Effect of Bronchoalveolar Lavage Fluid from *Pneumocystis carinii*-Infected Hosts on Phagocytic Activity of Alveolar Macrophages

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Received 9 October 2003/Returned for modification 20 November 2003/Accepted 12 January 2004

Alveolar macrophages from *Pneumocystis carinii*-infected rats are defective in phagocytosis. To investigate whether this defect is due to a certain factor present in *P. carinii*-infected lungs, alveolar macrophages from uninfected rats were incubated with bronchoalveolar lavage (BAL) fluid samples from *P. carinii*-infected rats. Alveolar macrophages treated with these BAL fluid samples became defective in phagocytosis but remained normal when treated with BAL fluid samples from noninfected or *Toxoplasma gondii*-infected rats. The suppressive activity of the BAL fluid samples from *P. carinii*-infected rats on phagocytosis was retained when the BAL fluid samples were passed through a filter with a pore size of 0.45 μm but was lost when the BAL fluid samples were digested with proteases such as trypsin, pepsin, papain, or endopeptidase Gly-C. Lipid fractions of these BAL fluid samples had no suppressive activity on phagocytosis. The suppressive activity of these BAL fluid samples was also lost when they were incubated with concanavalin A-agarose beads, suggesting that the inhibitor is a glycoprotein. The inhibitor was estimated to be larger than 100,000 Da by exclusion filtration. After binding to the concanavalin A-agarose beads, the inhibitor in BAL fluid samples and *P. carinii* lysate could be eluted with 200 mM methylmannose. Treatment of both the crude BAL fluid samples and *P. carinii* lysate and the 200 mM methylmannose eluate with antibody against the major surface glycoprotein of *P. carinii* eliminated their suppressive activity. These results suggest that the factor capable of suppressing the phagocytic activity of alveolar macrophages is *P. carinii* major surface glycoprotein or one or more of its derivatives.

An important function of alveolar macrophages is to clear foreign particles and infectious agents from the lung. Their role in the defense against *Pneumocystis carinii* infection is demonstrated by the observation that transtracheally inoculated *P. carinii* organisms are not cleared in alveolar macrophage-depleted rats (31). Administration of granulocyte-macrophage colony-stimulating factor, which activates alveolar macrophages (40), decreases the severity of *P. carinii* pneumonia (34). This finding also indicates the importance of alveolar macrophages in the clearance of *Pneumocystis* organisms.

The alveoli are constantly exposed to foreign material and microorganisms. The alveolar macrophage maintains a low level of activation to engulf and destroy these materials while inhibiting an overly reactive inflammatory reaction to foreign objects encountered. To accomplish this, alveolar macrophages inhibit antigen presentation by dendritic cells (20), limit the expansion of lymphocyte populations (38), and restrict pulmonary lymphocyte functions (19). When activated, alveolar macrophages become proinflammatory and produce interleukin-8, which attracts neutrophils and lymphocytes by chemotaxis, as well as interleukin-1 β , tumor necrosis factor alpha, interleukin-6, and granulocyte-macrophage colony-stimulating factor, which participate in granulomatous lung inflammation (6–8, 25, 34, 35).

Intact *Pneumocystis* organisms or the major surface glycoprotein of *P. carinii* can activate alveolar macrophages to release inflammatory mediators such as tumor necrosis factor

alpha and the eicosanoid metabolites prostaglandin E₂ and leukotriene B₄ (4, 17, 18). Vitronectin and fibronectin enhance macrophage activation and accumulate in the lung during *P. carinii* pneumonia (37). Alveolar macrophages are thought to interact with *Pneumocystis* organisms through the macrophage mannose receptor or the β -glucan receptor (30). It has been shown that a recombinant macrophage mannose receptor which consists of the ectodomain of the macrophage mannose receptor and the Fc portion of human immunoglobulin G1 binds to *Pneumocystis* organisms and causes an increase in phagocytosis of the organisms by macrophages (47). However, the interaction of *Pneumocystis* organisms with the macrophage mannose receptor does not stimulate the release of tumor necrosis factor alpha (30).

Although *Pneumocystis* organisms may activate alveolar macrophages, they are rarely seen intracellularly in alveolar macrophages from *P. carinii* pneumonia patients (11). With a *P. carinii* f. sp. *muris*-infected SCID mouse model, Chen et al. demonstrated that phagocytosis of *Pneumocystis* organisms by alveolar macrophages during *P. carinii* pneumonia is uncommon (8). Furthermore, phagocytosis of *Pneumocystis* organisms by macrophages is reduced in human immunodeficiency virus patients with *P. carinii* pneumonia, and the expression of the macrophage mannose receptor is decreased in these patients (24). Previous results suggest that *Pneumocystis* organisms cause alveolar macrophages to shed mannose receptors (12). These findings indicate that alveolar macrophages from *Pneumocystis*-infected hosts are defective in phagocytosis of *Pneumocystis* organisms. We have recently shown that the defect in phagocytosis during *P. carinii* pneumonia is not specific for *Pneumocystis* organisms because alveolar macrophages

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from *P. carinii*-infected rats are also defective in phagocytosis of fluorescein isothiocyanate-labeled latex beads (27).

In this study, we investigated whether the defect in phagocytosis by alveolar macrophages is due to the presence of a certain inhibitor that suppresses phagocytosis during *P. carinii* pneumonia. Protein fractions of *P. carinii* cell lysate and bronchoalveolar lavage (BAL) fluid samples from *P. carinii*-infected rats were found to suppress the phagocytic activity of normal alveolar macrophages. This suppressive effect could be inactivated by antibody against the major surface glycoprotein of *P. carinii*.

MATERIALS AND METHODS

Rat model of *P. carinii* pneumonia. Female Sprague-Dawley rats (140 to 160 g), obtained from Harlan (Indianapolis, Ind.), were divided into three different groups: normal (immunocompetent) rats, rats supplied with regular drinking water and not inoculated with *P. carinii*; Dex rats, rats immunosuppressed with dexamethasone at 1.8 mg/liter (0.36 mg/kg/day) in their drinking water; and Dex-Pc rats, rats immunosuppressed with dexamethasone for 7 days, transtracheally inoculated with *P. carinii* f. sp. *carinii* organisms, as previously characterized (52), and then maintained on an immunosuppressive regimen by being given the same dose of dexamethasone (0.36 mg/kg/day) in their drinking water. Transtracheal inoculation of *P. carinii* was performed as described previously (1).

Rat model of *Toxoplasma gondii* pneumonia. *T. gondii* tachyzoites of strain RH from the peritoneal exudate of infected rats were stored at -70°C prior to use in this study. Organisms were thawed at 37°C , washed twice in 1 ml of phosphate-buffered saline (PBS; 10 mM phosphate buffer, 0.9% NaCl, pH 7.4), and diluted with PBS to 500 or 50 tachyzoites/ml. Female Sprague-Dawley rats were treated with dexamethasone (0.36 mg/kg/day) in drinking water for 7 days prior to inoculation of organisms and maintained on dexamethasone for the remainder of the study as described above. Two hundred microliters of *T. gondii* inoculum was transtracheally inoculated into rats by the method of Bartlett et al. (1). After 3 weeks of infection, the animals were lavaged for alveolar macrophages as previously described (26). Impression smears of infected lung tissue were stained with hematoxylin and eosin, and *T. gondii* organisms in the impression smears were counted in 10 microscopic fields (magnification, $\times 1,000$).

Isolation of rat alveolar macrophages. Alveolar macrophages were isolated by bronchoalveolar lavage as described previously (26). Rat lungs were lavaged with sterile, pyrogen-free PBS. A minimum of 100 ml of lavage fluid was recovered from the lungs of each rat. The cells in the lavage fluid samples were pelleted by centrifugation at $300 \times g$ for 5 min at 25°C and then resuspended to $1.5 \times 10^6/\text{ml}$ in complete medium (RPMI 1640 supplemented with 10% fetal bovine serum, 1 mM pyruvate, 1% nonessential amino acids, 14 mM glucose, 17.9 mM NaHCO_3 , 10 mM HEPES, 100 U of penicillin per ml, and 0.1 mg of streptomycin per ml). Quantification of alveolar macrophages was achieved by counting with a hemacytometer, and identification of alveolar macrophages was based on the size of the cell, the presence of granules in the cytoplasm, and the reaction with macrophage-specific anti-reactive macrophage antigen antibody as described previously (26).

Preparation of BAL fluid samples. *P. carinii*-infected rats were sacrificed when they showed signs of *P. carinii* pneumonia and then scored for severity of infection. Moderate infections were defined as trophozoite scores of 3.5 to 4.3 or cyst scores of 2.0 to 4.0 in lung impression smears, and severe infections were defined as trophozoite scores of higher than 4.3 or cyst scores higher than 4.0, according to the scale of Bartlett et al. (1). Bronchoalveolar lavage of these rats and some of the Dex rats was performed as described previously (27). Only 10 ml of lavage fluid was recovered from these animals to minimize dilution of the alveolar lining fluid. The lavage fluid was centrifuged for 8 min at $3,000 \times g$ to remove cells and *P. carinii* organisms. An aliquot of the BAL fluid supernatants was stained for organisms with Giemsa stain and assessed for *Pneumocystis* organisms. Samples containing more than one trophozoite or cyst in 50 $1,000\times$ microscopic fields were rejected for use. The BAL fluid samples were stored at -70°C until used. The protein concentration in the supernatant was determined with the Coomassie Plus protein reagent (Pierce, Rockford, Ill.) and then adjusted to 0.5 mg/ml with PBS.

Preparation of *P. carinii* lysate. Isolation of *P. carinii* organisms from infected lungs was performed as described by Chin et al. (9). The isolated organisms were pelleted by centrifugation at $10,000 \times g$ for 10 min and then resuspended in 5 ml of PBS. After addition of 50 μl of protease inhibitor cocktail (Sigma Chemical Co., St. Louis, Mo.) and 25 mg of zymolyase, the organism suspension was soni-

cated 20 times (15 s each with a 1-min cooling interval on ice). This protease inhibitor cocktail contains six different protease inhibitors, including 4-(2-aminoethyl)benzenesulfonyl fluoride, pepstatin A, E-64, bestatin, leupeptin, and aprotinin. The *P. carinii* lysate was then centrifuged in an ultracentrifuge at $50,000 \times g$ for 1 h. The supernatant was stored at -70°C until used. The protein concentration in the supernatant was determined by the Coomassie Plus protein reagent (Pierce) and then adjusted to 0.5 mg/ml with PBS.

Filtration of BAL fluid and *P. carinii* lysate. Some BAL fluid samples from the Dex-Pc rats were passed through a filter (Nalgene, Rochester, N.Y.) with a pore size of 0.45 μm prior to assay for effect on phagocytosis. Ultrafiltration was performed to estimate the molecular size of the inhibitor. BAL fluid samples were first centrifuged at $10,000 \times g$ for 20 min to pellet insoluble materials. The clarified supernatant was filtered through three different ultrafiltration membranes (Amicon, Danvers, Mass.) with exclusion limits of 25,000, 50,000, and 100,000 Da.

Extraction of lipid from BAL fluid. Three milliliters of ether was added to 10 ml of BAL fluid from *P. carinii*-infected rats (Pc-BAL fluid samples). The mixture was vortexed for 3 min and then allowed to stand at room temperature. After the ether layer was separated from the aqueous layer, the ether layer was transferred to a new tube. The whole procedure was repeated four times until a total of 10 ml of ether was recovered. The lipid-containing ether was evaporated, and the dried lipid was dissolved in 10 ml of BAL fluid from the normal rats.

Digestion of BAL fluid and *P. carinii* lysate with DNase I or RNase A. DNase I was added to 1 ml of *P. carinii* lysate or Pc-BAL fluid samples to a final concentration of 1 μg per ml. The mixture was incubated at 37°C for 1 h and then assayed for effect on phagocytosis of normal alveolar macrophages. To determine whether the inhibitor is RNA, 1 ml of *P. carinii* lysate or Pc-BAL fluid samples was digested with a final concentration of 10 μg of RNase A per ml at 37°C for 1 h and then assayed for effect on phagocytosis.

Digestion of Pc-BAL fluid samples and *P. carinii* lysate with various proteases. One milliliter of *P. carinii* lysate or Pc-BAL fluid samples was digested with trypsin (10 $\mu\text{g}/\text{ml}$), pepsin (10 $\mu\text{g}/\text{ml}$), papain (25 $\mu\text{g}/\text{ml}$), or endoproteinase Glu-C (1.25 $\mu\text{g}/\text{ml}$) at the indicated final concentrations as recommended by the manufacturer. To digest with pepsin, the pH of the *P. carinii* lysate or Pc-BAL fluid samples was adjusted to 5.0. After pepsin digestion, the pH of the reaction mixture was adjusted back to 7.4. All protease digestions were done at 37°C for 2 h. The protease activity was then inhibited by adding 10 μl of the protease inhibitor cocktail mentioned above to each of the 1-ml *P. carinii* lysate or Pc-BAL fluid samples. The protease-digested *P. carinii* lysate or Pc-BAL fluid sample was then assayed for effect on phagocytosis.

Reaction of Pc-BAL fluid samples and *P. carinii* lysate with concanavalin A-agarose beads. Five milliliters of concanavalin A-conjugated agarose beads (Sigma Chemical Co., catalog no. C6904) was washed five times with $0.1\times$ PBS (diluted with normal saline) containing 1 mM CaCl_2 and then resuspended in 45 ml of *P. carinii* lysate or Pc-BAL fluid. This Pc-BAL fluid sample was centrifuged at $10,000 \times g$ for 20 min to pellet insoluble materials before being mixed with the concanavalin A-agarose beads. After being stirred overnight at 4°C , the concanavalin A-agarose beads were pelleted by centrifugation at $200 \times g$ for 5 min. The supernatant was saved and tested for effect on phagocytosis. Concanavalin A-agarose beads that had been incubated with *P. carinii* lysate or Pc-BAL fluid samples were then washed sequentially with 50 ml each of plain $0.1\times$ PBS or $0.1\times$ PBS containing different concentrations (0.2, 0.4, 0.6, 0.8, and 1.0 M) of methylmannose (Sigma Chemical Co.). Each wash fluid was dialyzed against distilled water and then lyophilized. The lyophilized substance was dissolved in 300 μl of PBS; 25 μl was used to assay for effect on phagocytosis by alveolar macrophages.

Reaction of Pc-BAL fluid samples and *P. carinii* lysate with antibody against the major surface glycoprotein of *P. carinii*. Twenty microliters of monoclonal antibody 5E12 (15) was added to 1 ml of *P. carinii* lysate or Pc-BAL fluid sample. The mixture was incubated at 37°C for 1 h and then assayed for effect on phagocytosis.

Phagocytosis assay. One million alveolar macrophages from normal or Dex rats in 1 ml of complete RPMI medium were incubated with 1 ml of BAL fluid (adjusted to 0.5 mg of protein per ml) from normal, Dex, or Dex-Pc rats or 1 ml of *P. carinii* lysate (also adjusted to 0.5 mg of protein per ml) for 12 h at 37°C and 5% CO_2 . After incubation with the BAL fluid, alveolar macrophages were pelleted and then resuspended in fresh medium to 1 million cells/ml. Phagocytosis was assessed with fluorescein isothiocyanate-labeled latex beads, since previous results indicated that *Pneumocystis* infection has similar effects on phagocytosis of radiolabeled *Pneumocystis* organisms and latex beads (27). Briefly, 50 million fluorescein isothiocyanate-labeled, 1- μm -diameter, carboxylated latex beads (Sigma Chemical Co.) were added to the macrophage suspension. The mixture was incubated at 37°C with 5% CO_2 for 2 h, with gentle agitation every

FIG. 1. Phagocytosis of latex beads by alveolar macrophages from *T. gondii*-infected rats. Immunosuppressed rats were transtracheally inoculated with 100 (Toxoplasma-100) or 10 (Toxoplasma-10) *T. gondii* tachyzoites. Three weeks after initiation of infection, alveolar macrophages and BAL fluid were recovered. (A) Alveolar macrophages from *T. gondii*-infected rats were able to phagocytose latex beads as efficiently as those from uninfected controls (dexamethasone treated). *T. gondii* burdens were higher in the animals that received 100 tachyzoites ($P < 0.05$), but this increased burden did not affect phagocytosis ability ($P > 0.05$ versus 10 tachyzoites). Open columns indicate levels of phagocytosis, and shaded columns indicate *T. gondii* organism burdens. (B) Alveolar macrophages from normal and dexamethasone-treated rats were incubated with 500 μ l of PBS (open columns) or BAL fluid (shaded columns) (500 μ l at 1 mg of protein per ml) from the most heavily *T. gondii*-infected rats from the experiments in panel A for 12 h and then assessed for the ability to phagocytose latex beads. Phagocytosis of latex beads by alveolar macrophages from normal and Dex rats was unaffected by incubation with BAL fluid samples from *T. gondii*-infected rats ($P > 0.05$).

10 min. Beads that were not phagocytosed were removed by centrifuging through 3 ml of fetal bovine serum in a 15-ml centrifuge tube at $300 \times g$ for 5 min at 25°C . Previous studies with confocal microscopy confirmed that this protocol results in determination of internalized beads (27).

To visualize phagocytosed beads, macrophages (30,000 to 50,000 cells) were fixed on a Superfrost⁺ slide (Fisher, Pittsburgh, Pa.) by cytospinning (Cytospin II; Shandon, Pittsburgh, Pa.) at 750 rpm for 5 min at 25°C , stained with Giemsa stain, and then examined on a fluorescent microscope (Olympus BH-2) at $400\times$ magnification. Beads in at least 300 macrophages from at least 50 random fields were counted, and an average number of fluorescein isothiocyanate-labeled latex beads per macrophage was determined. Separate assays were performed with alveolar macrophages and lavage fluid samples from at least three different rats.

Statistics. Statistical analysis was performed with the SigmaStat (Jandel Scientific, San Rafael, Calif.) software. Data were compared by analysis of variance followed by a two-tailed Student's *t* test, as described previously (27). A *P* value of <0.05 was considered significant.

RESULTS

Effect of *T. gondii* infection on phagocytosis of latex beads by alveolar macrophages. Previous results have indicated that the defect in phagocytosis seen in alveolar macrophages is not a result of immunosuppression by corticosteroids (27). It is not known whether an infection in an immunosuppressed animal leads to a generalized defect in phagocytosis due to a defect in the immune response cascades, or whether the defect is organism specific. To determine whether the defect in phagocytosis of alveolar macrophages is unique to *P. carinii* infection, the phagocytic activity of alveolar macrophages from *T. gondii*-infected rats was examined. Dexamethasone-treated rats were infected with *T. gondii* for 3 weeks by transtracheal inoculation of organisms. Alveolar macrophages from these animals were assessed for their ability to phagocytose fluorescein isothiocyanate-labeled latex beads.

In five rats from each condition, animals inoculated with 10 *T. gondii* tachyzoites for 21 days had *T. gondii* burdens of 4.4 ± 0.5 tachyzoites per 1,000 $\times$ microscopic field in lung impression smears, while those inoculated with 100 tachyzoites showed 12.1 ± 1.1 tachyzoites per field (Fig. 1A). Alveolar macrophages from immunosuppressed, uninfected rats phagocytosed

19.9 ± 2.0 latex beads ($n = 6$), and those from rats inoculated with either 10 or 100 *T. gondii* tachyzoites phagocytosed slightly more beads, indicating that they were not defective in phagocytosis. As shown in Fig. 1A, animals that received 10 tachyzoites had alveolar macrophages that phagocytosed an average of 21.0 ± 3.7 latex beads per cell ($n = 5$). Alveolar macrophages from animals that were inoculated with 100 tachyzoites were similarly active and engulfed 21.5 ± 2.5 beads per cell ($n = 5$, Fig. 1A). Although an increase in phagocytic activity was observed, this increase was not statistically significant ($P > 0.05$ versus control alveolar macrophages).

We also assessed the effect of BAL fluid samples from *T. gondii*-infected rats on phagocytosis by alveolar macrophages. The normal and Dex rats were lavaged for alveolar macrophages. These alveolar macrophages were incubated with lavage fluid from the rat most heavily infected with *T. gondii* as assessed by microscopic evaluation of impression smears of lung tissue from *T. gondii*-infected animals. As shown in Fig. 1B, BAL fluid from this *T. gondii*-infected rat had no effect on phagocytosis of latex beads by alveolar macrophages from normal or dexamethasone-treated animals. Alveolar macrophages from the normal rats treated with BAL fluid from *T. gondii*-infected rats phagocytosed 22.1 ± 3.7 beads/cell ($n = 4$), while control alveolar macrophages treated with PBS engulfed 21.1 ± 3.1 beads. Similarly, alveolar macrophages from the Dex rats phagocytosed 20.2 ± 3.2 beads/cell when incubated with BAL fluid from *T. gondii*-infected rats and 20.4 ± 4.2 beads/cell when incubated with PBS ($n = 4$).

The results of phagocytosis by alveolar macrophages exposed to *T. gondii* agree well with previously published results (28) that macrophages exposed to *T. gondii* were as phagocytically active as those that were not exposed. The observation that alveolar macrophages from dexamethasone-suppressed, *T. gondii*-infected rats were phagocytically normal implies that the defect in phagocytosis by alveolar macrophages during *P. carinii* pneumonia is organism specific.

FIG. 2. Effect of BAL fluid samples from normal, Dex, and Dex-Pc rats on phagocytosis by alveolar macrophages. (A) Alveolar macrophages from normal, Dex, and Dex-Pc rats were incubated with BAL fluid samples (0.5 mg of protein) from moderately (lightly shaded columns) or severely (darkly shaded columns) infected Dex-Pc rats. Compared to the PBS control (open columns), BAL fluid from both moderately and severely infected rats was able to inhibit phagocytosis of latex beads by alveolar macrophages from both normal and Dex rats ($P < 0.05$). (B) BAL fluid samples from normal and Dex rats were unable to restore the phagocytic activity of alveolar macrophages from Dex-Pc rats ($P > 0.05$). Alveolar macrophages from Dex-Pc rats were unable to phagocytose increased numbers of latex beads despite having been removed from the infected host for 24 h and incubated with BAL fluid samples from uninfected rats for 12 h.

Effect of BAL fluid samples from *P. carinii*-infected rats on phagocytosis of latex beads by alveolar macrophages. To determine whether the defect in phagocytosis of alveolar macrophages in *P. carinii* pneumonia is due to the presence of a factor in the lung of *P. carinii*-infected rats, alveolar macrophages from the normal, Dex, and Dex-Pc rats were incubated with PBS or BAL fluid from moderately or severely *P. carinii*-infected rats for 12 h and then assayed for phagocytosis of fluorescein isothiocyanate-labeled latex beads. Figure 2 shows that alveolar macrophages from normal rats treated with PBS phagocytosed an average of 22.3 ± 2.4 latex beads per cell, whereas those treated with BAL fluid samples from moderately infected rats phagocytosed an average of 16.2 ± 1.9 beads, a 23% decrease in phagocytic activity. The same cells treated with BAL fluid samples from severely infected rats phagocytosed even fewer beads, 13.1 ± 2.0 ; this is a 41% decrease in phagocytic activity compared to those treated with PBS. Similar results were obtained with alveolar macrophages from Dex rats. A 34% (average, 15.9 ± 1.4 beads) decrease in phagocytic activity was seen in those treated with BAL fluid samples from moderately infected rats, and a 48% (average, 12.5 ± 1.0 beads) decrease was seen in those treated with BAL fluid samples from severely infected rats compared to cells treated with PBS (average, 24.1 ± 2.3 beads, Fig. 2A).

Alveolar macrophages from the Dex-Pc rats did not become more defective in phagocytosis when they were treated with BAL fluid samples from either moderately or severely infected rats. As shown in Fig. 2A, cells treated with PBS phagocytosed 1.54 ± 0.2 beads, and those treated with BAL fluid samples from moderately infected and severely infected rats phagocytosed 1.1 ± 0.0 and 1.3 ± 0.2 beads per cell, respectively. Differences in phagocytosis were statistically significant for alveolar macrophages from normal and Dex rats treated with either BAL fluid preparation compared to those treated with PBS ($P < 0.05$). These results, as with all experiments in this study, represent the averages of at least three trials with alveolar macrophages and BAL fluid samples from three different rats.

Experiments were then performed to determine whether BAL fluid samples from the normal rats could restore the defect in phagocytosis of alveolar macrophages from the Dex-Pc rats. Alveolar macrophages from severely infected Dex-Pc rats were incubated with BAL fluid samples from normal or Dex-Pc rats in the same manner as described above. Phagocytosis assays revealed that these alveolar macrophages phagocytosed an average of 1.3 ± 0.1 beads/cell when treated with BAL fluid samples from the normal rats and 1.2 ± 0.2 beads/cell when treated with BAL fluid samples from the Dex-Pc rats ($P = 0.9897$), as shown in Fig. 2B. These levels of phagocytosis were as low as those of untreated alveolar macrophages from Dex-Pc rats, which phagocytosed an average of 1.0 ± 0.2 beads/cell (26). These results indicated that alveolar macrophages from the Dex-Pc rats remained defective in phagocytosis and that BAL fluid samples from normal rats were incapable of correcting the defect.

Effect of filtered BAL fluid samples on phagocytosis by alveolar macrophages. The BAL fluid samples used in the above experiments were centrifuged to remove intact organisms and cells but were not filtered to remove particulate matter, such as fragments of lysed organisms or cells. In order to determine whether *P. carinii* or host cell debris had any effect on phagocytosis by alveolar macrophages from dexamethasone-treated rats, the experiments described above were performed with BAL fluid samples that had been passed through a filter with a pore size of $0.45 \mu\text{m}$. Alveolar macrophages from the normal rats phagocytosed an average of 12.2 ± 2.2 latex beads per cell when treated with filtered BAL fluid from the Dex-Pc rats and engulfed 24.3 ± 2.8 and 22.4 ± 1.9 beads when treated with filtered BAL fluid samples from Dex and normal rats, respectively (Fig. 3, $P < 0.05$ for normal or Dex BAL fluid samples versus Pc-BAL fluid samples). Similar results were obtained with alveolar macrophages from the Dex rats (Fig. 3). When incubated with BAL fluid samples from the normal rats, they phagocytosed 22.9 ± 3.4 latex beads, while treatment with BAL fluid samples from the Dex rats resulted in engulfment of 20.7 ± 3.6 beads/cell. Filtered Pc-BAL fluid samples still had a

FIG. 3. Effect of filtered BAL fluid on phagocytosis of latex beads by alveolar macrophages. Normal and Dex rats were lavaged for alveolar macrophages, and these cells were incubated for 12 h with BAL fluid (0.5 mg of protein) that had been filtered through a 0.45- μ m filter. The alveolar macrophages were then assessed for their ability to phagocytose 1- μ m-diameter latex beads. Results are averages $\pm$ standard deviations from triplicate phagocytosis assays performed for each of five experiments for each condition. For alveolar macrophages from both normal and Dex rats, incubation with Pc-BAL fluid samples (darkly shaded columns) significantly reduced phagocytosis ($P < 0.05$ versus normal BAL fluid samples), while BAL fluid samples (lightly shaded columns) from immunosuppressed rats had no effect on phagocytosis ($P > 0.05$ versus normal BAL fluid samples).

suppressive effect on alveolar macrophages from the Dex rats (10.4 ± 2.1 beads/cell, Fig. 3). The results for all conditions and all BAL fluid samples ($n = 5$ each) were statistically the same for filtered and unfiltered BAL fluid samples. These results indicate that the suppressive factor in Pc-BAL fluid samples is not particulate matter greater than 0.45 μ m in size.

Effect of *P. carinii* lysate on phagocytosis. The finding that BAL fluid samples from Dex-Pc rats but not those from Dex rats suppressed the phagocytic activity of normal alveolar macrophages suggests that a *P. carinii* factor is responsible for this suppression. To examine this possibility, *P. carinii* lysates were assayed for effect on phagocytic activity of normal alveolar macrophages. Normal alveolar macrophages phagocytosed an average of 1.26 ± 0.4 latex beads per cell when they were treated with *P. carinii* lysate at a concentration of 0.5 mg of total protein macrophage mannose receptor. This is a 94.4% decrease in phagocytosis compared to control alveolar macrophages that were treated with PBS (Table 1).

Heat stability of the phagocytosis inhibitor. To determine whether the phagocytosis inhibitor is sensitive to heat, a tube containing 4 ml of *P. carinii* lysate or BAL fluid from *P. carinii*-infected rats was placed in a boiling-water bath. At 5, 10, 15, and 20 min, 1-ml aliquots of the lysate or BAL fluid were removed from the tube and then assayed for a suppressive effect on phagocytosis. The results for both *P. carinii* lysates and BAL fluid samples from Dex-Pc rats were similar for each boiling period. After 20 min of boiling, both *P. carinii* lysate and Pc-BAL fluid samples inhibited phagocytosis (2.2 ± 0.25 beads/cell for cells treated with boiled *P. carinii* lysate and 12.4 ± 2.0 beads/cell for those treated with boiled Pc-BAL fluid samples). These levels of suppression in phagocytosis were very similar to those of unboiled *P. carinii* lysate or Pc-BAL fluid samples (Table 1). The results of this experiment indicate that the inhibitor was heat resistant; boiling for 20 min did not inactivate its suppressive effect on phagocytosis.

Estimation of the molecular size of the inhibitor. Ultrafiltration was carried out to estimate the molecular size of the suppressive factor. BAL fluid samples were passed through exclusion filters to obtain filtrates with molecules of less than 25,000, 50,000, or 100,000 Da. One milliliter of each filtrate was assayed for suppressive effect on phagocytosis. Unfiltered BAL fluid from *P. carinii*-infected rats was assayed in the same manner to serve as the control. As shown in Table 1, unfiltered BAL fluid inhibited phagocytosis of normal alveolar macrophages by 41%, whereas Dex-Pc BAL fluid samples filtered through any of these three membranes lost the suppressive activity (3.5% inhibition for 25,000-Da exclusion, 2.8% inhibition for 50,000-Da exclusion, and 4.2% inhibition for 100,000-Da exclusion). As indicated in Table 1, the same results were obtained when *P. carinii* lysate was filtered through these ultrafiltration membranes (9.9% inhibition for exclusion of all molecules smaller than 100,000 Da), suggesting that the molecular size of the inhibitor is greater than 100,000 Da.

Determination of the nature of the inhibitor. To determine whether the inhibitor is a lipid, the lipid fraction in Pc-BAL fluid samples was extracted with ether. After evaporation of the ether, the extracted lipid fraction was dissolved in BAL fluid from the normal rats and then assayed for a suppressive effect on phagocytosis. As shown in Table 1, the lipid fraction of Pc-BAL fluid samples had no effect on phagocytosis (0% inhibition, 22.4 ± 2.4 beads/cell), indicating that the inhibitor is not a lipid. Pc-BAL fluid samples were then digested with

TABLE 1. Effect of treated BAL fluid from *P. carinii*-infected rats and *P. carinii* lysates on phagocytosis of normal alveolar macrophages^a

Treatment	Sample	Avg. no. of latex beads phagocytosed/cell $\pm$ SD	% Inhibition of phagocytosis
None	PBS	22.3 ± 2.4	0
	Lysate	$1.25 \pm 0.4^*$	94.4
	BAL fluid	$13.1 \pm 2.0^*$	41.0
Heat	Lysate	$2.23 \pm 0.25^*$	90
	BAL fluid	$12.4 \pm 2.0^*$	44.5
Filtered to remove proteins of $\leq 100,000$ Da	Lysate	20.1 ± 2.1	9.9
	BAL fluid	21.4 ± 3.1	4.2
Lipid fraction only	Lysate	Not assayed	Not applicable
	BAL fluid	22.4 ± 2.4	0
DNase I	Lysate	$1.72 \pm 0.1^*$	92.3
	BAL fluid	$11.8 \pm 1.1^*$	47.1
RNase A	Lysate	$1.32 \pm 0.2^*$	94.1
	BAL fluid	$11.4 \pm 0.9^*$	48.8
Protease	Lysate	20.8 ± 3.2	6.6
	BAL fluid	21.3 ± 2.5	4.39
Concanavalin A	Lysate	20.7 ± 4.5	6.9
	BAL fluid	21.6 ± 3.8	3.1
Incubated with anti-MSG ^b	Lysate	20.0 ± 3.3	10.2
	BAL fluid	21.4 ± 4.2	4.1

^a Lysates and BAL fluid were digested or treated with different enzymes or procedures and then tested for their inhibition of phagocytosis by normal alveolar macrophages. The results for protease treatments are for endopeptidase Lys-C, the protease least effective for eliminating the suppressive effect of the *P. carinii* sample. The results for filtered samples are representative of experiments to remove proteins of $\geq 25,000$, 50,000, or 100,000 Da. For all experiments, the results are averages and standard deviations of at least four experiments for each condition. The percent inhibition in each condition is in comparison to the level obtained with untreated normal alveolar macrophages incubated with PBS. Values that were significantly lower than control values ($P < 0.05$) are marked (*).

^b MSG, major surface glycoprotein.

DNase I, RNase A, and various kinds of proteases to determine whether the inhibitor was a DNA, RNA, or protein. Digestion of the BAL fluid with DNase I or RNase A did not affect the suppressive effect of the BAL fluid (47.1% inhibition for DNase-treated and 48.8% suppression for RNase A-treated samples, Table 1), whereas digestion of the BAL fluid with pepsin, trypsin, papain, or endopeptidase Glu-C inactivated the suppressive effect of the BAL fluid on phagocytosis (4.39% inhibition by endopeptidase Glu-C-digested Pc-BAL fluid samples, the least effective protease in eliminating suppression of phagocytosis; Table 1). The suppressive effect of *P. carinii* lysate was also not affected by DNase I or RNase A digestion but was inactivated by all the proteases mentioned above (Table 1). These results suggest that the inhibitor is a protein.

To further explore the nature of the inhibitor, Pc-BAL fluid samples and *P. carinii* lysate was mixed with concanavalin A-agarose beads overnight at 4°C. After removal of the beads, BAL fluid and *P. carinii* lysate were found to lose the suppressive effect on phagocytosis (3.1% and 6.9% inhibition, respectively; Table 1), suggesting that the inhibitor is a glycoprotein. To determine whether the inhibitor could be eluted off the concanavalin A-agarose beads, the beads were treated with PBS containing various concentrations of methylmannose, and 200 mM methylmannose in PBS was found to elute the inhibitor from the concanavalin A-agarose beads. This eluate was dialyzed against water, lyophilized, and then dissolved in PBS to a final protein concentration of 0.25 mg/ml; 1 ml of this solution was assayed for an effect on phagocytosis and found to cause a 76.3% inhibition (5.3 ± 0.8 beads/cell) in phagocytosis. Immunoblot analysis of the eluate with an antibody against the major surface glycoprotein revealed several reactive bands (data not shown).

Inactivation of the inhibitor with anti-major surface glycoprotein antibody. Based on the results that the inhibitor is a glycoprotein with a molecular size greater than 100,000 Da and that *P. carinii* lysate also has a suppressive effect on phagocytosis, we hypothesized that the inhibitor is the major surface glycoprotein of *P. carinii*. To prove this hypothesis, *P. carinii* lysate and Pc-BAL fluid samples were treated with monoclonal antibody 5E12 against the *P. carinii* major surface glycoprotein and then assayed for an effect on phagocytosis. Pc-BAL fluid samples and *P. carinii* lysate were found to lose the suppressive activity when they were treated with the anti-major surface glycoprotein antibody. Anti-major surface glycoprotein-treated Pc-BAL fluid samples caused only a 4.1% reduction in phagocytic activity, and anti-major surface glycoprotein-treated *P. carinii* lysate caused only a 10.2% inhibition of phagocytosis (Table 1).

DISCUSSION

Alveolar macrophages from *P. carinii*-infected hosts are defective in phagocytosis (27). This defect appears to be specific to *P. carinii* infection, as alveolar macrophages from *T. gondii*-infected rats were found to be normal in phagocytosis (Fig. 1). Some microorganisms can evade or resist phagocytosis by alveolar macrophages (3, 5, 29), but *P. carinii* appears to make alveolar macrophages defective in phagocytosis in order to avoid destruction. The demonstration that BAL fluid samples

from *P. carinii*-infected rats but not those from uninfected rats inhibited phagocytosis of alveolar macrophages (Fig. 2A) indicates that an inhibitor is present in *P. carinii*-infected lungs. The inhibitor is not the whole *P. carinii* organism because BAL fluid samples from *P. carinii*-infected rats were still able to inhibit phagocytosis when they were filtered through a membrane with a pore size of 0.45 μ m. The observation that *P. carinii* cell lysate also had the ability to inhibit phagocytosis by alveolar macrophages suggests that the inhibitor is derived from *P. carinii*, not from the host. The release of molecules by microorganisms to alter the alveolar macrophage response has been described. Endotoxin is a good example of a soluble bacterial product that stimulates local as well as systemic changes in immune response and acts to enhance phagocytosis of alveolar macrophages (13). *Cryptococcus neoformans* releases a laccase enzyme to protect it from oxygen radicals produced by alveolar macrophages by converting catecholamines to melanin, which acts as an antioxidant (33).

Several experiments were performed to determine the nature of the suppressive factor(s). The inhibitor is not a DNA or RNA because digestion with DNase I and RNase A did not affect the suppressive activity of *P. carinii* lysate or BAL fluid samples from *P. carinii*-infected rats (Table 1). The inhibitor is also not a lipid because the lipid fraction of the BAL fluid samples from *P. carinii*-infected rats had no suppressive activity (Table 1). Using an ultrafiltration membrane, we estimated the molecular size of the inhibitor as greater than 100,000 Da. The fact that the suppressive activity of *P. carinii* lysate and BAL fluid samples from *P. carinii*-infected rats was destroyed by protease digestion indicates that the inhibitor is a protein (Table 1). We believe that the inhibitor is a glycoprotein because it can bind to concanavalin A-agarose beads and that it is a mannose-bearing glycoprotein because it can be eluted off the concanavalin A-agarose beads with a buffer containing 200 mM methylmannose. These results suggested that the inhibitor is the major surface glycoprotein of *P. carinii* or a derivative of it. This speculation was confirmed by the result that anti-*P. carinii* major surface glycoprotein antibody inactivated the suppressive activity of *P. carinii* lysate and Pc-BAL fluid samples.

Inhibition of phagocytosis was greater in *P. carinii* lysates than in BAL fluid samples for all parameters tested. Also, more suppression remained in *P. carinii* lysate fractions treated by proteases, ultrafiltration, concanavalin A, and incubation with anti-major surface glycoprotein antibody (Table 1). These results indicate that there is a greater concentration of the suppressive factor(s) in *P. carinii* lysates than in Pc-BAL fluid samples. These results also support the idea that the suppressive factor is the major surface glycoprotein of *P. carinii*. The major surface glycoproteins are a highly expressed family of proteins on the organism surface, and they participate in organism attachment to host pneumocytes (44, 45), evasion of host responses by facilitating the binding of *Pneumocystis* organisms to host surfactant lipids and proteins (56), and disease pathogenesis (10, 16, 44, 45). The inhibitor may be the soluble form of major surface glycoprotein released from *P. carinii* described by Linke and Walzer (32) or major surface glycoprotein remnants of lysed *P. carinii* organisms.

In this study, dexamethasone was used to immunosuppress rats in order to develop *P. carinii* infection. Therefore, rats that were treated with dexamethasone but not infected with *P. ca-*

rinii were used as controls. The results showed that dexamethasone had no effect on the phagocytic activity of alveolar macrophages. This observation is contradictory to the previous report of Nakamura et al. in that dexamethasone was found to suppress the phagocytic activity of alveolar macrophages in their study (36). A careful comparison of the immunosuppression protocols revealed that Nakamura et al. used 4 to 50 times more dexamethasone (1.25 to 20 mg/kg/day) than we did in the present study, in which rats were immunosuppressed with 0.36 mg/kg/day. Another difference is that dexamethasone was given by a single subcutaneous injection in the study of Nakamura et al. In our study, dexamethasone was given orally by placing it in the drinking water. The dose that we used was probably sufficient to allow *P. carinii* to infect the rats but not to affect the phagocytic activity of alveolar macrophages.

Infection with *P. carinii* also brings many changes in the lung environment, including a decrease in phosphatidylcholine (46), an increase in sphingomyelin (48), and increases in surfactant proteins A and D (39, 43) and the adhesion proteins vitronectin and fibronectin (37). *Pneumocystis* infection also causes increases in the cytokines tumor necrosis factor alpha (23, 29, 50); interleukin-8, interleukin-10, and interleukin-12 (2, 51); gamma interferon (14, 51); the growth factor granulocyte-macrophage colony-stimulating factor (40); and the chemokine MCP-1 (2). An inflammatory response (51, 54), changes in macrophage and polymorphonuclear cell populations (11, 53, 55), and proliferation of organisms also occur during *Pneumocystis* infection. *Pneumocystis* organisms also interact with host cells and alveolar constituents to scavenge important factors such as fatty acids and lipids (21, 42). Whether any of these factors or actions has any impact on the suppressive effect of the inhibitor remains to be investigated.

The mechanisms by which the inhibitor renders alveolar macrophages defective in phagocytosis are unknown. One possibility is that the macrophage mannose receptors responsible for the phagocytosis of *P. carinii* organisms are saturated by *P. carinii* major surface glycoprotein that is released into the alveoli. In our previous studies, alveolar macrophages from *P. carinii*-infected rats were also found to be defective in phagocytosis of latex beads (27). Since latex beads are phagocytosed via the scavenger receptor (22, 41), it is likely that the scavenger receptors are also affected. Scavenger receptors may not bind major surface glycoprotein via the mannose moieties. It is possible that *P. carinii* major surface glycoprotein or gpA causes a deregulation in signal transduction which consequently turns off most, if not all, phagocytosis receptors. These possibilities are being investigated. We also found that the expression of the transcription factor GATA-2 is downregulated in alveolar macrophages during *P. carinii* infection (49) and that suppression of GATA-2 production in normal alveolar macrophages renders them defective in phagocytosis (27). In addition, overexpression of GATA-2 in alveolar macrophages from *P. carinii*-infected rats restored their phagocytic activity (27). Whether the inhibitor suppresses phagocytosis by downregulating GATA-2 expression is also being investigated.

ACKNOWLEDGMENTS

This study was supported by NIH grant RO1 HL65170.

We thank F. Gigliotti for anti-*P. carinii* major surface glycoprotein antibody and S. F. Queener for *T. gondii* organisms.

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