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A H Limper, J S Hoyte, J E Standing

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### Research Article

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# The Role of Alveolar Macrophages in *Pneumocystis carinii* Degradation and Clearance from the Lung

Andrew H. Limper,\*† Jacques S. Hoyte,\* and Joseph E. Standing\*

\*Thoracic Diseases Research Unit, Division of Pulmonary, Critical Care and Internal Medicine, Department of Medicine, and

†Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, Minnesota 55905

## Abstract

Although studies indicate that alveolar macrophages participate in host defense against *Pneumocystis carinii*, their role in organism degradation and clearance from the lung has not yet been established. We, therefore, quantified the uptake and degradation of  $^{35}\text{S}$ -labeled *P. carinii* by cultured macrophages, demonstrating significant degradation of *P. carinii* over 6 h. We further evaluated the role of macrophages in elimination of *P. carinii* from the living host. Rats received either intratracheal PBS, liposomal PBS (L-PBS), or liposomal dichloromethylene diphosphonate (L-Cl<sub>2</sub>MDP), a preparation which leads to selective depletion of macrophages. Over 72 h, L-Cl<sub>2</sub>MDP-treated animals had loss of > 85% of their alveolar macrophages. In contrast, L-PBS-treated rats had cellular differentials identical to rats receiving PBS. Macrophage-depleted rats and controls were next inoculated with *P. carinii* and organism clearance was determined after 24 h. *P. carinii* elimination was evaluated with both cyst counts and an ELISA directed against glycoprotein A (gpA), the major antigen of *P. carinii*. Both assays indicated that macrophage-depleted rats had substantial impairment of *P. carinii* clearance compared to L-PBS- or PBS-treated rats. These data provide the first direct evidence that macrophages mediate elimination of *P. carinii* from the living host. (J. Clin. Invest. 1997; 99:2110–2117.) Key words: *Pneumocystis carinii* • macrophage • liposome • phagocytosis • bisphosphonates

## Introduction

*Pneumocystis carinii* remains a significant cause of life-threatening pneumonia in immunocompromised patients, particularly those with AIDS, hematological or solid malignancies, organ transplantation, or inflammatory conditions treated with immunosuppressive agents (1–8). Host defenses against *P. carinii* are poorly understood, but involve a number of cellular mediators, most notably CD4 lymphocytes whose depletion promotes development of *P. carinii* pneumonia (9, 10). Additional

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Address correspondence to Andrew H. Limper, Thoracic Diseases Research Unit, 601A Guggenheim Building, Mayo Clinic and Foundation, Rochester, MN 55905. Phone: 507-284-2301; FAX: 507-284-4521; E-mail: limper.andrew@mayo.edu

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studies implicate roles for alveolar macrophages, CD8 lymphocytes, and neutrophils in host defense during *P. carinii* pneumonia (11–16).

Accumulating evidence indicates that alveolar macrophages provide a number of key functions during *P. carinii* infection (12, 13, 17–23). Ultrastructural and in vitro experiments demonstrate that macrophages bind and internalize *P. carinii* (12, 13, 17, 20, 24). Macrophage mannose receptors mediate uptake of the organisms through interaction with glycoprotein A (gpA),<sup>1</sup> a 120-kD antigenic complex present on the surface of *P. carinii* (17, 20, 25–32). Additional in vitro studies demonstrate that interaction of *P. carinii* with alveolar macrophages causes release of proinflammatory substances including reactive oxidants, arachidonic acid metabolites, and TNF- $\alpha$  (18, 19, 21, 33, 34).

The role of alveolar macrophages in *P. carinii* degradation and clearance from the lungs has not yet been established. This study was, therefore, undertaken to quantify the binding, uptake, and degradation of *P. carinii* organisms by macrophages. We further provide evidence that alveolar macrophages mediate degradation and clearance of *P. carinii* organisms from the intact host.

## Methods

**Materials.** Monoclonal antibody 5E12 recognizing *P. carinii* gpA was provided by Dr. Frank Gigliotti (University of Rochester, Rochester, New York) (35). Concanavalin A conjugated to horseradish peroxidase was obtained from E.Y. Laboratories, Inc. (San Mateo, CA). Ciprofloxacin was from Miles Pharmaceuticals (West Haven, CT). Initial samples of liposomal dichloromethylene diphosphonate were a kind gift from Drs. Yongmoon Han and Jim Cutler (Montana State University, Bozeman, MT) (36). Pamidronate was purchased from Ciba-Geigy Corp. (Suffern, NY).

**Preparation of *P. carinii*.** All animal studies were approved by the institutional animal care and utilization committee. *P. carinii* pneumonia was induced in Harlan Sprague-Dawley rats by immunosuppression with dexamethasone and transtracheal injection with *P. carinii* (37–40). Pathogen-free rats were provided with drinking water containing dexamethasone (2 mg/liter), tetracycline (500 mg/liter), and nystatin (200,000 U/liter) and fed an 8% protein diet (Teklad Premier Laboratory Diets, Madison, WI). Each week, the animals received oral ciprofloxacin (0.45 g/liter) for two consecutive days to reduce the risk of bacterial infections (41). After 5 d of immunosuppression, rats were transtracheally inoculated with *P. carinii* (~ 500,000 cysts) prepared by homogenizing infected rat lung. The rats were suppressed for an additional 4–8 wk, killed, and the lungs homogenized in 50 ml

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1. Abbreviations used in this paper: BAL, bronchoalveolar lavage; [ $^{14}\text{C}$ ]UDPG, [ $\text{glucose-}^{14}\text{C}$ ]uridine diphosphate glucose; gpA, the glycoprotein A surface antigenic complex of *P. carinii*; L-Cl<sub>2</sub>MDP, liposomal dichloromethylene diphosphonate; L-PAM, liposomal pamidronate disodium; L-PBS, liposomal PBS.

of HBSS using a laboratory blender. *P. carinii* were purified by differential filtration. The homogenates were exhaustively filtered through 10- $\mu$ M filters which retain lung cells but allow passage of *P. carinii* organisms. The filtrates were collected, centrifuged (1,500 g for 30 min), and the pellets resuspended in 5 ml of HBSS. Duplicate 10  $\mu$ l aliquots of suspension were spotted onto slides, stained with Diff Quick, and *P. carinii* quantified as described (40, 42). If other microorganisms were noted in the smear or on microbiologic culture the material was discarded.

**Preparation of gpA and monoclonal antibody to gpA.** The major surface antigenic complex of *P. carinii*, gpA, was purified by continuous-flow gel electrophoresis as we previously reported (20, 22). In brief, isolated *P. carinii* were solubilized in 125 mM Tris, 4% SDS, 4% 2-mercaptoethanol, 0.002% bromophenol blue, and 20% glycerol, pH 7.4. This extract was separated on a 10% polyacrylamide preparative tube gel (PrepCell Apparatus; Biorad, Hercules, CA). The gel was resolved over 48 h using 35-mA current and eluted with 25 mM Tris Base, 192 mM glycine, and 0.1% SDS. Fractions were analyzed by SDS-PAGE and silver staining on 4–15% gradient resolving gels. Fractions containing the complex migrating at 120 kD were pooled, dialyzed, and concentrated. Immunoblotting of the purified gpA complex was performed using monoclonal antibody 5E12 to verify the identity of the product (20, 35). Monoclonal 5E12 is a mouse anti-*P. carinii* IgM-recognizing gpA from rodents, ferrets, and humans (35).

A second monoclonal antibody, LM-1, recognizing gpA was generated in the following manner. Balb/c mice were immunized with purified gpA and the spleens fused with SP2/OAg14 myeloma cells using previously published protocols (43, 44). Fusions were cultured in 96-well plates in the presence of hypoxanthine aminopterin thymidine (HAT) selection medium, and growth-positive wells assessed by ELISA to gpA. Antibody-positive colonies were cloned by limiting dilution, recloned antibody secreting cells expanded in tissue culture, and grown as ascites tumors in pristane-primed Balb/c mice. Ascites fluid was harvested and treated with protease inhibitors including 2 mM PMSF, 10 mM EDTA, and 1 mM *N*-ethyl maleimide. Monoclonal IgG was purified from the ascites by protein A-Sepharose affinity chromatography.

**Determination of *P. carinii* binding, uptake, degradation, and viability.** The initial binding of radiolabeled *P. carinii* to macrophages was quantified as follows. Alveolar macrophages ( $1 \times 10^6$ ) were plated overnight in 24-well tissue culture dishes using mixed medium (RPMI/medium 199, 1:1) containing 20% FBS at 37°C. *P. carinii* were radio-labeled by incubating organisms ( $400 \times 10^6$ ) for 18 h in 2 ml DME with 250  $\mu$ Ci [<sup>35</sup>S]cysteine/methionine (Trans Label, NEN Research Products, Boston, MA). Next, radiolabeled *P. carinii* were washed with DME and BSA (1 mg/ml) to remove unincorporated label. <sup>35</sup>S-labeled *P. carinii* ( $4 \times 10^6$ /well) were then allowed to attach to macrophages in DME and BSA (1 mg/ml) at 37°C for 0–6 h. Unattached *P. carinii* were removed by washing, and the percentage of organisms associated with macrophages quantified.

To quantify *P. carinii* uptake and degradation by macrophages, *P. carinii* were similarly labeled overnight with [<sup>35</sup>S]cysteine/methionine (250  $\mu$ Ci, <sup>35</sup>S-Trans label; NEN) and washed to remove unincorporated label. <sup>35</sup>S-*P. carinii* were allowed to bind to the macrophages over 0–6 h. *P. carinii* uptake was determined as follows. Nonbound *P. carinii* were removed by washing. Bound but nonphagocytized *P. carinii* were removed from the macrophages by digestion with trypsin (2  $\mu$ g/ml) in EDTA (0.2 mg/ml) for 15 min on ice. The macrophages were then separated from the liberated *P. carinii* by differential centrifugation in which the macrophages, but not the released *P. carinii*, were pelleted at 400 g for 5 min. Phagocytized *P. carinii* were counted in the macrophage pellet. The supernatant was spun again (2,000 g for 10 min) to bring down nonbound *P. carinii*. *P. carinii* degradation was determined by the amount of soluble <sup>35</sup>S-label released into the medium. To correct for spontaneous release of <sup>35</sup>S-label from *P. carinii*, radiolabeled organisms were treated in an identical fashion without macrophages. Spontaneous release of <sup>35</sup>S was sub-

tracted from the amount released in the presence of macrophages to yield a net measurement of *P. carinii* degradation.

Additional experiments were undertaken to determine whether alveolar macrophages suppress *P. carinii* viability in culture. Alveolar macrophages ( $0.5 \times 10^6$ ) were plated overnight in 48-well tissue culture dishes in mixed medium with 20% FBS at 37°C. The next day, the macrophages were rinsed with DME containing BSA (1 mg/ml) and incubated with *P. carinii* organisms ( $4 \times 10^6$ ) in DME containing BSA for 6 h at 37°C. Bound *P. carinii* were released from the macrophages by treatment with trypsin as described above. In addition, internalized *P. carinii* were released by freezing and thawing the specimens twice and by passage through a 23-gauge needle. Next, *P. carinii* were separated free of the macrophage component by differential centrifugation in which the macrophage component was pelleted at 400 g for 5 min, and the free *P. carinii* subsequently collected by centrifugation at 2,000 g for 10 minutes. Control *P. carinii* were cultured in the absence of macrophages, and subjected to identical digestion, freeze-thaw, and centrifugation conditions. Subsequently, *P. carinii* viability was determined by the ability of aliquots of *P. carinii* to incorporate [<sup>14</sup>C]uridine diphosphate glucose ([<sup>14</sup>C]UDPG; NEC 403; NEN) into cell wall  $\beta$ -glucan (45). Following the differential centrifugation, the *P. carinii* pellets were resuspended in 30  $\mu$ l of 50 mM Hepes, containing 0.05% sodium deoxycholate, pH 7.40. To each 30- $\mu$ l aliquot was added 20  $\mu$ l of 1 mM cold UDPG and 1 mM EDTA containing 0.25  $\mu$ Ci of [<sup>14</sup>C]UDPG. The reactions were incubated at 37°C for 4 h. Aliquots were spotted and dried onto glass filter paper discs, washed twice with 20% trichloroacetic acid, twice with dry acetone, and counted. To verify that macrophage components were not significantly contaminating the final preparation nor contributing to measured incorporation of [<sup>14</sup>C]UDPG, macrophages were also cultured in the absence of *P. carinii* and processed in an identical manner.

**Generation of liposomal bisphosphonate compounds.** Liposomes containing bisphosphonates are selectively phagocytized by alveolar macrophages resulting in depletion of these cells from the lung (46). Liposomes were generated containing either dichloromethylene diphosphonate or pamidronate disodium according to the method of Van Rooijen (47). In brief, multilamellar liposomes were formed from 75 ml of L- $\alpha$ -phosphatidyl choline (100 mg/ml) and 11 ml of cholesterol (100 mg/ml) dissolved in methanol and chloroform (1:1 vol). After removal of the solvent by low-vacuum rotary evaporation, the lipid film was dissolved in chloroform (10 ml). The thin lipid film formed after a second evaporation was dispersed for 15 min in 10 ml of PBS containing either 1.89 g of dichloromethylene diphosphonate to form liposomal dichloromethylene diphosphonate (L-Cl<sub>2</sub>MDP) liposomes or 120 mg of pamidronate disodium to form liposomal pamidronate disodium (L-PAM) liposomes, respectively. These suspensions were subjected to four freeze-thaw cycles, incubated for 2 h (25°C), and sonicated for 3 min. After incubation for an additional 2 h, the liposomes were diluted in PBS (90 ml) and centrifuged at 100,000 g for 30 min. The liposomes were either used immediately or stored under nitrogen at 4°C for up to 2 wk. For control liposomes, liposomes were formed in the presence of PBS alone in an identical fashion (liposomal PBS [L-PBS]).

**Role of alveolar macrophages in *P. carinii* clearance in vivo.** Rats were administered equipotent doses of either L-Cl<sub>2</sub>MDP (0.2 ml) or L-PAM (0.6 ml) or corresponding volumes of PBS or L-PBS by intratracheal installation (48, 49). After 72 h, macrophage depletion was assessed by whole-lung lavage (50 ml). The lavage was processed and differential counts performed on at least 200 consecutive cells on cytopreparation smears (50). Initial studies indicated that liposomal bisphosphonate treatment eliminated most alveolar macrophages from the lavage resulting in the formation of morphologically distinct remnant macrophages. Remnant macrophages were defined as those cells which did not possess clear evidence of cytoplasm and a limiting cellular membrane, and characteristically consisted of only a condensed nuclear body.

To evaluate the role of macrophages in *P. carinii* clearance from

an intact host, rats were depleted of alveolar macrophages by intratracheal instillation of L-CL<sub>2</sub>MDP, L-PBS, or PBS (0.2 ml). After 72 h, the animals were challenged transtracheally with *P. carinii* ( $1 \times 10^7$ ). After an additional 24 h, rats were killed and whole-lung lavage performed with 50 ml of HBSS. The BAL was centrifuged (800 g for 10 min) to pellet inflammatory cells and associated *P. carinii*. Pellets were resuspended in 1 ml of HBSS. Cytopreparation smears were prepared from equivalent quantities of BAL suspension (150  $\mu$ l) and stained with methenamine silver to visualize *P. carinii* cysts. Cyst burdens were counted in 40 contiguous oil immersion fields from each slide as previously described (1). The coefficient of variation for this method is  $\sim 7.5\%$  (1).

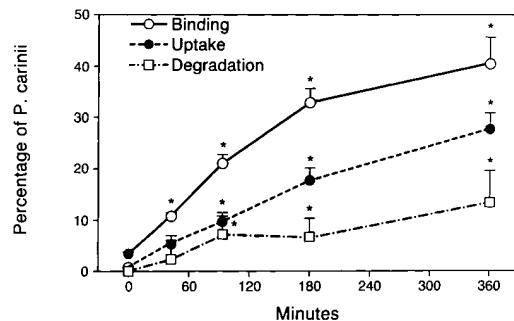
**Determination of total *P. carinii* gpA by ELISA.** As a further measure of *P. carinii* degradation and clearance from the lung, an ELISA was developed which measures total *P. carinii* gpA present in specimens. Animals received intratracheal injection of either PBS, L-PBS, or L-PAM (0.6 ml each). After 72 h of macrophage depletion, the animals were transtracheally inoculated with *P. carinii* ( $1 \times 10^7$ ). After an additional 24 h, animals underwent BAL (50 ml), and the BAL centrifuged (800 g for 10 min) to pellet inflammatory cells and associated *P. carinii*. The pellets were dissolved in 1% SDS with 15 mM dithiothreitol. Subsequently, samples were treated with iodoacetamide (30 mM), and mixed micelles formed by adding 1% Triton X-100 (4:1 by vol). The gpA contained in the samples was quantified by capture ELISA. Monoclonal antibody recognizing gpA (monoclonal LM-1; 15  $\mu$ g/ml in 0.1 M NaHCO<sub>3</sub>) was coated onto plastic plates overnight at 4°C. The plates were washed and blocked with 1% TWEEN-20 in TBS for 1 h. After washing, samples were applied to the wells, incubated at room temperature for 2 h, and washed extensively. Next, the wells were treated with concanavalin A conjugated to horseradish peroxidase (25  $\mu$ g/ml) for 2 h, again washed, and the ELISA developed with *o*-phenylene diamine substrate.

**Statistical analysis.** Differences between multiple data groups were first assessed by ANOVA. Paired data groups were compared using Student's *t* tests. Statistical testing was performed using the Statview II statistical package (Abacus Concepts, Inc., Berkeley, CA), with  $P \leq 0.05$  defining a statistically significant difference.

## Results

**Alveolar macrophages bind, take up, and degrade, *P. carinii* in culture.** We first determined the ability of cultured alveolar macrophages to mediate the binding, uptake, and degradation of *P. carinii* (Fig. 1). *P. carinii* were radiolabeled and macrophage-mediated binding, uptake, and degradation of *P. carinii* ( $P < 0.05$  each compared to that parameter measured at time zero). Each parameter increased over the 6 h of study. These data demonstrate that normal alveolar macrophages in culture mediate not only the binding and uptake of *P. carinii*, but also degrade the organisms.

Additional experiments were performed to further determine whether alveolar macrophages suppress *P. carinii* viability. *P. carinii* viability was determined by the ability of *P. carinii* to incorporate [<sup>14</sup>C]UDPG into cell wall  $\beta$ -glucan after incubation in the presence or absence of macrophages. Over 6 h of culture, macrophages substantially reduced the viability of *P. carinii* by  $78.1 \pm 15.2\%$  ( $P = 0.001$  compared to control *P. carinii* viability in the absence of macrophages). Macrophages cultured in the absence of *P. carinii*, were lysed and submitted to identical differential centrifugation separation, yielding negligible product and no significant incorporation of [<sup>14</sup>C]UDPG. These experiments demonstrate that macrophages suppress



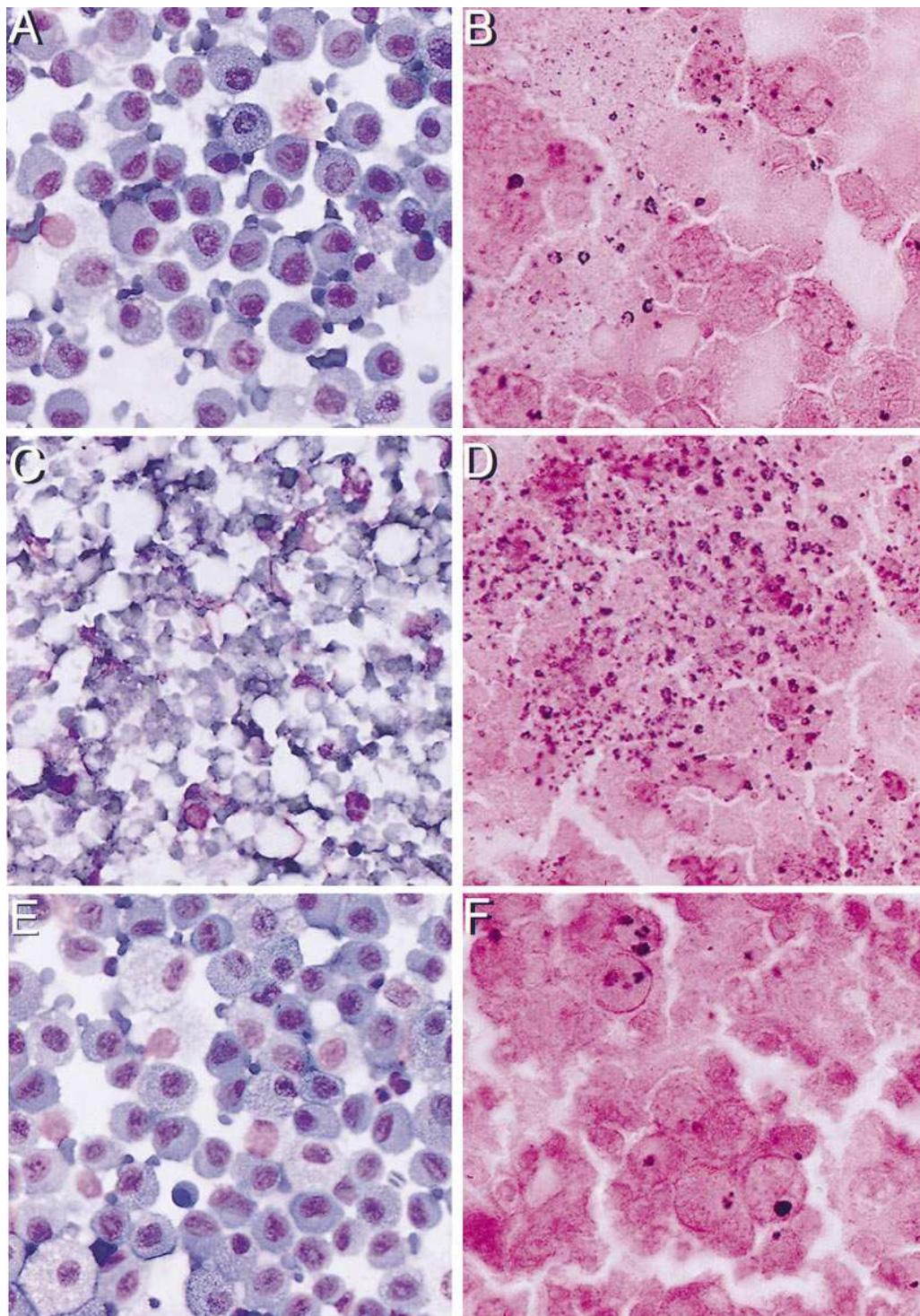
**Figure 1.** Alveolar macrophages mediate uptake and degradation of *P. carinii*. To determine whether alveolar macrophages mediate uptake and degradation of *P. carinii*, organisms were <sup>35</sup>S-labeled and allowed to interact with normal alveolar macrophages in culture. At the times specified, the percentage of *P. carinii* bound (cpm associated with the macrophages after washing), percentage of *P. carinii* phagocytized (cpm not released after trypsin digestion), and the percentage of *P. carinii* degraded (free cpm released into the media) were determined. At all times after 60 min, *P. carinii* binding, uptake, and degradation were statistically greater than at time zero. (\* $P < 0.05$ ). Shown is mean  $\pm$  SEM of four determinations.

*P. carinii* viability and further confirm that alveolar macrophages eliminate *P. carinii* in culture.

**Liposomal bisphosphonates deplete alveolar macrophages from the lung.** Liposomal administration of bisphosphonates has been used to selectively deplete macrophages and thereby study their function in a variety of experimental settings (36, 46–48). Macrophages phagocytized lecithin–cholesterol liposomes, the bisphosphonate is released, chelating intracellular calcium, and thereby resulting in the death of the cell. To study the role of alveolar macrophages in mediating clearance of *P. carinii* from the lower respiratory tract of rats, liposomes containing dichloromethylene bisphosphonate (L-Cl<sub>2</sub>MDP) or pamidronate disodium (L-PAM) were administered to normal rats. Administration of both agents effectively eliminated most of the recoverable alveolar macrophages as assessed by whole-lung BAL. Cellular differentials counts obtained from L-Cl<sub>2</sub>MDP-treated animals contained only  $12.7 \pm 1.2\%$  intact alveolar macrophages, compared to  $95.3 \pm 0.9\%$  intact macrophages in PBS-treated animals ( $P = 0.0001$ ; Fig. 2, A, C, and E, and Fig. 3). L-PBS animals exhibited cellular differential counts virtually identical to PBS treated animals. Interestingly, depletion of alveolar macrophages with liposomal dichloromethylene bisphosphonate did not induce a significant recruitment of neutrophils ( $4.0 \pm 2.5\%$  in L-Cl<sub>2</sub>MDP treated rats versus  $2.3 \pm 1.3\%$  in PBS controls,  $P = 0.59$ ).

Treatment of rats with equipotent doses of L-PAM yielded similar degrees of macrophage depletion. L-PAM treated animals had  $17.1 \pm 4.5\%$  intact alveolar macrophages,  $72.4 \pm 5.3\%$  remnant macrophages,  $8.1 \pm 1.1\%$  neutrophils,  $1.3 \pm 1.0\%$  lymphocytes, and  $1.0 \pm 0.7\%$  eosinophils. Taken together, these data indicate that liposomal bisphosphonates selectively eliminate the majority of alveolar macrophages.

These studies indicated that liposomal bisphosphonate treatment eliminates most alveolar macrophages from the lavage and results in the formation of remnant macrophages. Remnant macrophages were defined as those cells that did not possess clear evidence of cytoplasm or a limiting cellular mem-

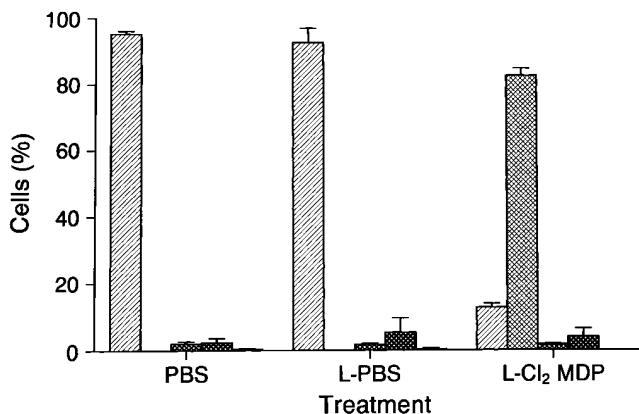


**Figure 2.** Liposomal dichloromethylene diphosphonate eliminates alveolar macrophages from the lung and impairs clearance of *P. carinii*. (A) Control rats were treated with intratracheal PBS, killed after 72 h, and lower respiratory tract cellular populations assessed by bronchoalveolar lavage. Control rats treated with PBS exhibited typical lavage differential counts composed primarily of intact alveolar macrophages. (Modified Wright Giemsa staining;  $\times 100$ .) (B) Control rats that had received PBS 72 h previously were inoculated with *P. carinii* ( $1 \times 10^7$ ). The next day, the rats were lavaged and *P. carinii* cysts burdens determined. Rats treated with PBS had effective elimination of *P. carinii* cysts. (Methenamine silver staining;  $\times 160$ .) (C) Additional rats were treated with intratracheal L-Cl<sub>2</sub>MDP, killed after 72 h, and assessed by bronchoalveolar lavage. L-Cl<sub>2</sub>MDP treated rats had substantial reduction of alveolar macrophages. Numerous remnant macrophages consisting primarily of a condensed nuclear bodies without definable cytoplasmic membranes were present. (Modified Wright Giemsa Staining;  $\times 100$ .) (D) Rats depleted of macrophages with L-Cl<sub>2</sub>MDP 72 h previously, were inoculated with *P. carinii*, and the cysts burdens determined the next day by bronchoalveolar lavage. Macrophage depleted rats had substantial impairment of *P. carinii* clearance. (Methenamine silver staining;  $\times 160$ .) (E) Rats treated with intratracheal L-PBS (liposome controls) exhibited lavage cellular differential counts predominantly containing normal macrophages. These cellular differentials of L-PBS treated rats were indistinguishable from rats receiving PBS alone. (Modified Wright Giemsa staining;  $\times 100$ .) (F) Rats which had received L-PBS 72 h previously were inoculated with *P. carinii*, and the *P. carinii* cysts burdens determined the next day. Animals treated with L-PBS (liposome controls) also exhibited effective elimination of *P. carinii* from the lungs. (Methenamine silver staining;  $\times 160$ .)

taining normal macrophages. These cellular differentials of L-PBS treated rats were indistinguishable from rats receiving PBS alone. (Modified Wright Giemsa staining;  $\times 100$ .) (F) Rats which had received L-PBS 72 h previously were inoculated with *P. carinii*, and the *P. carinii* cysts burdens determined the next day. Animals treated with L-PBS (liposome controls) also exhibited effective elimination of *P. carinii* from the lungs. (Methenamine silver staining;  $\times 160$ .)

brane, and characteristically consisted of only a condensed nuclear body. To further verify that the remnant macrophages and any residual macrophages were nonviable, in additional experiments, cells collected by bronchoalveolar lavage 72 h af-

ter L-PAM administration were assessed for viability by the criterion that they contained a cytoplasm that could exclude trypan blue dye (0.4%). In these experiments, remnant macrophages exhibited no definable cytoplasm capable of dye ex-

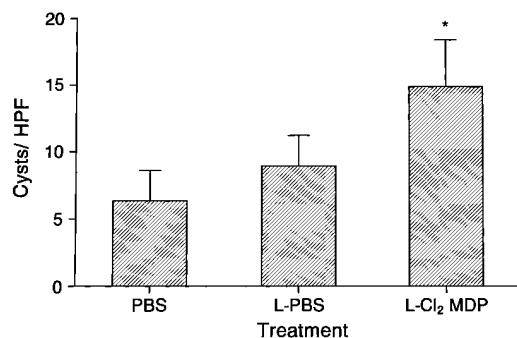


**Figure 3.** Liposomal dichloromethylene diphosphonate eliminates alveolar macrophages from the lung. Cellular differential counts obtained from whole-lung lavage of rats treated with either PBS, L-PBS, or L-Cl<sub>2</sub>MDP. Rats treated with L-Cl<sub>2</sub>MDP had substantial elimination of alveolar macrophages. In addition, macrophage elimination with L-Cl<sub>2</sub>MDP did not result in significant recruitment of inflammatory cells including neutrophils. Shown is mean $\pm$ SEM from three determinations. (Viable alveolar macrophages, ; remnant macrophages, ; lymphocytes, ; neutrophils, ; eosinophils, ).

clusion. In addition,  $\sim 80\%$  of the remaining residual alveolar macrophages that did possess a limiting cellular membrane after L-PAM administration were unable to exclude trypan blue dye and were hence nonviable. Thus, liposomal bisphosphonate substantially eliminates viable alveolar macrophages from the lower respiratory tract.

**Macrophages mediate clearance of *P. carinii* from the lower respiratory tract.** We next employed the macrophage depletion technique to evaluate the role of alveolar macrophages in clearance of *P. carinii* from the lung. Rats were depleted of alveolar macrophages 72 h after treatment with L-Cl<sub>2</sub>MDP. Subsequently, the animals received transtracheal challenge with *P. carinii* organisms. The next day, *P. carinii* clearance was assessed by whole-lung BAL and standardized cyst counts on silver-stained cytopreparation smears (Fig. 2, B, D, and F). Macrophage-depleted rats exhibited  $2.4\pm 0.6$ -fold greater *P. carinii* cyst numbers compared to control rats that received PBS treatment alone (PBS controls;  $P = 0.027$ ), and  $1.7\pm 0.4$ -fold greater *P. carinii* cyst numbers compared to rats who received L-PBS (liposomal controls;  $P = 0.036$ ) (Fig. 4). Rats who received L-PBS had minimally increased organism burdens, which were not significantly different than PBS controls ( $P = 0.16$ ). Therefore, macrophage-depleted animals had significantly impaired ability to clear *P. carinii* cysts from the lower respiratory tract.

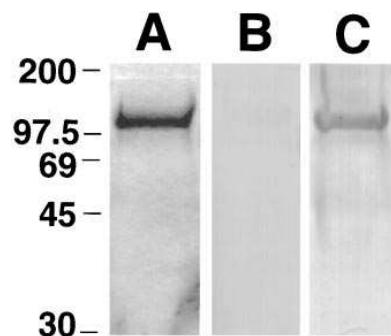
**Determination of *P. carinii* burden by measuring organism-associated gpA.** Traditional methods to quantify *P. carinii* organism burdens have relied upon counting stained cytopreparation smears or histologic sections, both of which are laborious and which may possess inter observer variability. We therefore sought to develop a second independent means to measure *P. carinii* organism burden and to apply this method to evaluating the role of alveolar macrophages in *P. carinii* clearance from the lower respiratory tract. *P. carinii* gpA is the prominent antigen present on the surface of both cysts and trophozoite forms of the organism (20, 51). As such, quantitation of gpA content serves as a useful measure of total organism bur-



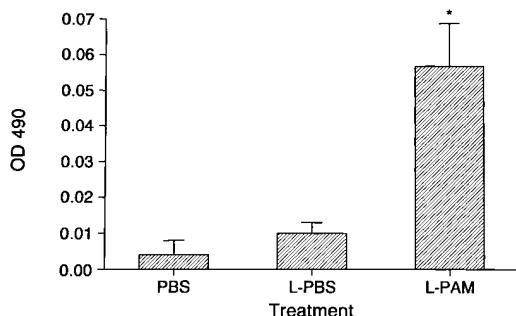
**Figure 4.** Rats depleted of macrophages have impaired clearance of *P. carinii* cysts. Rats received either PBS, L-PBS, or L-Cl<sub>2</sub>MDP. After 72 h, the rats were again anesthetized and received transtracheal inoculation of *P. carinii* ( $1 \times 10^7$  organisms). 24 h later the rats were killed and whole-lung lavage performed. *P. carinii* cysts were counted in 40 contiguous high-powered fields on silver stained cytopreparation smears. Rats treated with L-Cl<sub>2</sub>MDP had substantial impairment of *P. carinii* clearance as measured by higher levels of cysts counts in the lavage. (\* $P < 0.05$  compared to PBS controls). Rats treated with L-PBS had only minimal impairment of *P. carinii* elimination, which was not significantly different from control rats receiving PBS alone. Shown is mean $\pm$ SEM of three determinations.

den. To accomplish this, a mouse monoclonal antibody LM-1 was generated to purified gpA and characterized. LM-1, an IgG1k monoclonal antibody, reacted with purified gpA in immunoblot analysis (Fig. 5). LM-1 was subsequently used to develop a gpA ELISA to quantify *P. carinii* organism burden in whole lung BAL specimens. This ELISA detected the gpA content of 100 to 10,000 *P. carinii* organisms per sample of extract (100  $\mu$ l). Appropriate dilutions were performed to target the linear range of the assay in all subsequent studies.

The gpA ELISA was then used to further establish the role of macrophages in *P. carinii* clearance from the lower respiratory tract (Fig. 6). Rats were macrophage-depleted over 72 h by treatment with L-PAM and received transtracheal inoculation with purified *P. carinii* organisms. The next day *P. carinii* organism burden was assessed by BAL and gpA analysis. BAL



**Figure 5.** Generation of a mouse monoclonal antibody recognizing gpA, the major surface antigenic complex of *P. carinii*. Balb/c mice were immunized with purified gpA, the spleens fused with SP2/OAg14 myeloma, and reactive cells cloned by limiting dilution to generate monoclonal antibody LM-1. (A) The gpA antigenic complex was purified by continuous elution preparative gel electrophoresis. Shown is purified gpA complex (Coomassie Brilliant Blue staining) migrating at  $\sim 120$  kD. (B) Nonimmune mouse IgG failed to react with purified *P. carinii* gpA blotted onto nitrocellulose. (C) Monoclonal antibody LM-1 reacted with the purified gpA protein complex.



**Figure 6.** Determination of *P. carinii* burden in rats depleted of alveolar macrophages using a gpA ELISA. Rats received either intratracheal PBS, L-PBS, or L-PAM (0.6 ml). After 72 h of macrophage depletion, the rats were transtracheally inoculated with *P. carinii* ( $1 \times 10^7$  organisms). 24 h later, animals were killed, lavaged, and organism burdens determined using a gpA ELISA, as described in Methods. Rats treated with L-PAM had substantial impairment of *P. carinii* clearance as measured by higher levels of gpA in the lavage. (\* $P < 0.05$  compared to PBS control). Rats treated with L-PBS were not significantly different from controls receiving PBS alone. Shown is the mean  $\pm$  SEM of four determinations.

from macrophage-depleted rats exhibited  $14.3 \pm 2.9$ -fold greater gpA content compared to control rats who received PBS alone (PBS controls;  $P = 0.013$ ), and  $8.1 \pm 1.7$ -fold greater gpA content compared to rats who received L-PBS (liposomal controls;  $P = 0.008$ ). Rats who received L-PBS had minimally increased gpA content that was not significantly different than the gpA level found in BAL from PBS control rats ( $P = 0.24$ ). These results further suggest that intact macrophage function mediates the degradation and clearance of *P. carinii* organisms from the lower respiratory tract.

## Discussion

Recognition, uptake, and degradation of *P. carinii* by immune effector cells is essential for clearance of this organism from the lower respiratory tract. As a principal phagocyte occupying the airspaces, alveolar macrophages represent an integral component of host defense against this organism. The current study demonstrates that isolated alveolar macrophages possess the ability to not only bind and phagocytize *P. carinii*, but also to degrade and reduce the viability of the organism in culture. In addition, we demonstrate for the first time that macrophage function is necessary for clearance of *P. carinii* from the lungs of an intact mammalian host.

Before this study, methods to assess *P. carinii* degradation by alveolar macrophages are lacking in the literature. The current study provides a convenient method to monitor *P. carinii* degradation as  $^{35}\text{S}$ -radiolabeled amino-acids are degraded and released into the culture media. Using this method, we observed significant degradation of *P. carinii* over 6 h of culture with macrophages. It is possible, however, that some  $^{35}\text{S}$ -radio-labeled amino-acids released during degradation of *P. carinii* might be partially taken up and reincorporated into macrophage proteins. Such potential reutilization of  $^{35}\text{S}$ -radiolabeled amino-acids by macrophages might underestimate *P. carinii* degradation, and the actual amount of *P. carinii* degraded by alveolar macrophages may possibly be even greater than re-

ported. However, even if this was true, our conclusion that alveolar macrophages significantly degrade *P. carinii* in culture remains valid. The degradation of *P. carinii* and the impairment of its viability by alveolar macrophages in culture further supports a central role for these phagocytes in host defense against this organism.

Previous studies further indicate that cultured macrophages bind and phagocytize *P. carinii* organisms through specific receptor-ligand interactions. Ezekowitz et al. (17) demonstrated that macrophage mannose receptors mediate *P. carinii* uptake. Investigations from our laboratory indicate that macrophage mannose receptors recognize *P. carinii* by interacting with the mannose-rich gpA antigenic complex on the surface of the organism (20). Additional investigations indicate that the alveolar adhesive glycoproteins fibronectin, vitronectin, surfactant protein-D, and surfactant protein A bind to *P. carinii* and enhance interaction of the organism with macrophages (22, 23, 51–54). Ultrastructural studies have further suggested that *P. carinii* may be degraded within macrophages (13). However, these previous studies have not provided quantitative evidence of *P. carinii* degradation, nor have they investigated the effects of macrophage function on the organism within a living host.

Our current study evaluated the interaction of *P. carinii* with macrophages both in an isolated culture system, and further used the liposome suicide technique to analyze macrophage activity in the intact mammalian host. This latter method allows selective elimination of alveolar macrophages, and provides convincing evidence that macrophages degrade *P. carinii* and mediate organism clearance. This unique methodological approach should have wide applicability for studying macrophage function in a variety of lung diseases.

Our study provides a model of acute challenge and clearance of *P. carinii* from the lung by alveolar macrophages. Over the time course of these studies, no mortality was observed in macrophage-depleted animals challenged with *P. carinii*. Infection-related mortality was not observed in macrophage-depleted animals up to 10 d later. Assessing *P. carinii*-related mortality in macrophage-depleted animals over longer periods would be necessary to determine the role of alveolar macrophages in chronic models of *P. carinii* infection. Accordingly, additional experiments were performed in an attempt to chronically deplete rats by repeatedly injecting liposomal bisphosphonates at weekly intervals. However, on repeated injections, the lungs became infiltrated with neutrophils and monocytoid cells, and a chronic model of selective macrophage depletion without recruitment of additional inflammatory cells could not be established. Regardless, this study does provide firm evidence that alveolar macrophages can phagocytize and degrade *P. carinii* and substantially mediate the clearance of *P. carinii* from the lower respiratory tract after acute challenge with the organism.

Beyond the binding and phagocytosis of the organisms, alveolar macrophages are stimulated by *P. carinii* to release a number of inflammatory substances (18, 19, 21, 22). Interaction of surface-associated immunoglobulin on *P. carinii* with macrophage Fc receptors promotes the generation and release of eicosanoid metabolites (18). In contrast, fungal  $\beta$ -glucan in the *P. carinii* cell wall stimulates the macrophage release of reactive oxidant species and generation of the potent proinflammatory cytokine TNF $\alpha$  (19, 21). TNF $\alpha$  expression is enhanced in macrophages recovered from patients with *P. carinii* pneu-

monia, and is necessary for optimal elimination of *P. carinii* from the lungs of experimental animals (55, 56). Other studies further suggest that TNF $\alpha$  binds to the cell wall of *P. carinii* and may also exert a direct toxic effect upon the organism (41, 57).

Most clinical settings associated with *P. carinii* pneumonia are characterized by dysfunction of multiple components of immune surveillance including not only lymphocyte function, but also deficits in macrophages and other cells as well (1, 8, 10). The function of alveolar macrophages is impaired in many conditions associated with the development of *P. carinii* pneumonia (8, 14, 24). For instance, although HIV is well established to deplete CD4 lymphocytes, this agent also infects macrophages and monocyteid cells, resulting in impaired mannose receptor function and aberrant uptake of *P. carinii* (24). In addition, patients without AIDS who develop *P. carinii* pneumonia frequently do so as a complication of prolonged systemic corticosteroid therapy (8). Again, although corticosteroids have profound effects on lymphocytic immunity, these agents also substantially impair macrophage activation (10, 58–60). Functional impairment in the monocyte–macrophage system has also been documented in patients with advanced malignancies (61). Macrophage dysfunction may contribute in a synergistic fashion to other immunodepression such as CD4 lymphocytic immunity in promoting development of *P. carinii* pneumonia in various immunocompromised patient populations.

The quantification of organism burdens in patients and animal models of *P. carinii* pneumonia continues to be a challenging aspect of these types of investigations (1). Traditional methods based on counting cysts or trophozoites are time consuming, laborious, and may contain interobserver variability. Herein, we report a sensitive and specific ELISA-based assay to quantify *P. carinii* through measurement of gpA, its major surface antigen. *P. carinii* gpA is present on both cyst and trophozoite forms of the organism, and can serve as a marker for total numbers of organisms present within a given sample (20, 51). This method was applied to whole-lung BAL sampling, which gives a summary reflection of total organism burden in the *P. carinii* infected lung. This convenient method should prove useful for measuring *P. carinii* organism burden in other applications, such as in therapeutic drug trials, or in studies of *P. carinii* proliferation using *in vitro* culture systems.

In summary, we have demonstrated that alveolar macrophages bind, take up, and degrade *P. carinii* in tissue culture and also mediate clearance of the organisms from the lower respiratory tract of an intact host. The interactions of *P. carinii* organisms with alveolar macrophages represents a significant component of host recognition and defense against *P. carinii* infection.

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