Depletion of phagocytes in the reticuloendothelial system causes increased inflammation and mortality in rabbits with *Pseudomonas aeruginosa* pneumonia

Kiyoyasu Kurahashi,1,2,3 Teiji Sawa,2 Maria Ota,2 Osamu Kajikawa,3 Keelung Hong,4 Thomas R. Martin,3 and Jeanine P. Wiener-Kronish5

1Department of Anesthesiology and Critical Care Medicine, Yokohama City University Graduate School of Medicine, Yokohama, Japan; 2Department of Anesthesia, The University of California at San Francisco, San Francisco, California; 3Medical Research Service, Veterans Affairs Puget Sound Health Care System and the Division of Pulmonary and Critical Care Medicine, Department of Medicine, University of Washington School of Medicine, Seattle, Washington; 4Department of Biopharmaceutical Sciences, The University of California at San Francisco, San Francisco, California; and 5Department of Anesthesia and Perioperative Care, Department of Medicine, and Cardiovascular Research Institute, The University of California at San Francisco, San Francisco, California

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Kurahashi K, Sawa T, Ota M, Kajikawa O, Hong K, Martin TR, Wiener-Kronish JP. Depletion of phagocytes in the reticuloendothelial system causes increased inflammation and mortality in rabbits with *Pseudomonas aeruginosa* pneumonia. *Am J Physiol Lung Cell Mol Physiol* 296: L198–L209, 2009. First published November 21, 2008; doi:10.1152/ajplung.90472.2008.—Phagocytes of the reticuloendothelial system are important in clearing systemic infection; however, the role of the reticuloendothelial system in the response to localized infection is not well-documented. The major goals of this study were to investigate the roles of phagocytes in the reticuloendothelial system in terms of bacterial clearance and inflammatory modulation in sepsis caused by *Pseudomonas* pneumonia. Macrophages in liver and spleen were depleted by administering liposome encapsulated dichloromethylene diphosphonate (clodronate) intravenously 36 h before the instillation of *Pseudomonas aeruginosa* into the lungs of anesthetized rabbits. Blood samples were analyzed for bacteria and cytokine concentrations. Lung injury was assessed by the bidirectional flux of albumin and by wet-to-dry weight ratios. Blood pressure and cardiac outputs decreased more rapidly and bacteremia occurred earlier in the clodronate-treated rabbits compared with the nondepleted rabbits. Plasma TNF-α (1.08 ± 0.54 vs. 0.08 ± 0.02 ng/ml) and IL-8 (6.8 ± 1.5 vs. 0.0 ± 0.0 ng/ml) were higher in the depleted rabbits. The concentration of IL-10 in liver in the macrophage-depleted rabbits was significantly lower than in normal rabbits at 5 h. Treatment of macrophage-depleted rabbits with intravenous IL-10 reduced plasma proinflammatory cytokine concentrations and reduced the decline in blood pressure and cardiac output. These results show that macrophages in the reticuloendothelial system have critical roles in controlling systemic bacterial infection and reducing systemic inflammation, thereby limiting the systemic effects of a severe pulmonary bacterial infection.

Address for reprint requests and other correspondence: K. Kurahashi, Dept. of Anesthesiology and Critical Care Medicine, Yokohama City Univ. Graduate School of Medicine, 3-9 Fukaura, Kanazawa-ku, Yokohama, 236-0004, Japan (e-mail: kiyok@med.yokohama-cu.ac.jp).

NOSOCOMIAL PNEUMONIA OCCURS IN ~25% of mechanically ventilated patients (13, 39). *Pseudomonas aeruginosa* is now one of the most common (18, 33) and most lethal (6, 15, 19) pathogens causing nosocomial pneumonia. One possible reason for the high mortality associated with *P. aeruginosa* pneumonia is the fact that pneumonia due to *P. aeruginosa* often leads to bacteremia (12, 14, 21). Clinical isolates of *P. aeruginosa* that are positive for type III secretion system cause more cytotoxicity to macrophage cell lines than those strains that are negative for type III secretion system (3). The intrapulmonary instillation of *P. aeruginosa* strains that produce ExoU, a toxin secreted via the type III secretion system, promotes entry of bacteria into the circulation as well as the transport of inflammatory mediators from the infected air spaces into the circulation, leading to lethal hypotension and acidosis (37). Local as well as systemic defense mechanisms are therefore important in the responses to intrapulmonary *P. aeruginosa*.

The reticuloendothelial system provides important defenses against systemic bacteremia. A number of early studies showed that intravenously administered bacteria, including *Escherichia coli* (2), *Staphylococcus aureus* (2), *Salmonella enteritidis* (5), *Listeria monocytogenes* (42), and *Streptococcus pneumoniae* (11), are rapidly taken up by the reticuloendothelial system. However, the effect of the reticuloendothelial system in the host response to localized infection has not been studied in detail. In addition to scavenging bacteria, phagocytes in the reticuloendothelial system secrete inflammatory mediators in response to bacterial contact or cytokines that have leaked from the primary sites of infection. Fox-Dewhurst et al. (22) showed using an *E. coli* pneumonia model that when the bacterial inoculum in the air space exceeds the capacity of the pulmonary defense system, compartmentalization fails and severe systemic inflammation occurs, suggesting that the reticuloendothelial system might have an important role in severe local pulmonary infections.

The aims of this study were to determine the role of the reticuloendothelial system in the early physiological and inflammatory response to localized infection in the lungs caused by *Pseudomonas* pneumonia. We depleted phagocytes in the reticuloendothelial system of anesthetized rabbits using liposome encapsulated dichloromethylene diphosphonate (clodronate-liposomes) and then instilled *P. aeruginosa* into the air spaces to produce bacteremia. We evaluated lung injury, the number of circulating bacteria, hemodynamic and blood gas...

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changes, and cytokines in the blood, liver, and spleen. Because IL-10 is an important immunomodulatory cytokine produced by mononuclear cells of the reticuloendothelial system, we measured IL-10 levels in the clodronate-treated animals and then treated the animals with human recombinant IL-10 (rhIL-10). The IL-10 levels were lower and inflammatory responses were greater in the clodronate-treated animals, and treatment with rhIL-10 reduced inflammation and improved inflammatory responses. These results provide novel evidence about the role of phagocytes in the reticuloendothelial system in localized bacterial pneumonia and suggest that the reticuloendothelial system contributes not only to the clearance of systemic bacteria, but also to the pathogenesis of the systemic anti-inflammatory response syndrome that accompanies localized bacterial pneumonia.

MATERIALS AND METHODS

Reagents. rhIL-10 was provided by the Schering-Plough Research Institute (Kenilworth, NJ) and stored at –80°C until used. In our previous study (37), a 50 μg/kg dose of the rhIL-10 was found to be effective in blocking the biological effects of TNF-α in rabbits with P. aeruginosa pneumonia.

Dichloromethylenediphosphonate (clodronate) was provided by Boehringer Mannheim (Mannheim, Germany). Clodronate was dissolved in distilled water at a concentration of 250 mM and stored at 4°C. Bovine brain phosphatidylserine (PS) and egg phosphatidylcholine (PC) were purchased from Avanti Polar Lipids (Alabaster, AL). Cholesterol was purchased from Sigma Chemical (St. Louis, MO). Clodronate-encapsulated liposomes were prepared using the modified reverse evaporation technique (62). In brief, PS, PC, and cholesterol were mixed together at a molar ratio of 1:6:4 in a round-bottom flask, and the solvent was removed under reduced pressure by a rotary evaporator. Clodronate was encapsulated by the liposomes when the lipids were redissolved in the clodronate solution. This suspension was then sterilized by filters and centrifuged at 40,000 rpm for 1 h to separate the liposomes from the clodronate solution. The liposomes were collected and resuspended in 20 ml of Ringer lactate solution (Baxter, Deerfield, IL) and stored at 4°C until use.

Bacteria. A strain of P. aeruginosa, PA103, provided by Dara Frank, PhD, was used in these experiments. This bacterial strain has been previously characterized as to its exoproducts and ability to cause lung injury (35, 48). The bacterial solution was prepared as described previously (26, 35, 68). Briefly, frozen bacterial stock was inoculated into a deferrated dialysate of trypticase soy broth supplemented with 10 mM nitrotriacetic acid (Sigma Chemical), 1% glyceral, and 100 mM monosodium glutamate, and grown at 33°C for 13 h in a shaking incubator. Cultures were spun at 8,500 g for 10 min, and the bacterial pellet was washed three times with PBS before the addition of the bacteria to the instillate. The bacterial solution was diluted to 1.0 × 10⁸ colony-forming units (CFU)/ml. The number of bacteria added to the instillate was confirmed by serial dilution and culture of the bacterial suspension.

The protocol for animal studies. The protocols for all animal experiments were approved by the Animal Research Committee of the University of California at San Francisco. A total of 46 specific pathogen-free male New Zealand White rabbits (range of body wt, 3.5–4.5 kg; Western Oregon Rabbit, Philomath, OR) were used for these experiments (Table 1).

Depletion of phagocytes in the reticuloendothelial system. Rabbits described as depleted were injected intravenously with clodronate-liposomes 36 h before the acute experiments to eliminate the macrophages in the reticuloendothelial system. Naito et al. (47) and Van Rooijen and Sanders (64) have shown that this method successfully depletes macrophages in the reticuloendothelial system. This method is advantageous because nonphagocytic cells are not affected by this drug (64). In addition, previous studies have shown that the number of lymphocytes and the number and function of neutrophils are not affected by intravenous clodronate-liposomes (9, 55). In brief, 2.5 ml/kg body wt of stored liposome suspension (see above) was injected intravenously. Additional rabbits were injected with the same volume (2.5 ml/kg body wt) of vehicle (Ringer lactate solution). We used Ringer lactate as a control because empty liposomes can reduce the phagocytic activity of the reticuloendothelial system so that liposome-treated control animals might have abnormal macrophage function (54, 64).

Confirmation of macrophage depletion. Four rabbits (Table 1) were injected with colloidal carbon solution (carbon black dispersion no. 8; Sanford, Lewisburg, TN) to evaluate the efficacy of the depletion of phagocytes in the reticuloendothelial system. Two rabbits were injected with clodronate-liposomes, and another two rabbits were injected with vehicle 36 h before the injection of the carbon solution. Colloidal carbon solution (100 mg/kg body wt) was given intravenously to these four rabbits under general anesthesia. One hour after the injection of the carbon solution, each rabbit was more deeply anesthetized, a thoracotomy was performed, and a catheter was inserted into the ascending aorta through the apex of the heart. To wash out blood, the aortic catheter was perfused for 2 min with warmed PBS (37°C) at a pressure of 140 mmHg, after which the liver and spleen were harvested and fixed with 10% formalin (Fisher Scientific, Fair Lawn, NJ). Tissue samples were prepared for light microscopy using hematoxylin-eosin staining, and the amount of phagocyte depletion was assessed by comparing the amount of carbon black in the photomicrographs of the clodronate-liposome-injected rabbits with those of the vehicle-injected rabbits using computer software (NIH Image; National Institutes of Health, Bethesda, MD).

Quantitative evaluation of alveolar macrophage population. Lung sections taken from four rabbits were used to confirm the depletion of macrophages. Two additional rabbits were similarly treated (Table 1).

Table 1. Number of animals studied

<table>
<thead>
<tr>
<th>Pretreatment*</th>
<th>clod⁻ (Depleted) Saline</th>
<th>clod⁻ (Normal) Saline</th>
<th>clod⁻ (Depleted) IL-10</th>
<th>clod⁻ (Normal) IL-10</th>
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<td>0</td>
<td>0</td>
<td>4</td>
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<tr>
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<td>4</td>
<td>0</td>
<td>0</td>
<td>8</td>
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<tr>
<td>³⁻⁴⁻⁻⁻⁻⁻⁻ Bacteria in the organs*</td>
<td>18</td>
<td>18</td>
<td>5</td>
<td>5</td>
<td>46</td>
</tr>
</tbody>
</table>

*Experimental rabbits were pretreated with clodronate-liposomes (clod⁻) or Ringer lactate solution (clod⁻) 36 h before the experiment. In addition, some animals were pretreated with IL-10 or normal saline (saline) 30 min before bacterial instillation. †Efficacy of macrophage depletion in our model was confirmed histologically 1 h after injection of colloidal carbon solution. Number of alveolar macrophages present was also evaluated using the lungs of these animals. ‡Major experiments described in the experimental protocols. §Cytokine concentrations were measured in the liver, spleen, and lungs before (0 h) and 5 h after bacterial inoculation (5 h). *Total number of bacteria in the lungs and spleens were determined by counting radioactivity of the organs after injection of radiolabeled bacteria.

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and then used to evaluate the number of alveolar macrophages present. Lung sections harvested 36 h after the administration of either clodronate-liposomes or vehicle were processed for immunohistochemistry using an anti-rabbit macrophage antibody. Paraffin-embedded lung tissue was sectioned into 5-µm slices and water-mounted onto charged Superfrost Plus glass slides (Anatomical Pathology, Pittsburgh, PA). Slides were deparaffinized by washing twice in xylene for 5 min and rehydrated by washing twice in 100% ethanol for 3 min, twice in 95% ethanol for 3 min, and once in deionized water (diH₂O) for 5 min. Endogenous peroxidases were blocked by incubating slides with Peroxo-Block (00-2015; Invitrogen, Carlsbad, CA) for 30 min and rinsing twice with PBS for 5 min. Antigens were retrieved by treating the slices with proteinase K (S3020; Dako, Carpinteria, CA) for 5 min at room temperature and then washed twice in PBS for 5 min and blocked with 1.5% normal horse serum (Vector Laboratories, Burlingame, CA). Alveolar macrophages were visualized using mouse anti-rabbit alveolar macrophage antibody, clone RAM11 (M0633; Dako). Tissue was incubated with the primary antibody or an isotype control antibody (Purified Mouse IgG1, 16-4714-82; eBioscience, San Diego, CA) at 1:100 in a moist chamber overnight at 4°C. The slides were rinsed twice with PBS for 5 min, labeled with biotinylated anti-mouse IgG (Vectastain Elite ABC Kit, PK-6102; Vector Laboratories) for 30 min at room temperature, rinsed twice with PBS for 5 min, covered with avidin and biotinylated horseradish peroxidase macromolecular complex (ABC) reagent for 30 min, rinsed twice with PBS for 5 min, and treated with diaminobenzidine substrate solution (DAB tablets, D4418; Sigma Chemical) for 10 min. Slides were rinsed in running diH₂O for 5 min, three times with PBS for 3 min, counterstained with Methyl Green (4800-30-18; Trevigen, Gaithersburg, MD) for 5 min, and rinsed in running diH₂O for 5 min. Tissue samples were dehydrated in 95% ethanol twice, 100% ethanol twice, and then immersed twice in xylene for 5 min and mounted with a coverslip using Permount (SP15-100; Fisher Scientific).

The number of labeled cells was counted in five randomly selected low-magnification (×10) fields in each slide without knowledge of the animal groups.

**Animal preparation for acute experiments.** Anesthesia was induced with an intravenous injection of sodium pentobarbital (25 mg/kg; Abbott Laboratories, North Chicago, IL). After an infiltration of local anesthetic, a tracheotomy was performed, and an endotracheal tube [4.0-in. inner diameter (ID); Smiths Industries, Keene, NH] was inserted. Mechanical ventilation was maintained by a constant volume pump (Harvard Apparatus, Millis, MA) with an inspired oxygen fraction (FiO₂) of 1.0 at a tidal volume of 20 ml/kg body wt and positive end-expiratory pressure of 3 cmH₂O. The respiratory rate was adjusted to maintain an arterial carbon dioxide pressure between 35 and 45 Torr. Rabbits were anesthetized with 0.5% halothane (Halocarbon Laboratories, River Edge, NJ) and paralyzed with a continuous infusion of 0.2 mg/h pancuronium bromide (Elkins-Sinn, Cherry Hill, NJ). A heat and moisture exchanger (Humid-Vent Mini; Gibeck, Indianapolis, IN) was used to minimize the loss of humidity and heat. The right carotid artery was catheterized to monitor blood pressure and to sample arterial blood. A 5-Fr thermodilution catheter (Swan-Ganz; Baxter, Irvine, CA) was inserted in the pulmonary artery via the right femoral vein for measurement of cardiac output. After these procedures, rabbits were placed in the right lateral decubitus position and observed for 30 min to obtain baseline hemodynamic data. Each rabbit received an intravenous injection of 131I-albumin (Merck Frost, Kirkland, Québec, Canada) as the vascular protein tracer 30 min after the surgical preparation as previously described (35, 68). At time 0, rabbits were treated with a lobar inoculum of 1.0 × 10⁶ CFU/ml PA103 in a 5% BSA solution (Sigma Chemical) that contained 0.5 μCi of 125I-labeled albumin (Merck Frost). The bacterial inoculum was instilled into the right lower lobe over 20 min through a 0.86-mm ID polyethylene tube (Becton Dickinson, Sparks, MD) inserted into the right lower lobe via the tracheotomy.

Systemic blood pressure, airway pressure, and central venous pressure were recorded continuously on a polygraph (model 7; Grass Instrument, Quincy, MA). Arterial and mixed venous blood gas analyses were performed with a blood gas analyzer (model 178; Ciba Corning Diagnostics, Medfield, MA). Cardiac output was calculated by a cardiac output computer (model 9520A; American Edwards Laboratories, Irvine, CA) using the thermodilution method with a 3-ml injectate of ice-cold Ringer lactate solution (0−4°C). Every animal received a continuous intravenous infusion of 4 ml·kg⁻¹·h⁻¹ Ringer lactate solution throughout the experimental period. Blood was sampled every 15 min and used for bacterial cultures and isotope and cytokine measurements.

**Distribution of animals.** Twenty rabbits were used for the acute experiments in which physiological changes and lung injury were assessed (Table 1). The rabbits were divided into two main groups comprised of two subgroups each (5 rabbits in each subgroup). The depleted group (n = 10) was injected intravenously with clodronate-liposomes 36 h before the experiment, whereas the normal group (n = 10) was injected with the same volume of Ringer lactate solution as a vehicle. Five rabbits from the depleted group and five rabbits from the normal group received 50 μg/kg rhIL-10 intravenously over 30 min, 30 min before bacterial inoculation (subgroups: depleted/IL-10, n = 5; normal/IL-10, n = 5). Ten remaining rabbits received the same volume of normal saline as a vehicle before the bacterial inoculation (depleted/vehicle, n = 5; normal/vehicle, n = 5).

**Measurement of lung injury.** The quantity of the alveolar protein tracers that moved from the air spaces of the lung into the systemic circulation was calculated as previously reported (45, 67). Briefly, the radioactivity counts in the plasma (per milliliter) were multiplied by the estimated plasma volume [body weight in grams × 0.07 (1 − hematocrit/100)], and this value was expressed as a percentage of the total radioactivity instilled in the lung at the beginning of the experiment.

Eight hours after the bacterial instillation, each rabbit was deeply anesthetized and then euthanized by transecting the abdominal aorta. The lungs were removed via a sternotomy, and the left mainstem bronchus was clamped. A 0.86-mm ID polyethylene tube (Becton Dickinson) was placed into the right lower lobe to retrieve the remaining alveolar liquid for measurements of radioactivity and protein concentration as previously described (35, 68). The right and left lungs were homogenized separately in a sterile fashion, and the homogenates were used for bacterial culture, measurement of radioactivity, and wet-to-dry weight ratios (W/D).

**Extraction of cytokines from the liver, spleen, and lungs.** Twelve additional rabbits were used for measurement of cytokines (Table 1). The preparation of the rabbits was the same as in the acute experiment, but radioisotopes were not administered. Both the depleted (n = 4) and normal (n = 4) rabbits were deeply anesthetized 5 h after the instillation of the P. aeruginosa and thoracotomies were performed. Organs were harvested from another four rabbits, depleted (n = 2) and normal (n = 2) (Table 1), prepared again in the same manner but euthanized before the bacterial inoculation (0 h). These rabbits were used for histology as well as cytokine measurements. A catheter was placed in the ascending aorta, and each rabbit was perfused with PBS solution containing 0.1% EDTA for 2 min at 140 mmHg pressure immediately after the euthanasia. Small pieces of liver, spleen, and both lungs were taken and weighed. Lysis buffer, made from 0.5% Triton X-100, 150 mM sodium chloride, 15 mM Tris, 1 mM calcium chloride, and 1 mM magnesium chloride, was adjusted to pH 7.40. Protease inhibitor (Boehringer Mannheim) and the lysis buffer were added in a ratio of 500 μl to 100 mg of tissue. The tissue was then homogenized on ice for 10 s with a tissue homogenizer (Tissue-Tearor; BioSpec, Bartlesville, OK) and incubated for 30 min on ice. The homogenate was then centrifuged at 2,500 rpm for 10 min, and the supernatant was passed through a 0.45-μm syringe filter (Nalge Nunc International, Rochester, NY), and stored at −80°C until used.

**References.**
**Bacteriology.** A quantitative analysis of bacteremia was performed using arterial blood. One hundred microliters of arterial blood was obtained every 15 min and spread on tryptic soy agar with 5% sheep blood (Remel, Lenexa, KS). To quantify the bacteria in the infected lungs, right lung homogenates were diluted with known quantities of sterile PBS and streaked onto agar plates. Bacterial colonies were counted on the agar plate after 16 h of incubation at 37°C. The quantity of bacteria was determined by multiplying the number of colonies by the dilution ratio.

**Determination of cytokines by bioassay or ELISA.** The biological TFN-α assay was performed using a mouse sarcoma cell line (WEHI-13 VAR, ATCC CRL-2148; American Type Culture Collection, Manassas, VA) as we (37) reported previously. The WEHI-13 VAR cell assay is 100 times more sensitive than the previous L929 cell assay (17, 31). The TFN-α activity of each sample was calculated using a standard curve obtained with rabbit TFN-α (PharMingen, San Diego, CA). The lower limit of detection for this assay was 1.0 pg/ml.

Cytokines were measured with rabbit-specific immunoassays using goat polyclonal IgG raised against either recombinant rabbit (rRab)-IL-8, rRab-growth regulated oncogene (GRO), rRab-macrophage chemotactic protein-1 (MCP-1), or rRab-IL-10, as previously described (28, 29). The lower limits of detection for these ELISAs were as follows: IL-8, 100 pg/ml; GRO, 100 pg/ml; MCP-1, 100 pg/ml; and IL-10, 300 pg/ml. When cytokines in tissue extracts were measured, the cytokine concentrations were divided by the protein concentration of the sample, measured using the Bradford assay (500–0001; Bio-Rad, Hercules, CA).

**Total bacteria in the livers and spleens.** To determine whether the numbers of live and dead bacteria were similar in the livers and spleens of the control and macrophage-depleted animals, we labeled the bacteria with [125I]methionine (Amersham, Little Chalfont, Buckinghamshire, England). Ten minutes after instilling the labeled bacteria in the albumin solution, animals, normal/vehicle (n = 4) and depleted/vehicle (n = 4), were euthanized and perfused with PBS to wash out blood as for the extraction of cytokines from the liver, spleen, and lungs section (see above). Four pieces of liver and spleen (20–25 mg) were harvested, weighed separately, and digested with 350 μl of solubilizer (Solvable; Packard, Meriden, CT) at 60°C for 2 h. This solution was then decolorized with 2 aliquots of 35 μl of 30% hydrogen peroxide, and then the β-emission was counted using a liquid scintillation analyzer (1900CA; Packard) after adding 3.3 ml of LSC cocktail (Ultima Gold; Packard). The measurements [counts/min (CPM)] were then divided by the weight of the tissue in the sample. This measurement represents the sum of live and dead bacteria in the liver and spleen.

**Statistical analysis.** Data obtained at a single time during the experiment were analyzed with an unpaired Student’s t-test and presented as means ± SE. Data obtained repeatedly during the course of the experiment were converted into a single variable for each animal. We used linear regression analysis to compare the average slopes of the lines for each parameter between the clodronate treated and the untreated, control animals [e.g., for mean arterial pressure (MAP), cardiac output, and base excess (BE)]. For 125I-albumin in blood and bacteria in blood, the y-axis was converted to a log10 scale before applying linear regression, and then the times to reach a specific threshold were compared. The thresholds for the 125I-albumin in blood were analyzed at 10% and 20% of the instilled intrapulmonary dose, which are about the values at 8 h when rabbits are infected with a noncytotoxic strain of *P. aeruginosa* and a cytotoxic strain, respectively, according to our previous study (37). The threshold for bacteria in blood was analyzed at 1 × 10^2 CFU/ml because this bacterial concentration in blood has been reported in patients with gram-negative bacteremia with high incidence of death (25, 69). Because the repeated measurements were performed on systemic data following acute airway infection, we included data at 1 h and thereafter, when systemic responses occur. Some animals died after 5 h; therefore, we analyzed these data from 1 to 5 h. Plasma cytokine concentrations were only compared at 6 h. P < 0.05 was considered statistically significant.

**RESULTS**

**Confirmation of depletion of macrophages.** Carbon particles injected intravenously were ingested by phagocytes in the reticuloendothelial system, allowing detection of these cells. The liver and spleens of the clodronate-liposome-treated rabbits contained relatively few carbon particles compared with the liver and spleens taken from the control rabbits (Fig. 1). The quantities of carbon in the liver and spleens of the clodronate-liposome-treated rabbits were 5.4% ± 1.5% and 1.1% ± 0.2%, respectively, of the quantity present in normal rabbits. In contrast, the number of alveolar macrophages in the lungs of the clodronate-liposome-treated rabbits (30 ± 2.8/low-magnification field) did not differ from the number in the vehicle-treated rabbits (26 ± 9.5-low-magnification field) (P = 0.45; Fig. 1, E and F).

**Hemodynamics.** MAP decreased gradually in the normal rabbits 2 h after the bacterial inoculation (Fig. 2A). The MAP of the macrophage-depleted rabbits remained at the baseline value for the first 2 h; however, after 2 h, it decreased more rapidly (P = 0.0004) than that of the normal rabbits. Some rabbits died after 5 h in the macrophage-depleted group, and no rabbit survived to the end of the 8-h experiment in the macrophage-depleted group. In contrast, only one rabbit died between 7 and 8 h after the infection in the normal group.

**Airway pressure changes.** Peak airway pressure was initially about 18–21 cmH2O in each group and increased after the bacterial instillation, reaching 28–31 cmH2O in both groups at the end of the experiment.

**Bacterial-induced lung injury assessed with bidirectional protein movement.** The quantity of 125I-albumin tracer that left the air spaces and entered the circulation was measured by repeated sampling of arterial blood and expressed as the percentage of the instilled tracer that was present in circulating plasma (Fig. 3A). The percentage of the 125I-albumin tracer in plasma increased in a time-dependent manner in both the macrophage-depleted and normal rabbits. The increase started earlier and the slope was steeper in the macrophage-depleted group than the normal group, and thus, in the macrophage-depleted rabbits, it took significantly less time for the percentage of 125I-albumin in blood to reach 10% (P = 0.0097) or 20% (P = 0.0032) of the instilled 125I-albumin tracer compared with normal rabbits. The plasma 125I-albumin levels in the noninfected rabbits (control), both depleted and nondepleted, were low and identical (data not shown). The percentage of the 125I-albumin tracer that appeared in the urine at the end of the experiment was low in both groups (0.04% ± 0.05% and 0.19% ± 0.16%, in macrophage-depleted and nondepleted rabbits,
groups, respectively). The percentage of the plasma $^{131}$I-albumin tracer that was injected intravenously 30 min before the bacterial inoculation declined in a time-dependent manner in both groups, slightly faster in the normal group compared with the depleted group (Fig. 3B). Therefore, the macrophage depletion protocol did not have a major effect on the rate of albumin clearance from plasma. There was no difference in the entry of $^{131}$I-albumin from the plasma into the lungs in macrophage-depleted and nondepleted rabbits (Table 2). These values were both higher than in uninfected rabbits ventilated for 8 h (0.24 ± 0.10), but the difference did not reach statistical significance. Thus, in the macrophage-depleted rabbits with pneumonia, there was a significant increase in the movement of albumin from the alveolar space to the vascular space but no increase in movement from the vascular space into the lungs.

**Bacterial-induced lung edema.** Lung edema was measured as the lung W/D. There was no difference in the W/D of the instilled lung between macrophage-depleted and nondepleted rabbits (Table 2). However, these values were both significantly ($P < 0.01$) elevated compared with the W/D of right lungs of uninfected rabbits ventilated for 8 h in the right lateral decubitus position (6.42 ± 0.44).

**Assessment of bacteremia and bacteria in the instilled lung.** We assessed the degree of bacteremia every 15 min by obtaining blood samples of arterial blood. In the normal rabbits, bacteremia was detected between 3 and 4 h after the instillation of bacteria, and the number of bacterial colonies increased rapidly (Fig. 3C). The onset of bacteremia occurred ~2 h after the beginning of the protein leak out of the lungs, measured by $^{125}$I-albumin leakage (Fig. 3A). In contrast, bacteremia was detected earlier in the macrophage-depleted group of rabbits, between 1 and 2 h after the instillation, and the time to reach a bacterial threshold of $1 \times 10^2$ CFU/ml in blood was significantly shorter in the macrophage-depleted rabbits compared with the normal rabbits (Fig. 3C). There was no statistical difference ($P = 0.97$) in the number of bacteria remaining in the instilled lungs at the end of the experiments between the depleted and normal rabbits (Table 2).

**Number of bacteria in the liver and spleen.** The number of bacteria in the liver and spleen was measured using radiolabeled bacteria. The number of bacteria in the livers ($P < 0.05$) and spleens ($P < 0.0001$) were significantly less in the macrophage-depleted rabbits compared with the number in the normal rabbits (Fig. 3D).
Pathogenesis of pneumonia.

Fig. 2. Hemodynamics and acid-base status of rabbits. Displayed are mean arterial pressure (MAP; A), % change of cardiac output from the baseline values (B), and base excess (C). Values are means ± SE. The initial number of rabbits was 5 in each group. Some rabbits died before the end of the 8-h experiment, and the number of surviving animals is presented in parentheses. The bacterial suspension was instilled at 0 h. *P < 0.05 compared with the normal group.

**DISCUSSION**

The major goal of this study was to investigate the role of phagocytes in the reticuloendothelial system during *P. aeruginosa* pneumonia and sepsis using the systemic macrophage depletion method of Van Rooijen and Sanders (64). This study documents that the depletion of macrophages/phagocytes in the reticuloendothelial system leads to more prominent bacteremia and systemic inflammation during *P. aeruginosa* pneumonia.

Near the end of the experiment, the number of surviving rabbits in the macrophage-depleted group was significantly lower than in the normal rabbits (Fig. 5A). The IL-10 concentration in the liver 5 h after infection was significantly higher than in the normal group (P < 0.05) (Fig. 5A). The IL-10 concentration in the spleen in macrophage-depleted rabbits was significantly lower than in normal rabbits (P < 0.05). The IL-10 concentration in the liver 5 h after infection in the macrophage-depleted rabbits was significantly lower than in normal rabbits (P < 0.001; Fig. 5B).

**Effect of exogenous IL-10 in macrophage-depleted and normal rabbits.** Pretreatment of rabbits with IL-10 caused significant improvement in the physiology and bacteriology of the macrophage-depleted rabbits. Compared with the depleted/vehicle group, the depleted/IL-10 group had a significantly less rapid decline in MAP (P = 0.0082) and cardiac output (P = 0.036) (Table 3). There was a trend for the IL-10 treatment to delay the appearance of the alveolar 125I-albumin tracer in plasma compared with depleted/vehicle rabbits (Fig. 3A). There was also a trend toward a longer time to reach a bacteremia threshold of 1 × 10^2 CFU/ml in the depleted/IL-10 group compared with the depleted/vehicle group (P = 0.15; Fig. 3C). Finally, the concentrations of proinflammatory cytokines in the plasma were reduced with IL-10 administration (TNF-α, P = 0.09; IL-8, P < 0.05; Fig. 4A). In contrast, IL-10 pretreatment did not affect the quantity of bacteria in the instilled lungs at the end of the experiment (P = 0.73) nor did it improve the lung endothelial protein permeability (131I-albumin leak) (Table 2).

In the normal rabbits, IL-10 treatment decreased the slope of the decline in MAP and cardiac output compared with the normal/vehicle rabbits (Table 3). In contrast, there were no appreciable differences in the lung injury as assessed by 131I-albumin leak (P = 0.56), W/D (P = 0.40; Table 2), or by 125I-albumin leak (data not shown) between the normal/IL-10 and normal/vehicle groups.
Depletion of phagocytes in the reticuloendothelial system promotes early and augmented bacteremia in rabbits with pneumonia and shifts cytokine balance toward the inflammatory side. Our results suggest that macrophages in the reticuloendothelial system limit inflammation by ingesting bacteria that enter the circulation and/or by augmenting the clearance of bacteria by other cell types. In addition, the data suggest that systemic release of IL-10 by macrophages has a role in limiting systemic inflammation.

**Macrophage depletion and systemic inflammation.** Some reports have shown a reduction of circulating proinflammatory cytokines in macrophage-depleted rabbits. Bautista et al. (1) reported that the elimination of Kupffer cells suppressed TNF-α release in response to intravenous administration of LPS in vivo. Luster et al. (41) showed that hepatic TNF-α secretion was reduced in mice that had Kupffer cells depleted and were then treated with intravenous injections of LPS. Thus Kupffer cells appear to be a major source of circulating TNF-α in LPS-treated mice. Fujimoto et al. (23) showed that systemic macrophage depletion caused a decreased TNF-α concentration in plasma in mice with bacterial pneumonia. In that study, the cytokine measurements were done only once in the course of pneumonia, so the dynamic inflammatory responses were not studied. Emmanuilidis et al. (16) demonstrated that Kupffer cells also are a major source of systemic IL-10 in mice with septic peritonitis. They showed that the concentration of TNF-α in the systemic circulation was higher in mice that were depleted of Kupffer cells using clodronate-liposomes, suggesting a counterregulatory role for Kupffer cells in mice with abdominal infections. Kono et al. (34) provided direct evidence that serum IL-10 levels were lower in rats with peritonitis when Kupffer cells were depleted and that the IL-10 concentration further declines when phagocytes in the reticuloendothelial system are depleted and that the IL-10 concentration further declines after the infection, exaggerating the differences with normal rabbits (Fig. 5B). These findings support and extend the work of Emmanuilidis et al. (16) and Kono et al. (34). In addition,
The decrease in anti-inflammatory cytokines produced by the reticuloendothelial system and the increase in proinflammatory cytokines in the circulation of the depleted rabbits provide support for an important role for macrophages of the reticuloendothelial system in animals with sepsis from localized pneumonia. In contrast to TNF-α and IL-8, there was no difference in the plasma concentration of GRO in the macrophage-depleted and nondepleted rabbits (Fig. 4C), suggesting that cells other than macrophages in the reticuloendothelial system are the source of this chemokine. Finally, a delay in the appearance of MCP-1 in the systemic circulation and then a

**Fig. 4.** A–D: concentration of cytokines in the plasma. Displayed are means ± SE of 5 rabbits in each group. *P < 0.05 compared with the normal rabbits. GRO, growth-regulated oncogene; MCP-1, monocyte chemoattractant protein-1.

the concentrations of proinflammatory cytokines, including TNF-α and IL-8, in the systemic circulation increase in bacterial pneumonia when macrophages in the reticuloendothelial system are depleted (Fig. 4, A and B).

**Fig. 5.** Concentrations of TNF-α and IL-10 in the liver, spleen, and lungs. Liver, spleen, or lungs of normal or macrophage-depleted rabbits were harvested 5 h after the inoculation of bacteria and then homogenized and lysed. The supernatant of the lysed homogenate was assessed for TNF-α (A) and IL-10 (B) concentrations. Data are shown as means ± SE of each cytokine concentration divided by total protein concentration in the supernatant. *P < 0.05 compared with the value at 0 h. §P < 0.05 compared with the value in normal rabbits.
large increase of this chemokine at a later time in the macrophage-depleted rabbits compared with the nondepleted rabbits (Fig. 4D) suggests that there are important sources of MCP-1 aside from macrophages in the reticuloendothelial system and that macrophages in the reticuloendothelial system have a regulatory function on the production of this chemokine.

When macrophages of the reticuloendothelial system were present, IL-8 was not detectable in plasma, whereas GRO and MCP-1 were found at early times after infection. This could be partly explained by selectivity in translocation of chemokines from the alveolar compartment into the circulation. Quinton et al. (56) showed a selective translocation of cytokine-induced neutrophil chemoattractant (CINC) from the lungs to the systemic circulation in rats after intratracheal LPS, whereas another CXC chemokine, macrophage inflammatory protein (MIP)-2, remained in the air spaces. In addition, the Duffy antigen binds to plasma chemokines in the circulation and functions as a chemokine reservoir that regulates inflammation (40). Thus the plasma free cytokine concentrations are the net result of several factors: 1) cytokine production in the air space; 2) translocation of cytokines from the alveolar compartment to the circulation; 3) binding to Duffy antigen or other nonfunctional receptors in the circulation; and 4) cytokine production in the circulation. The high concentration of TNF-α and IL-8 in the macrophage-depleted group (Fig. 4, A and B) could be explained by the extent of lung injury (Fig. 3A), which allowed these cytokines to escape from the lungs into the circulation and exceed the binding capacity of red blood cells and peripheral tissues. In addition, the high number of circulating bacteria could also have been a direct stimulus for TNF-α or IL-8 production in blood.

Macrophage depletion caused more severe bacteremia. The macrophage-depleted rabbits had more bacteria in their blood than nondepleted rabbits in this acute *P. aeruginosa* air space infection. In the air space, cells of the monocyte/macrophage lineage as well as migrating polymorphonuclear leukocytes (PMNs) are important in the clearance of bacteria. In the systemic circulation, PMNs play a central role in the clearance of bacteria (10, 63, 65). Our data show that phagocytes in the reticuloendothelial system also have a critical role in controlling bacteria that reach the systemic circulation. Gregory et al. (24) reported that a neutrophil-Kupffer cell interaction improved host defense in systemic bacterial infections. They showed that the uptake of bacteria by the liver at 10 min postinfection was reduced from ~60% of the intravenous inoculum in normal mice to ~15% in mice rendered Kupffer-cell deficient. The reduced number of bacteria in the livers and spleens in our macrophage-depleted rabbits compared with the number of bacteria in the normal rabbits (Fig. 3D) is consistent with the study of Gregory et al. (24). The earlier and more severe bacteremia in the macrophage-depleted rabbits in the present study (Fig. 3C) is consistent with loss of phagocytosis and killing of circulating bacteria by phagocytes of the reticuloendothelial system. The data suggest that the reticuloendothelial system has an important early role in clearing bacteria that reach the bloodstream from a localized site of infection because bacteremia was not detectable in the first 3 h after infection in normal rabbits (Fig. 3C), even though intrapulmonary alveolar was already detectable in the circulation (Fig. 3A).

**Macrophage depletion caused hemodynamic instability.** In the present study, macrophage-depleted rabbits had more severe physiological aberrations following *P. aeruginosa* pneumonia than the nondepleted rabbits, consistent with the earlier onset of bacteremia. Macrophage-depleted rabbits had lower systemic blood pressure and lower cardiac output than nondepleted rabbits. These findings were associated with higher plasma concentrations of TNF-α than in the normal rabbits. TNF-α has been implicated in a number of studies as an important mediator of myocardial dysfunction, as indicated by the following parameters: depression of maximum extent and peak velocity of myocyte shortening in vitro (36); decreased left atrial force and velocity of contraction in an isolated heart (27); hypotension, tachycardia, and decrease in cardiac index (20); and decreased left ventricular systolic contractility and...
diastolic function (49). Our findings are consistent with these previous studies because higher plasma concentrations of TNF-α were associated with hemodynamic instability in the macrophage-depleted rabbits.

**Macrophage depletion caused more lung injury.** Macrophage-depleted rabbits had more severe lung epithelial injury than normal rabbits, as indicated by an increase in the transalveolar flux of 125I-albumin into plasma (Fig. 3A), which is a reflection of lung epithelial injury (45, 67). We found that the radioactivity in urine was low, suggesting that the difference in the quantity of 125I-albumin in the circulation is not likely to be explained by the difference in the rate of the clearance of the tracer from the circulation into the urine. Furthermore, macrophages in the reticuloendothelial system in the normal rabbits could be responsible for the lower concentration of the alveolar tracer in plasma compared with the macrophage-depleted rabbits because of uptake of 125I-albumin by macrophages. Berthiaume et al. (4) evaluated the clearance of the albumin from the air spaces following instillation of 125I-albumin into sheep lungs. They found that the quantity of 125I-albumin present in the phagocytic cells of lungs was less than 1% of the instilled amount. In the present experiment, our data on the clearance of 131I-albumin from plasma of normal and macrophage-depleted rabbits (Fig. 3B) suggests that systemic macrophages have only a small effect on the clearance of albumin from plasma. Therefore, it is unlikely that the higher concentrations of 131I-albumin in plasma of clodronate-treated rabbits with pneumonia can be explained by slower systemic clearance of 131I-albumin or reduced passage of 131I-albumin into urine. The differences in the amount of alveolar protein tracer in plasma are most likely a reflection of differences in alveolar epithelial injury.

Even though the macrophage-depleted rabbits did not have an increase in lung endothelial injury as measured by 131I-albumin flux into the lungs, the increased epithelial permeability might have promoted the leak of TNF-α from the air spaces into the systemic circulation, as we (37) have shown in a prior study, resulting in higher concentration of circulating TNF-α (Fig. 4). The mechanism for the increased epithelial permeability is not clear because the number of bacteria in the lungs was not increased in the macrophage-depleted rabbits (Table 2). The data suggest that systemic bacteremia, or the uncontrolled systemic inflammatory response, has a previously unrecognized effect on lung epithelial permeability. Further studies are warranted to clarify the mechanisms involved.

In all groups, mechanical ventilation was maintained with an FiO2 of 1.0 at a tidal volume of 20 ml/kg body wt and positive end-expiratory pressure of 3 cmH2O. Prolonged exposure to a high FiO2 can cause hyperoxia-induced lung injury; however, the duration of this study was only 8 h, so the potential adverse effect of hyperoxia was limited. With the tidal volume, peak airway pressure was initially 18–21 cmH2O and rose to 28–31 cmH2O at the end of the experiment, and there was no difference in the airway pressure between groups. Importantly, all of the rabbits were treated with the same FiO2, and ventilator settings, so these variables cannot explain the differences in the lung injury between the groups.

**Methods of depleting phagocytes in the reticuloendothelial system.** There are many ways to eliminate macrophages or to suppress their functions; however, some methods have adverse effects that affect immune responses. Silica, carrageenan, dextran sulfate, and gadolinium stimulate macrophages to produce inflammatory mediators such as IL-1β (58, 60), TNF-α (30, 53, 58), IL-6 (66), and nitric oxide (NO; Refs. 8, 32). Carrageenan and dextran sulfate also have important effects on lymphocytes (50, 52). In contrast, the clodronate-liposomes method is advantageous because nonphagocytic cells are not affected by this drug (64). In addition, clodronate-liposomes do not affect the number of lymphocytes or the number and function of neutrophils (9, 55). The efficacy of the depletion is also very important. Clodronate-liposomes are efficient in depleting systemic macrophages (46, 51), and the depletion of macrophages was almost complete in the liver and spleen in the present study (Fig. 1, A–D). In contrast, the number of alveolar macrophages was not affected by intravenous clodronate-liposome administration (Fig. 1, E and F).

We did not evaluate the potential for LPS contamination of the clodronate-liposomes, but the fact that the clodronate-liposomes were given 36 h before the experiment suggests that the effects of contaminating LPS would be minimal by the time of the experiment (43, 44). In fact, plasma cytokine concentrations including TNF-α, IL-8, GRO, and MCP-1 at the beginning of the experiment (before instillation of bacteria) were almost undetectable (Fig. 4).

**Effect of exogenous IL-10 in sepsis.** A number of studies have shown that the administration of IL-10 leads to suppression of innate immunity. Laichalk et al. (38) examined the ability of PMNs to phagocytose and kill *E. coli* in vitro and showed that IL-10 inhibited phagocytosis and killing. Rolloides et al. (57) investigated the antibacterial and antifungal activities of human PMNs against *S. aureus* and *Candida albicans* and showed that IL-10 suppressed the ability of PMNs to phagocytose these organisms. Capsoni et al. (7) investigated the ability of IL-10 to modulate the activities of human neutrophils in vitro. They showed that culture of PMNs with IL-10 downregulated their capacity to produce O2 and decreased their capacity to lyse antibody-coated sheep erythrocytes. Steinhauser et al. (61) showed that when an antibody blocking IL-10 was administered to mice with sepsis due to *P. aeruginosa* pneumonia, the mice had improved survival and increased clearance of bacteria from the lungs.

In contrast, we have previously shown that treatment of rabbits (37) and mice (59) with recombinant IL-10 before *P. aeruginosa* air space infections decreased the number of bacteria in the circulation and mortality. In the present study, the administration of exogenous rhIL-10 to the macrophage-depleted rabbits was associated with significantly slower decline in blood pressure and cardiac output (Table 3). Administration of rhIL-10 slowed the leakage of 125I-albumin into the systemic circulation and reduced the increase in bacteremia in animals whose phagocytes in the reticuloendothelial system were depleted. These results support and extend the work of Kono et al. (34), who reported that blocking IL-10 using an anti-IL-10 antibody aggravated lung injury and mortality associated with cecal ligation and puncture.

**Conclusions.** In conclusion, this study suggests that macrophages of the reticuloendothelial system have two important functions in rabbits with severe *Pseudomonas* pneumonia. First, systemic macrophages clear bacteria that leak from the lungs during the early phase of infection, thereby minimizing early bacteremia and systemic cytokine production. Second, reticuloendothelial macrophages may produce a counterregu-
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Latory response, which includes the production of IL-10. Treatments such as exogenous IL-10, which enhance this counter-inflammatory response, are associated with better hemodynamics when systemic macrophage responses are impaired. Systemic defense mechanisms mediated by phagocytes of the reticuloendothelial system are crucial in sepsis induced by localized bacterial pneumonia.

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