface tension. In accordance with other reports, cellular uptake of PFC in our experiments was not associated with morphological signs of cell damage, and leukocyte viability was not impaired (4, 25, 44). We provide for the first time ultrastructural images of bacterial adherence to and uptake by leukocytes exposed to pure PFC. Neither process was influenced qualitatively, with membrane-bound and intracellular E. coli visible in controls as well as in PFC-treated cells. In this context, we repeatedly observed bacteria inside of cytoplasmic PFH vacuoles, presumably resulting from a fusion of E. coli phagosomes with ingested PFC particles. A similar phenomenon was reported in an in vivo study showing rodent liver Kupffer cells that contained S. aureus within phagolysosomes of a PFC emulsion (4). The interaction between PFH and E. coli did not result in an altered bacterial morphology in our experiments. In contrast, Jung et al. (23) described damage to gram-negative bacteria with loss of cell wall integrity, outer membrane blebbing, and vacuolization due to in vitro perfluorooctylbromide exposure. The reason for these different findings is not clear, but impurities in PFC liquids of non-medical grade have been shown to have deleterious effects on bacteria.

Phagocytosis was not impaired by PFH in any treatment group. Conditions for phagocytosis were optimized with a bacteria-to-leukocyte ratio of 40:1 and a neutrophil concentration about 10-fold higher than that reported to be critical for bacterial killing (3–4 × 10⁵/ml) (28). However, the excess of E. coli with a high probability of bacteria-leukocyte contact did not conceal an underlying PFH effect, since phagocytosis was similar in treatment groups and controls with the use of a bacteria-to-leukocyte ratio as low as 4:1. Also, the assay sensitivity was adequate to detect quantitative differences in FITC-E. coli ingestion as intracellular fluorescence decreased dramatically with lowering of the bacteria-to-leukocyte ratio and was also reduced by the phagocytosis inhibitor cytochalasin D. Although PFH did not interfere with bacterial phagocytosis, E. coli adherence was moderately inhibited by PFH exposure during bacterial challenge (PFH-4h+). Therefore, one could speculate that PFH physically constrained bacterial binding to a certain fraction of respective cell membrane receptors but did not affect pathways responsible for leukocyte phagocytosis. The underlying mechanisms, however, are unclear.

Respective in vitro studies that evaluated PFC emulsions (PFCE) as intravascular oxygen carriers gave conflicting results with respect to phagocytosis. PFCE compromised leukocyte uptake of serum-coated plastic beads, whereas another group found decreased chemotaxis while phagocytosis and bactericidal activity of human neutrophils were not impaired (26, 48). However, the emulsifying agent Pluronic F-68 used for PFCE preparation in these and other studies was found to be, at least in part, responsible for the interaction with immune cell function. An in vivo study (4) analyzed bacterial blood clearance in rats given PFCE intravenously. Elimination of gram-positive S. aureus was not influenced by PFCE, but...
removal of gram-negative *P. aeruginosa* was substantially suppressed. The effect of the emulsifier Pluronic itself was not evaluated. The authors discussed an “overload effect” of PFC-containing phagocytes with a decreased number of bacterial receptors on plasma membrane or mechanical obstruction to organelle motion, bacterial uptake, and killing. We observed morphologically phagocytic cells containing PFC vacuoles that were nevertheless capable of ingesting bacteria as also mentioned in the study by Brain et al. (4). This finding, together with an unaltered phagocytic capacity after incubation with nonemulsified PFH, argues against the theory of immune cells being in a refractory state during bacterial challenge after exposure to this particular PFC. In contrast to our results, two other studies found phagocytosis to be impaired by pure PFC. Perfluorooctylbromide decreased ex vivo neutrophil phagocytosis of *Pseudomonas aeruginosa* as well as ingestion of opsonized sheep erythrocytes in vitro (12, 35). We speculate that the nature of the particular PFC may account for this contradiction, since cellular effects have been shown to increase with lipid solubility that is clearly lower for PFH compared with perfluorooctylbromide (31, 52).

To date, there are no reports regarding the influence of PFC on bacteria-induced respiratory burst, which is a crucial part of the host defense against microorganisms. Previously, somewhat conflicting results have been published concerning the effects of PFC on stimulated ROS generation, showing decreased production of hydrogen peroxide in neutrophils and macrophages, unaltered leukocyte responses, or even a promotion of LPS-induced respiratory burst (26, 30, 37, 44, 47). Although stimulated mean ROS generation per cell decreased moderately in two treatment groups in our experiments, at the same time a greater proportion of cells was recruited to produce oxygen radicals, probably resulting in a maintained overall respiratory burst capacity. Our data indicate that, upon bacterial challenge, PFH should not substantially compromise oxidant defense mechanisms involved in the elimination of *E. coli*. We did not evaluate intracellular bacterial killing, but this was demonstrated to be unaffected even when phagocytosis was impaired considerably (35). The contact of whole blood leukocytes with plastic is known to cause a certain induction of cellular ROS production (14). In the absence of *E. coli* stimulation, we found low basal ROS generation in both PMN and monocytes to be decreased by PFH in all treatment groups. Most likely, this results from a diminished contact between leukocytes and plastic tubes in the presence of 30% (vol/vol) PFH.

Until now, there was no clinical evidence that application of PFC is associated with an increased rate of nosocomial infection. In studies on both adult and infant patients, acute lung injury due to pneumonia was treated with liquid ventilation, and secondary pneumonia was not reported to be an adverse effect in PFC treatment groups (17, 24, 27). There is one study that found liquid ventilation to increase pulmonary bacterial counts in rats with *P. aeruginosa*-induced pneumonia (35). In contrast, inoculation of *P. multocida* in the lungs of rabbits treated with PFC resulted in a 10-fold reduction in pulmonary bacterial recovery after 24 h (41). Dickson and coworkers (9, 10) found that liquid ventilation together with antibiotics in rats with pneumococcal pneumonia strikingly improved survival compared with antibiotics alone. These data and the fact that PFC per se do not augment in vitro bacterial growth suggest that nosocomial pneumonia is not increased by liquid ventilation with PFH (29, 40). There are no animal trials using PFH in the setting of bacterial lung infection, and the experimental protocols of all studies performed in this field were too short to allow development of VAP (3, 8, 13, 45). However, our in vitro results support the assumption that despite previously demonstrated anti-inflammatory effects (15, 25), PFH does not seem to impair lung bactericidal defense, and therefore our initial hypothesis cannot be confirmed.

Nevertheless, we are aware that in addition to adherence, phagocytosis, and oxidative killing, bacterial elimination involves a concerted action of humoral and cellular mechanisms such as antibody opsonization, leukocyte chemotaxis, and proteolytic degradation that were not evaluated in our experiments. Even if PFH did not impair cellular defense mechanisms against *E. coli*, completely different results might be observed for other bacterial species. For example, PFCE differentially influenced blood clearance of gram-positive and gram-negative bacteria (4), and the role of ROS in bacterial killing has been shown to depend on the particular bacterial species (34). Furthermore, the significance of our findings is limited by the use of peripheral blood leukocytes. Although this is an established in vitro model to study inflammatory processes, cellular neutrophil and monocyte function changes significantly after cells leave the circulation and migrate to the lung (6, 22). Consequently, a definite statement regarding an influence of PFH on immune competence is not possible from an in vitro study.

Liquid ventilation with PFC did not result in improved survival or reduced need for ventilatory support in clinical trials of ARDS patients (17, 24). However, alternative forms of PFC application such as aerosol or vapor administration have shown promising effects without causing typical problems and shortcomings of liquid ventilation. In this context, vaporized PFH was associated with improved oxygenation and lung mechanics in experimental ARDS, protected animals from ventilator-induced lung injury, and improved lung histology in oleic acid-injured pigs to a far greater degree compared with liquid ventilation (3, 8, 13, 45). Whether the anti-inflammatory effects of PFH shown by us in recent in vitro studies contribute to this organoprotection remains unclear (15, 25).

In summary, we provide for the first time evidence that interaction of the perfluorocarbon PFH with human leukocytes exposed to *E. coli* does not result in reduced phagocytosis capacity and reduced bacteria-induced respiratory burst. Our in vitro findings provide further data regarding the safety of PFH application in vivo, suggesting that key functions of the innate host defense are not compromised by PFH treatment.

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