Polyamine-mediated Apoptosis of Alveolar Macrophages during Pneumocystis Pneumonia*

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The number of alveolar macrophages is decreased during Pneumocystis pneumonia (Pcp), partly because of activation of apoptosis in these cells. This apoptosis occurs in both rat and mouse models of Pcp. Bronchoalveolar lavage (BAL) fluids from Pneumocystis-infected animals were found to contain high levels of polyamines, including spermidine, N3-acetyl spermine, and N3-acetylspermidine. These BAL fluids and exogenous polyamines were able to induce apoptosis in alveolar macrophages. Apoptosis of alveolar macrophages during infection, after incubation with BAL fluids from Pneumocystis-infected animals, or after incubation with polyamines was marked by an increase in intracellular reactive oxygen species, activation of caspases-3 and -9, DNA fragmentation, and leakage of mitochondrial cytochrome c into the cytoplasm. When polyamines were depleted from the BAL fluids of infected animals, the ability of these BAL fluids to induce apoptosis was lost. Interestingly, the apoptosis inducing activity of the polyamine-depleted BAL fluids was restored when polyamines were added back. The results of this study suggested that Pneumocystis infection results in accumulation of high levels of polyamines in the lung. These polyamines activate apoptosis of alveolar macrophages, perhaps because of the ROS that are produced during polyamine metabolism.

Pneumocystis-infected lungs usually contain much alveolar exudate and numerous inflammatory cells in perivascular and peribronchiolar areas (1–3). The infection also causes changes in the lung. One such change is significantly increased levels of surfactant proteins A and D (4, 5). These collectins are implicated in the attachment of Pneumocystis to alveolar epithelial cells during infection (4–6) and evasion of the host immune response (7). In contrast, surfactant proteins B (8) and C (4, 9) are down-regulated in their expression. Macrophage mannose receptor expression is also down-regulated (10), although the shed form of the mannose receptor is increased (11), which may help the organism evade host immune responses (7, 10, 11). The expression of the transcription factor GATA-2 is reduced in alveolar macrophages during Pcp (12), and this GATA-2 down-regulation is correlated with the dysfunction of alveolar macrophages (13). There is a decrease in plasma S-adenosylmethionine levels during the infection (14). The infection also causes erosion of type I pneumocytes and proliferation of type II epithelial cells (15). These changes indicate that Pneumocystis mediates many alterations in pulmonary and systemic environments in an effort to survive in the host.

In addition to these changes, the number of alveolar macrophages is decreased during Pcp in humans (16–20) and in a rat model of infection (21). This change may be due to decreased precursor cell recruitment or maturation, increased efflux of cells from the lung, increased apoptosis rate, or a combination of these factors. Apoptosis is a normal event in development or tissue turnover (22) and can also be a response to disease states or infectious agents (23–25). However, some infectious agents, especially those that survive and replicate inside host cells, inhibit apoptosis of the infected cells (26–28).

Apoptosis of cells occurs via two pathways, the extrinsic and the intrinsic. These two pathways are initiated through different stimuli and employ different cascades but are often both active (29) and converge at the point of the caspase-3 protease activation. The extrinsic pathway is stimulated by the binding of ligands or specific antibodies to “death domain” receptors, including TNF-α receptors 1 or 2 (30), Fas (also called CD95 or APO-1), TRAIL-R1 and -R2, death receptor 3, and death receptor 6. The pro-apoptotic signals are transduced to the cytoplasm of the cell by adapter proteins that are recruited to the cytoplasmic portions of the death receptors. The bound adapter proteins then activate the first in a series of cytoplasmic proteases, the initiator caspases-8 and -10; they in turn activate downstream caspases, including executioner caspases-3, -6, and -7. These proteases lead to cell death by digesting crucial cell proteins.

The intrinsic (mitochondrial) pathway is activated by signals within the cell or by cleavage of Bid by the activated form of caspase-8 or -10 from the extrinsic pathway (29). Other stimuli

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2 The abbreviations used are: Pcp, Pneumocystis pneumonia; BAL, bronchoalveolar lavage; ROS, reactive oxygen species; SSAT, spermine and spermidine acetyltransferase; APAO, acetylpolamine oxidase; ALF, alveolar lining fluid; LP5, lipopolysaccharide; H2DCFDA, 2′,7′-dichlorofluorescein diacetate; HPLC, high pressure liquid chromatography; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; BisTris, 2-[bis(2-hydroxyethyl)aminol]-2-(hydroxymethyl)prop-1-3 diol; PBS, phosphate-buffered saline; TUNEL, terminal dUTP nick-end labeling; Dex, dexamethasone; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; TNF, tumor necrosis factor.
that may injure the mitochondrial membrane include polyamines or acetylpolyamine oxidase products (31, 32). The damage to the mitochondrial membrane or the formation of pores by the pro-apoptotic members of the Bcl-2 family induces a change in the mitochondrial membrane potential and the impairment of mitochondrial respiration, causing leakage of pro-apoptotic factors, such as cytochrome c, from the mitochondria to the cytoplasm. Cytochrome c binds to Apaf-1, which in turn recruits and activates caspase-9 to form the apoptosome. Activation of the mitochondrial pathway also results in cleavage of caspase-3, with subsequent activation of the other executioner caspases, just as with the extrinsic pathway. Many infections and disease processes induce apoptosis of host cells. Pneumocystis may also enhance its survival by stimulating apoptosis in host alveolar macrophages.

One possible trigger for apoptosis is increased polyamine levels. Polyamines are implicated in the initiation of apoptosis of several cell types, either through direct action (32) or via the reactive oxygen species (ROS) such as H$_2$O$_2$ (11) produced during several cell types, either through direct action (32) or via the reductive potential of the cytoplasm and cause mitochondrial membrane damage and leakage of pro-apoptotic factors. The primary polyamines putrescine, spermine, and spermidine are all implicated in initiation of apoptosis, but spermine and N$^4$-acetyl spermine are more often involved in the direct toxic effects of polyamines on the membrane potential of the mitochondria (32, 34). Spermine can directly activate caspase-3 in macrophage-like cell lines (35). Polyamines can affect the activity of transcription factors such as NF-$\kappa$B (36) and AP-1 (37), which in turn control the transcription of many other genes. Polyamines can also repress the transcription of genes through the stabilization of highly condensed chromatin (38).

We hypothesized that alveolar macrophage numbers were reduced during Pcp because of increased apoptosis of these cells and that abnormally high levels of polyamines were a stimulus for this apoptosis. The results of this study indicated that alveolar macrophages from animals with Pcp underwent apoptosis via the intrinsic pathway, with mitochondrial damage and activation of caspases-3 and -9. Furthermore, polyamines were increased in the lungs of animals with Pcp, and exogenous polyamines induced apoptosis similar to that seen in Pcp. Finally, depletion of polyamines from BAL fluids of animals with Pcp suppressed their ability to induce ROS production and apoptosis of alveolar macrophages.

**EXPERIMENTAL PROCEDURES**

**Reagents—**Antibodies to the activated form of caspases-3 and -9 and GAPDH were purchased from Cell Signaling Technologies (Beverly, MA). Procasapase-9 antibody was purchased from Abcam (Cambridge, MA). Bleomycin, spermidine, spermine, N$^4$-acetyl spermidine, N$^4$-acetyl spermine, and all other chemical reagents were purchased from Sigma, except where noted. The purity of purchased polyamines was assessed by HPLC using the method of Merali (39) and was determined to be greater than 99% for each polyamine.

**Analysis of Pneumocystis Pneumonia in Rats and Mice—**Animals were allowed to develop Pneumocystis pneumonia over a period of 6–10 weeks. At the end of the study period, or when animals became agonal as assessed by veterinarians, they were sacrificed by injection of 0.8 ml/kg ketamine mixture (ketamine hydrochloride (80 mg/ml), atropine (0.38 mg/ml), and acepromazine (1.76 mg/ml)) and transection of the aorta.
Giemsa-stained lung impression smears were used to assess trophozoite burden in infected animals. Trophozoites were counted in at least 20 random ×1000 microscopic fields for each sample. Cyst burdens were similarly determined from silver-stained impression smears. Results were expressed as averages ± S.D. for three independent experiments; each experiment used 8–10 animals of each condition.

**Isolation and Enumeration of Rat and Mouse Alveolar Macrophages from BAL Fluids**—Alveolar macrophages were isolated from rats and mice as described previously (21, 44). Briefly, sterile, pyrogen-free 0.9% saline (7–9 ml for rats and 1 ml for mice per lavage) was instilled into and recovered from rat lungs via a 14-gauge or mouse lungs via a 16-gauge angiocath (BD Biosciences). A total of 100 ml of saline was recovered from each rat, whereas 10 ml of saline was recovered from each mouse. For isolation of alveolar macrophages from BAL fluids of each condition was denatured in 6 μl of 4× Laemmli buffer (Invitrogen) by boiling for 10 min. The proteins in each sample were electrophoresed at 100 V on a 10% BisTris polyacrylamide gel (NuPAGE, Invitrogen) and transferred to polyvinylidene difluoride membranes. The membranes were blocked and then incubated with 1:5000 dilution of a rabbit anti-cleaved caspase-3 antibody or anti-cleaved caspase-9 antibody in blocking buffer (100 mm TBST, 5% nonfat milk). After washing the blot in 100 mm TBST three times, an HRP-conjugated mouse anti-rabbit IgG (1:2000 dilution in blocking buffer; BD Biosciences) was added for 1 h. The blot was washed again and the activated caspase-3 or caspase-9 was visualized by enhanced chemiluminescence (Amersham Biosciences). To ensure even loading, the constitutively expressed GAPDH protein was assessed in an identical manner.

**TUNEL Assay**—Alveolar macrophages from Normal rats (1 × 10⁴) or mice (5 × 10⁵) were allowed to adhere to a slide, fixed in 4% paraformaldehyde for 25 min, washed twice in PBS for 5 min each, and permeabilized in PBS containing 0.2% Triton X-100 for 5 min. After rinsing again in PBS, the slides were covered with equilibration buffer (200 mm potassium cacodylate, 25 mm Tris-HCl, pH 6.6, 0.2 mL dithiothreitol, 0.25 mg/mL bovine serum albumin, 2.5 mm cobalt chloride) for 5 min and then incubated with the biotinylated nucleotide mix (1:100) and terminal deoxynucleotide transferase (1:100) for 60 min at 37 °C from the DeadEnd colorimetric TUNEL assay kit (Promega, Madison, WI). The slides were washed in 2× SSC for 15 min, rinsed in PBS, and then treated with 0.3% H₂O₂ for 5 min to inactivate the endogenous peroxidase activity. The H₂O₂ was washed off with PBS, and the slides were incubated in 100 μl of streptavidin-HRP (1:500) for 30 min. After washing in PBS to remove the excess streptavidin-HRP, the slides were incubated with diaminobenzidine substrate (1:50), diaminobenzidine chromagen (1:20), and 5 μl of H₂O₂ until the stain was visible. At least 300 alveolar macrophages were counted from each slide, and averages ± S.D. were obtained for at least 5 animals or 10 independent alveolar macrophage aliquots from each condition.

**Cytosolic Cytochrome c in Alveolar Macrophages**—One million alveolar macrophages were tested for cytosolic cytochrome c levels using the Function ELISA cytochrome c kit (Active Motif, Carlsbad, CA). The macrophages, in 500 μl of lysis buffer (supplied with the kit), were homogenized in a glass pestle (20 strokes) and then centrifuged to pellet cellular debris. One hundred microliters of the supernatant was assayed with capture ELISA. The cellular extract was bound to a cytochrome c antibody attached to the ELISA plate, followed by reaction with a
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biotinylated detecting antibody to cytochrome c and with streptavidin-horseradish peroxidase (HRP). The signal was detected by oxidation of trimethylbenzidine substrate by HRP, and the color produced was read at 450 nm on a V_{max} kinetic microplate reader (Molecular Devices, Sunnyvale, CA). Experimental values were converted to nanograms/ml using a cytochrome c standard curve. Averages ± S.D. were obtained from quadruple replicates in each assay of at least five separate experiments.

Polyamine Determination—Spermine, spermidine, N\textsuperscript{1}-acetyl spermine, and N\textsuperscript{11}-acetyl spermidine levels in BAL fluids and alveolar macrophages were assessed. Briefly, BAL fluid samples or alveolar macrophages from animals were sonicated for a total of 2 min in 15-s intervals on ice. Nuclei and cell ghosts in the sonicated samples were removed by centrifugation at 2,000 × g for 5 min. The clarified BAL fluids and alveolar macrophage cell lysates were boiled in a water bath for 2 min. The boiled samples were clarified by centrifugation at 2,000 × g for 5 min, and the final supernatant was used for polyamine determination by HPLC using the AccQ.Fluor kit (Waters, Milford, Mass.) as described previously (47, 48). Results were averages ± S.D. for three independent assessments from different animals, expressed in micromolars for BAL fluids and pmol/10\textsuperscript{6} cells for alveolar macrophage lysates.

Some BAL fluids were depleted of polyamines using an ion exchange chromatography procedure described by Hawel and Byus (49). The ion exchange resin, Bio-Rex 70 (Bio-Rad), was prepared by adding 19 mm sodium carbonate buffer, pH 9.2, for 1 h with gentle mixing and then packed in 40-mm columns. Samples were tested for polyamine content before and after passage through the column.

BAL Fluid Lipopolysaccharide (LPS) Determination—BAL fluids from Normal, Dex, and Dex-Pc rats before and after polyamine depletion were assessed for LPS using the Limulus amebocyte lysate kit (Cambrex, Walkersville, MD) according to the manufacturer’s instructions. 1,3-β-Glucan is a principal constituent of the Pneumocystis cell wall (50) and may produce a false-positive signal in Limulus amebocyte lysate-based LPS assays. BAL fluids (diluted 1:4 in pyrogen-free saline) were mixed 1:1 with a β-glucan blocker (Cambrex) to prevent any interfering signal prior to LPS assessment. Results are presented as average enzyme units of LPS/ml of BAL fluid ± S.D. of three independent trials in each of four independent samples of each condition were run in triplicate.

ROS Determination—ROS were assayed using 2',7' dichlorodihydrofluorescein diacetate (H\textsubscript{2}DCFDA) (Invitrogen), which is a cell-permeable indicator for ROS. It is cleaved to a nonfluorescent diol by intracellular esterases and then oxidized by ROS to its fluorescent form. The H\textsubscript{2}DCFDA (10 μM) solution was incubated with macrophages at 37 °C for 2 h prior to the 24-h incubation with BAL or polyamines. The cells (1 × 10\textsuperscript{6}) were centrifuged at 300 × g; the supernatant was removed, and 1% paraformaldehyde in PBS was added. The macrophages were analyzed on an Epics 500 flow cytometer (Beckman Coulter, Miami, FL) with a forward and side scatter gate applied to select the macrophages. The median whole cell fluorescein isothiocyanate fluorescence of the oxidized H\textsubscript{2}DCFDA was captured for the population.

RESULTS

Alveolar Macrophage Number in a Rat and Two Mouse Models of Pcp—Previous studies show that the number of alveolar macrophages decreases by 60% as early as 7 days after the initiation of Pneumocystis infection in rats and remains low for the duration of the infection (21). To determine whether the reduction in alveolar macrophage number during Pcp is a general response to Pneumocystis infection in rodents, alveolar macrophage number in both the dexamethasone-suppressed and the anti-CD4 suppressed mouse models of infection was determined. Dex and Dex-Pc BALB/c mice were sacrificed every 7 days after initiation of immunosuppression, and the alveolar macrophages in BAL fluids were counted. The changes in alveolar macrophage number in mice showed a different profile from that of Dex-Pc rats (21). In Dex-Pc rats, there was an immediate decrease in alveolar macrophages numbers after Pneumocystis infection, whereas in mice there was an initial increase followed by a rapid and dramatic decrease in the alveolar macrophage numbers (Fig. 1A). The maximum increase in alveolar macrophage number was noted between days 21 and 28, with 69.7 and 69.8% increases over those of Dex controls, respectively. At these time points, the trophozoite and cyst scores were still low, as compared with those late in infection, but were increasing (Fig. 1A). By day 35, the number of alveolar macrophages began to decrease, falling below that of the uninfected controls after day 42. By day 63, when the study was terminated, the alveolar macrophage number in the Pneumocystis-infected group was 58.4% (p < 0.0001) below that of the control group (Fig. 1A), very similar to the degree of loss in the rat model of infection (21). At this point in infection, the organism burden was high, with many trophozoites (124.4 ± 13.1/1000× field) and cysts (21.6 ± 1.9/1000× field). Dexamethasone treatment alone did not reduce the alveolar macrophage number significantly (Fig. 1A).

To further verify that dexamethasone was not the cause of alveolar macrophage decrease, another mouse model was used. Immunosuppression was achieved by injecting BALB/c mice with an anti-CD4 cell antibody (GK1.5). At least five mice of each immunosuppressed group were sacrificed every 7 days and assessed for Pneumocystis organisms (both trophozoites and cysts) and alveolar macrophage number. Normal mice were sacrificed at day −3 and day 0 to determine the normal number of alveolar macrophages (~8 × 10\textsuperscript{4}). As seen in Fig. 1B, alveolar macrophage number in CD4-dep. mice remained relatively constant (~8 × 10\textsuperscript{4}/mouse) throughout the study. In CD4-Pc mice, alveolar macrophage number increased 1.25-fold by day 7, 1.5-fold by day 14, and peaked (1.65-fold) at day 21 (Fig. 1B). The number then decreased by 25% from the peak value by day 35 and continued to decrease over time; it was 44% below normal levels by day 42 and reached a nadir of 31% of normal values by day 49 (Fig. 1B). The number of macrophages stayed low for the remainder of the study period. This degree of decrease in alveolar macrophage number was about the same as that observed in Dex-Pc rats.
Both trophozoite and cyst forms of the organism increased slowly with time during the early stage (day 0–28) of infection, whereas the alveolar macrophage increased over this same time period. Trophozoite scores increased from an average of 0.4 ± 0.1 organisms per 1000 × microscopic field at day 7 to ∼118.5 ± 12.2 at day 63, and cyst scores increased from 0.2 ± 0.03 at day 14 to ∼79.2 ± 6.5 at day 63 when alveolar macrophage number was at its lowest level. Alveolar macrophage number began to decrease (day 28) before the organism burden started to rise rapidly at day 42. Because dexamethasone was not used as the immunosuppressant in this study, the decrease in alveolar macrophage number was because of Pcp.

Reduced Viability of Alveolar Macrophages during Pcp—XTT in vitro toxicity assay of alveolar macrophages from Normal, Dex, and Dex-Pc rats isolated after 7 days of infection showed that Pneumocystis infection increased the number of dead cells as compared with Normal and Dex controls. Normal alveolar macrophages were used as the control sample, representing 100% viability. However, 25.8% of alveolar macrophages from Dex-Pc rats were dead (74.2 ± 2.1% viable cells) as compared with Normal samples. Dexamethasone treatment alone reduced the number of viable alveolar macrophages by only 3% (96.6 ± 1.9% viable cells as compared with Normal controls), indicating that Pneumocystis infection specifically increased the number of dying cells. These results imply that the decrease in alveolar macrophage number during infection was due in part to increased cell death.

Similar results were obtained in the mouse models of infection. Alveolar macrophages showed increased cell death both early and late in infection in the CD4-dep. model of infection. On day 21 the alveolar macrophages from CD4-Pc mice showed a viability of 81.6 ± 4.0%, as compared with CD4-dep. and noninfected mice (p < 0.05). On day 42, infected mice had only 72.9 ± 5.2% viable alveolar macrophages (p < 0.05) as compared with immunosuppressed controls. Similar results were seen both early and late in infection for the Dex-Pc mouse model of infection (data not shown).

Increased Chromosomal DNA Fragmentation in Alveolar Macrophages during Pcp—To determine whether the increased cell death noted above was because of apoptosis, alveolar macrophages from Normal, Dex, and Dex-Pc rats were assessed for signs of programmed cell death by TUNEL assay. After 10 days of infection, Dex-Pc rats already showed 33.6 ± 3.9% of alveolar macrophages positive for TUNEL assay, indicating chromosomal fragmentation (Table 1). This number was increased to 35.8 ± 4.7% at 28 days after initiation of infection. In contrast, less than 1.5% of alveolar macrophages from Normal and Dex rats were positive for the assay at both time points. Treatment of normal alveolar macrophages with 400 μg/ml bleomycin for 24 h caused ∼45% of cell to undergo apoptosis (Table 1).

Similar results were obtained in mice. At 10 and 28 days, alveolar macrophages from Normal and CD4-dep. mice had a low TUNEL-positive rates (Table 1). CD4-Pc mice showed significantly higher levels (13.9–14.9-fold increase, p < 0.05 versus CD4-dep.) of alveolar macrophage apoptosis at 10 and 28 days of infection, even though the alveolar macrophage number was higher than that in the control animals at this time point. At 49 days of infection, the TUNEL positive alveolar macrophages were further increased to 18.7-fold over that of immunosuppressed controls (p < 0.05 versus immunosuppressed control, p > 0.05 versus CD4-Pc at 28 days infection).

Immunosuppression of mice with dexamethasone resulted in apoptosis rates similar to those of mice immunosuppressed with the anti-CD4+ antibody. Alveolar macrophages from Dex control mice had low apoptosis rates at all time points, whereas 10- and 28-day-infected Dex-Pc mice had 18.0- and 16.5-fold (10 and 28 days, respectively, p < 0.05 versus control) more apoptotic alveolar macrophages. At 49 days of infection,
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**TABLE 1**

Percent of alveolar macrophages undergoing apoptosis

| Animal | Normal | Immunosuppressed | Pcp | Bleomycin-treated |
|--------|--------|------------------|-----|-------------------|
| Rat, 10 days | 1.0 ± 0.2 | 1.1 ± 0.3 | 33.6 ± 3.9f | 45.1 ± 6.7f |
| Rat, 28 days | 1.1 ± 0.5 | 1.4 ± 0.6 | 35.8 ± 4.7f | 42.9 ± 5.6f |
| CD4-depleted, 10 days | 2.2 ± 0.3 | 2.1 ± 0.4 | 30.8 ± 4.2f | 46.7 ± 3.9f |
| CD4-depleted, 28 days | 1.9 ± 0.2 | 2.0 ± 0.5 | 27.8 ± 4.0f | NAf |
| CD4-depleted, 49 days | NA | 2.2 ± 0.3 | 41.3 ± 3.4f | NA |
| Dex mouse, 10 days | 1.7 ± 0.2 | 1.8 ± 0.3 | 32.4 ± 4.0f | 42.3 ± 5.1f |
| Dex mouse, 28 days | 2.0 ± 0.4 | 2.1 ± 0.1 | 29.2 ± 3.3f | 38.5 ± 5.3f |
| Dex mouse, 49 days | NA | 1.9 ± 0.1 | 42.6 ± 4.2f | NA |

*There were 8–10 animals per group.

f Immunosuppressed indicates Dex-suppressed rats or mice or anti-CD4 antibody-treated mice.

Pcp indicates immunosuppressed animals with *Pneumocystis* infection.

Bleomycin-treated indicates alveolar macrophages from normal animals treated with 400 μg/ml for 24 h.

Value indicates p < 0.05 versus immunosuppressed animals of same model.

NA indicates not assessed.

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Dex-Pc mice had 18.8-fold more apoptotic alveolar macrophages (p < 0.05 versus control). The results indicate that in mouse models of Pcp, alveolar macrophages are undergoing apoptosis at increased rates even when the alveolar macrophage numbers are higher than in the controls. This may reflect a species difference in their ability to recruit macrophages to the alveoli during Pcp.

**Increased Caspase-3 Activation in Alveolar Macrophages during Pcp**—To confirm apoptosis of alveolar macrophages during Pcp, soluble proteins from alveolar macrophages were examined for caspase-3 activation by immunoblots using antibodies specific for the cleaved (active) form of caspase-3. Fig. 2 shows that alveolar macrophages from Normal and Dex rats had low levels of caspase-3 activation, whereas those from Dex-Pc rats had significantly greater signal for the activated form of this enzyme.

Caspase-3 activation was also assessed in alveolar macrophages from CD4-depleted mice and those from CD4-depleted mice infected with *Pneumocystis* for 10, 28, or 49 days (Fig. 2). In parallel with the alveolar macrophage viability results, alveolar macrophages from infected mice at all time points showed increased levels of activated caspase-3, whereas those from CD4-depleted control mice had very low levels of caspase-3 activation. Similar results were obtained from alveolar macrophages isolated from Normal, Dex, and Dex-Pc mice (data not shown). For Dex-Pc mice, alveolar macrophages were obtained from animals that had been infected for 28–35 days, when alveolar macrophage numbers were still high. This result indicated that apoptosis of alveolar macrophages in Pcp mice preceded the precipitous drop in alveolar macrophage numbers.

**FIGURE 3. Caspase-9 activation in alveolar macrophages during Pcp.**

A, alveolar macrophages from Normal, Dex, and Dex-Pc rats were lysed, and proteins were separated by SDS-PAGE. Western blot analysis for the cleaved (activated) form of caspase-3 was performed, with GAPDH used to control for protein loading and for alveolar macrophages from individual rats and are pooled alveolar macrophages from three mice. Results are representative of three independent Western blot analyses.

**Increased Caspase-9 Activation during Pcp in Rats and Mice**—In a similar fashion to caspase-3, activated caspase-9 levels were assessed. In this case, immunoprecipitation with a procaspase-9 antibody was performed prior to Western blot analysis using an antibody specific for the cleaved (active) form of caspase-9. Activated caspase-9 was not seen in alveolar macrophages from Normal and Dex rats but was clearly observed in those from Dex-Pc rats that had been infected for 35 days (Fig. 3A). Results also indicated that alveolar macrophages from CD4-depleted mice had undetectable levels of activated caspase-9, whereas those from CD4-Pc mice had considerable levels of cleaved caspase-9 at both 4 and 7 weeks of infection (Fig. 3B).

These results were also seen in the Dex-Pc mouse model, where alveolar macrophages from Normal and Dex-suppressed mice had no activated caspase-9, but Dex-Pc mice had high levels of caspase-9 (data not shown). Interestingly, caspases-8 and -10 were not activated in any of the three animal models used for these studies (data not shown). This result suggested that activation of the caspase cascade took
place through the intrinsic pathway, via the mitochondria and caspase-9, rather than through the extrinsic pathway, via caspases 8 and 10.

**Induction of Alveolar Macrophage Apoptosis by BAL Fluids**—To determine whether the apoptosis was induced by certain factors in the lung during Pcp, 1 million alveolar macrophages from Normal or Dex rats were incubated with 1 ml of PBS or cell-free BAL fluids from Normal, Dex, and Dex-Pc rats and then assessed for apoptosis. As a positive control, the same alveolar macrophages were treated with media containing 400 µg/ml bleomycin, a substance known to induce apoptosis in alveolar macrophages. In all experiments, BAL fluids and alveolar macrophages were from different animals. After 24 h of incubation, the cells were harvested and assessed for apoptosis by TUNEL assay.

The results showed that apoptosis was induced in alveolar macrophages from Normal or Dex rats only by BAL fluids from Dex-Pc animals (Table 2). BAL fluids from Normal rats induced only 1.1 ± 0.5% of alveolar macrophages from Normal and 1.2 ± 0.2% of those from Dex rats to undergo apoptosis. Similar levels of apoptosis were seen in cells incubated with BAL fluids from Dex rats. In contrast, incubation of alveolar macrophages from Normal and Dex rats with BAL fluids from Dex-Pc rats induced ~15-fold more apoptosis (p < 0.05 versus Normal BAL fluids, for both) (Table 2). Bleomycin induced rates of apoptosis roughly equal to those of alveolar macrophages from Dex-Pc rats. In a similar fashion, alveolar macrophages from Normal and CD4-dep. mice were stimulated to undergo apoptosis by cell-free BAL fluids from CD4-Pc mice and bleomycin (15.2- and 45-fold increases, respectively, over control, p < 0.05 for each versus control; Table 2).

Some normal alveolar macrophages were incubated with a mixed population of viable *Pneumocystis* organisms at a multiplicity of infection of 10 in place of the Dex-Pc BAL fluids. The organisms alone did not induce apoptosis (1.4 ± 0.3%). These results indicated that soluble factors released into the alveoli during the infection, but not intact *Pneumocystis* organisms, induced the apoptosis.

**Dilution of ALF by Bronchoalveolar Lavage**—Because urea concentrations in serum and ALF are the same, the concentration in the serum divided by that in the BAL fluid of the same animal would be the fold dilution of the ALF by the lavage process. Serum samples from Normal rats were found to have 54.9 ± 4.1 mg/dl of urea, and BAL fluids from the same rats had 1.42 mg/dl urea, representing a 38.6-fold dilution of the ALF. Urea concentration in the serum samples of Dex rats was determined to 52.2 ± 3.9 mg/dl and that in BAL fluids from these rats was 1.46 ± 0.1 mg/dl; therefore, the ALF from Dex rats was diluted by 35.8-fold. Urea concentrations in sera and BAL fluids from Dex-Pc rats were determined to be 51.5 ± 0.6 and 2.59 ± 0.2 mg/dl, respectively, indicating a 19.9-fold dilution by the lavage. The reduced dilution of ALF in the Dex-Pc rats reflected the increased ALF volume in these animals caused by the influx of the proteinaceous exudate during the infection.

In CD4- cell-depleted mouse model, the dilution of ALF in infected mice was also lower than in immunosuppressed mice. CD4-dep. mice had 54.7 ± 4.8 mg/dl serum urea and 3.16 ± 0.3 mg/dl BAL fluid urea, for an ALF dilution of 17.3-fold. In the infected mice, the dilution was 11.4-fold, with serum containing 65.8 ± 7.1 mg/dl of urea and BAL fluids containing 5.74 ± 0.4 mg/dl of urea. The ALF dilution factors were lower in mice than in rats perhaps because of the difference in volume of saline instilled and recovered and the inherent differences in lung volumes between the two species.

**Polyamine and Acetylated Polyamine Levels in ALF and Alveolar Macrophages from Dex-Pc Rats**—Because polyamines can stimulate apoptosis (32), the levels of these compounds in BAL fluids and alveolar macrophages from rats with Pcp were assessed. BAL fluid polyamine levels were then corrected for dilution of ALF during the lavage process. The results indicate that Pcp in rats leads to increased polyamine levels in the ALF (Table 3). Spermidine was increased 27.2-fold after only 10 days that Pcp in rats leads to increased polyamine levels in the ALF. Serum samples from Normal rats were found to have 54.9 ± 4.1 mg/dl of urea, and BAL fluids from the same rats had 1.42 mg/dl urea, representing a 38.6-fold dilution of the ALF. Urea concentration in the serum samples of Dex rats was determined to 52.2 ± 3.9 mg/dl and that in BAL fluids from these rats was 1.46 ± 0.1 mg/dl; therefore, the ALF from Dex rats was diluted by 35.8-fold. Urea concentrations in sera and BAL fluids from Dex-Pc rats were determined to be 51.5 ± 0.6 and 2.59 ± 0.2 mg/dl, respectively, indicating a 19.9-fold dilution by the lavage. The reduced dilution of ALF in the Dex-Pc rats reflected the increased ALF volume in these animals caused by the influx of the proteinaceous exudate during the infection.

In CD4- cell-depleted mouse model, the dilution of ALF in infected mice was also lower than in immunosuppressed mice. CD4-dep. mice had 54.7 ± 4.8 mg/dl serum urea and 3.16 ± 0.3 mg/dl BAL fluid urea, for an ALF dilution of 17.3-fold. In the infected mice, the dilution was 11.4-fold, with serum containing 65.8 ± 7.1 mg/dl of urea and BAL fluids containing 5.74 ± 0.4 mg/dl of urea. The ALF dilution factors were lower in mice than in rats perhaps because of the difference in volume of saline instilled and recovered and the inherent differences in lung volumes between the two species.
present in higher concentrations than the nonacetylated versions of each polyamine.

To determine intracellular polyamine levels in alveolar macrophages, 2 × 10⁶ alveolar macrophages from Normal, Dex, and Dex-Pc rats or 4 × 10⁶ alveolar macrophages from CD4-dep. and CD4-dep-Pc mice were assayed for polyamine levels. Spermidine levels in alveolar macrophages from Dex-Pc rats were increased ~7-fold as compared with those from Dex rats (Table 4). There was no significant difference in spermine levels in alveolar macrophages from Normal and Dex rats. Similar degrees of increase in N³-acetylspermidine (~5-fold) and N⁴-acetylspermine (~7-fold) levels were observed in alveolar macrophages from Dex-Pc rats as compared with those from Dex rats, but there was no significant difference in the levels of N⁴-acetylspermidine and N⁴-acetylspermine in alveolar macrophages from Normal and Dex rats. Spermine levels were not increased during Pcp in BAL fluids or in alveolar macrophages (data not shown).

In infected mice, alveolar macrophage polyamine levels were also increased. Alveolar macrophages from uninfected mice had low levels of spermidine, N⁴-acetylspermidine, and N⁴-acetylspermine (99.2 ± 44, 68.1 ± 22, and 995 ± 45 μM, respectively). Pneumocystis-infected mice had much higher levels of these same polyamines with 5.4-, 7.26-, and 6.9-fold increases for spermidine, N⁴-acetylspermidine, and N⁴-acetylspermine, respectively. All these differences were statistically significant, p < 0.05. These results agreed well with those in rats with Pcp, indicating that increased intracellular polyamines are common to both models of Pcp.

**Polyamines and Apoptosis of Alveolar Macrophages in Vitro and in Vivo**—To link high polyamine levels with alveolar macrophage apoptosis, 1 million alveolar macrophages from Normal rats were incubated in complete media at 37 °C, 5% CO₂ supplemented with saline or polyamines in saline. Final concentrations of polyamines were 1 mM or 100 μM. Because the highest polyamine concentration in the ALF samples for Dex-Pc rats was 650 μM (Table 3), these two concentrations used in these studies were within physiologic ranges and sufficient to confirm that levels of ALF polyamines during Pcp are able to induce apoptosis. The polyamines used included spermine, spermidine, N⁴-acetylspermidine, N⁴-acetylspermine, or all of these together. Although N⁴-acetylspermidine was measured in the lungs of infected rats and mice, this polyamine is not available commercially; therefore, N⁸-acetylspermidine was used for exogenous addition of this acetylated polyamine for *in vitro* and *in vivo* experiments. After 24 h of incubation with polyamines, the cells were lysed, and Western blot analysis was performed to detect the activated form of caspase-3. GAPDH protein was also probed as an indicator of equal protein loading. N, normal; Sd, spermidine; Sm, spermine; N-Sd, N⁴-acetylspermidine; N-Sm, N⁴-acetylspermine; all, a mixture of all four polyamines described.

**TABLE 4**

| Condition*  | Spermidine | Acetylspermidine | Acetylspermine |
|-------------|------------|------------------|----------------|
| Normal      | 55.1 ± 7.8 | 170 ± 11         | 1070 ± 25      |
| Dex rat, 40 days | 62.3 ± 12 | 165 ± 17         | 1100 ± 45      |
| Dex-Pc rat, 40 days | 420 ± 45 | 820 ± 50*        | 7790 ± 66*     |
| CD4-dep. mouse, 49 days | 99.2 ± 44 | 68.1 ± 22        | 995 ± 45       |
| CD4-dep.-Pc mouse, 49 days | 540 ± 50* | 500 ± 55*        | 6960 ± 180*    |

* n = 8–10 animals of each condition were assessed.
* Value indicates p < 0.05 versus immunosuppressed at the same time point.
from Dex-Pc rats were then assessed for their ability to induce apoptosis. After 24 h of incubation with alveolar macrophages from Normal or Dex rats, activated caspase-3 levels in the treated cells were assessed. The results showed that polyamine-depleted BAL fluids from Dex-Pc rats lost their ability to induce apoptosis in alveolar macrophages as measured by caspase-3 activation (Fig. 5, top), suggesting that polyamines were a source of apoptotic stimulus in alveolar macrophages incubated with BAL fluids from Dex-Pc rats.

To confirm that polyamines induce apoptosis in alveolar macrophages, polyamine-depleted BAL fluids were supplemented with spermine, spermidine, N⁸-acetyl spermidine, and N⁴-acetyl spermine to concentrations roughly equivalent to those of the samples prior to polyamine depletion. These polyamine-supplemented BAL fluids were then assessed for their ability to induce apoptosis in normal alveolar macrophages. As shown in Fig. 5, bottom, Dex-Pc BAL fluids induced apoptosis as evidenced by strong activation of caspase-3, whereas polyamine-depleted Dex-Pc BAL fluids from the same rat did not induce caspase-3 activation in the alveolar macrophages. When the polyamine-depleted Dex-Pc BAL fluid was supplemented with 50 μM of the four different polyamines, activated caspase-3 was again detected in the alveolar macrophages treated with this BAL fluid. This result indicated that the polyamines in the Dex-Pc BAL fluids stimulate alveolar macrophage apoptosis.

The method used to deplete polyamines from BAL fluids was not specific to polyamines; any cationic substances could have been removed from the samples. Therefore, LPS levels before and after ion exchange chromatography were determined. Results of LPS assay showed that BAL fluids from Normal (2.1 ± 0.4 EU/ml) and Dex (2.5 ± 0.5 EU/ml) rats had very low levels of LPS, and this level was not changed by the polyamine depletion protocol. Dex-Pc rats had 4.1 ± 0.6 EU/ml LPS before and 3.7 ± 0.5 EU/ml LPS after the ion exchange chromatography. Stained lung sections from the animals used in these studies showed no bacteria or other extraneous microorganisms; therefore, the increase in LPS may be due to an incomplete blocking of the β-glucan in the Dex-Pc samples. Because apoptosis was reduced in alveolar macrophages incubated with the depleted samples and LPS levels remained unchanged, LPS was not the source of the apoptotic stimulus.

**DISCUSSION**

In this study, we found that alveolar macrophages undergo apoptosis during Pcp in rats and mice, making immune responses to *Pneumocystis* less effective. As early as 7 days after initiation of the infection in rats, alveolar macrophages are reduced by 60% (21). At this same time point, the apoptosis rate of alveolar macrophages in rats with Pcp was increased ~30.5-fold as compared with immunosuppressed rats (Table 1). This phenomenon also took place in both steroid-immunosuppressed and CD4⁺ lymphocyte-depleted mouse models of Pcp (Table 1). It is likely that this programmed cell death also occurs in humans with Pcp, as the decrease in alveolar macrophage number also occurs in these patients (16–20).

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**TABLE 5**

Induction of apoptosis in alveolar macrophages in vivo by polyamines

| Treatment | Alveolar macrophages (×10⁶) | TUNEL positive macrophages (%) | Cytochrome c (ng/ml/10⁶ cells) |
|-----------|-------------------------------|-------------------------------|-----------------------------|
| Dex       | 4.3 ± 0.1                     | 1.4 ± 0.2                     | 1.3 ± 0.3                   |
| Dex + 100 μM polyamines* | 2.2 ± 0.1³ | 17 ± 2.4⁴ | 5.0 ± 0.7⁹ |
| Dex-Pc    | 2.0 ± 0.2²                  | 32 ± 2.2⁸                    | 5.5 ± 0.4⁶                  |
| Dex-Pc + 100 μM polyamines | 0.8 ± 0.1⁴ | 37 ± 3.1⁵ | 6.6 ± 0.8⁶⁵ |

*100 μM polyamines consisted of 100 μM each of spermine, spermidine, acetyl spermine, and acetyl spermidine. n = 3 independent assays/condition.

* Value indicates p < 0.05 versus Dex.

* Value indicates p < 0.05 versus Dex-Pc (infected for 7 days) and Dex + 100 μM polyamines.

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**FIGURE 5.** Loss of apoptosis-inducing activity of polyamine-depleted BAL fluids from Dex-Pc rats. A, normal rat alveolar macrophages were incubated for 24 h with Normal or Dex-Pc BAL fluids, or Dex-Pc BAL fluids depleted of polyamines. Soluble proteins were then probed by Western blotting for activated caspase-3 as an indicator of apoptosis. GAPDH protein levels were similarly assessed to show equal protein loading. B, to confirm that polyamines were the apoptosis stimulant in the BAL fluids, polyamine-depleted Dex-Pc BAL fluids were supplemented with 50 μM spermine, N⁸-acetyl spermidine, and N⁴-acetyl spermine to restore polyamines to pre-depletion levels. Activated caspase-3 was assessed in soluble protein samples of alveolar macrophages incubated with these BAL fluids for 24 h.
Results of this study indicated that mice lost alveolar macrophages later in the infection than rats (Fig. 1, A and B). This may reflect differences in the inherent susceptibility of the two species to Pneumocystis. BALB/c mice require four times the dose of corticosteroid for twice as long compared with rats in order to render them susceptible to Pneumocystis (41). In these mice, alveolar macrophage numbers initially increased, similar to that seen in immunocompetent rats challenged with a bolus of Pneumocystis carinii (21). Starting at 28 days of infection, the alveolar macrophage number dropped precipitously, falling below the level of those animals on immunosuppressive regimen alone by day 35. The alveolar macrophage number continued to drop as the infection intensified. This decrease in alveolar macrophage number was not because of the steroid immunosuppression, because a similar pattern was observed in mice immunosuppressed by CD4⁺ lymphocyte depletion (Fig. 1B).

Increased apoptosis of alveolar macrophages was apparent prior to the decrease in alveolar macrophage number in both the corticosteroid-suppressed and CD4-dep. mouse models as DNA fragmentation (Table 1) as well as caspase-3 and -9 activation (Fig. 2 and 3) occurred during the early stages of infection. It is possible that there was an increased recruitment of macrophages or differentiation of monocytes so that alveolar macrophage number remained high in the early stages of Pcp in mice. Our results indicated that apoptosis contributed to ~60% loss of alveolar macrophages during Pcp. This 60% loss of alveolar macrophages by apoptosis is physiologically significant because suppression of this apoptosis by administration of the caspase-9 inhibitor results in decreased organism burdens and increased survival of infected animals (52).

The alveolar macrophage number drops abruptly in both rat (21) and mouse (Fig. 1, A and B) models of Pcp, whereas the number of organisms increases gradually over time in both models. The slow increase in organism burden in these animals agrees with previous observations in humans (53, 54) and is seemingly independent of the alveolar macrophage reduction. It is possible that the organism burden is controlled by more than just the alveolar macrophage number, or that the decreased phagocytic ability (10, 55) of the alveolar macrophages in Pcp renders these cells unable to control organism number even when in higher numbers.

The results of this study indicated that the mechanisms of apoptosis in rats and mice were similar, despite the time difference, suggesting that apoptosis of alveolar macrophages induced by Pneumocystis is common to all infected hosts. These results were based on lavaged alveolar macrophages. Our previous results show that the number of lavaged macrophages correlates well with the number of macrophages counted in lung sections of both uninfected and infected animals (21); therefore, the lavaged macrophages do not represent a certain subpopulation of alveolar macrophages from the lung.

Alveolar macrophages also undergo apoptosis during Bordetella pertussis infection in mice (23). In this infection, apoptosis is mediated by the secreted adenylate cyclase-hemolysin toxin (23, 56) that penetrates phagocytic cells and raises the intracellular levels of cyclic AMP (57, 58). Neisseria gonorrhoeae has also been shown to induce apoptosis in epithelial cells and phagocytes in vitro (25). This apoptosis is mediated through a structural protein, PorB, in the outer bacterial cell wall (25). It is believed that PorB forms a pore structure on the cell membrane of the target cell, with subsequent rises in cytosolic Ca²⁺ and activation of caspase-3 (35, 59). Mycobacterium tuberculosis also induces apoptosis in host macrophages (24, 60). This induction of apoptosis is initiated by the interaction of mycobacterial cell wall constituents with the Toll-like receptor 2 and is mediated through the production of TNF-α (61, 62).

Paradoxically, M. tuberculosis also inhibits the apoptosis of macrophages (26, 61). This inhibition may be due to the production of the anti-apoptotic soluble TNF-α receptor-2, which neutralizes the pro-apoptotic activity of TNF-α (26), or the up-regulation of NF-κB, which is anti-apoptotic through the production of interleukin-10 (61). Likewise, macrophages infected by Toxoplasma gondii are resistant to many apoptotic stimuli (27, 63). As an obligate intracellular parasite, the inhibition of apoptosis in T. gondii-infected cells provides a survival advantage for the organism. Both NF-κB activity (64, 65) and up-regulation of anti-apoptotic factors are implicated in this inhibition (64). Escherichia coli K1 can survive in monocytes and macrophage cell lines and can promote their survival despite treatment with staurosporine (28), which causes apoptosis. This inhibition of apoptosis by E. coli K1 is mediated by OmpA (outer membrane protein A), via inhibition of phosphatidylserine exposure on the cell surface, as well as inhibition of cytochrome c leakage from the mitochondria (28).

In Pcp-induced apoptosis of alveolar macrophages, we observed increased cytosolic cytochrome c levels, activation of caspase-9, and DNA fragmentation (Tables 1 and 2 and Fig. 3). Collectively, these results suggested that the intrinsic pathway was of primary importance in the apoptosis of alveolar macrophages induced by Pneumocystis infection. The observation that caspase-8 and -10 were not activated suggests that other factors such as TNF-α and FasL that activate the extrinsic pathway are not involved in the induction of this apoptosis.

Our results also showed that BAL fluid polyamine levels increased during Pcp as early as 10 days after initiation of infection (Table 3). The increase in polyamines during Pcp correlated with the induction of apoptosis in alveolar macrophages (Table 1). In addition, ROS levels were elevated in alveolar macrophages of rats with Pcp and those treated with BAL fluids from Dex-Pc rats or polyamines (Table 6). Based on these results, we hypothesize that apoptosis of alveolar macrophages during Pcp is induced by polyamines through ROS-mediated damage to the mitochondria. This type of apoptosis induction is also seen during Helicobacter pylori infection, during which apoptosis of macrophages is triggered by increased polyamine (31, 66) and ROS levels (31). This apoptosis is also characterized by mitochondrial damage and activation of caspase-9 (31, 66), similar to what is seen in alveolar macrophages during Pcp. Polyamines can stimulate apoptosis of cells directly (32, 34, 35) or through the generation of ROS (67, 68). Data of the current study suggest that polyamines induce apoptosis of alveolar macrophages via ROS during Pcp (Table 6).

ROS may be generated by the catabolism of polyamines by SSAT and APAO (33). SSAT converts spermine and spermidine to N⁴-acetylsperrmine and N⁴-acetylsperrmidine, respec-
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