The Journal of Immunology

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J Immunol 2000; 165:3934-3940; ;
doi: 10.4049/jimmunol.165.7.3934
http://www.jimmunol.org/content/165/7/3934

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Distinct Effects of Surfactant Protein A or D Deficiency During Bacterial Infection on the Lung[1]

Ann Marie LeVine,[2]* Jeffrey A. Whitsett, Jodie A. Gwozdz, Theresa R. Richardson, James H. Fisher,† Michael S. Burhans, and Thomas R. Korfhagen

Mice lacking surfactant protein (SP)-A (SP-A$^{-/-}$) or SP-D (SP-D$^{-/-}$) and wild-type mice were infected with group B streptococcus or *Haemophilus influenzae* by intratracheal instillation. Although decreased killing of group B streptococcus and *H. influenzae* was observed in SP-A$^{-/-}$ mice but not in SP-D$^{-/-}$ mice, deficiency of either SP-A or SP-D was associated with increased inflammation and inflammatory cell recruitment in the lung after infection. Deficient uptake of bacteria by alveolar macrophages was observed in both SP-A- and SP-D-deficient mice. Isolated alveolar macrophages from SP-A$^{-/-}$ mice generated significantly less, whereas those from SP-D$^{-/-}$ mice generated significantly greater superoxide and hydrogen peroxide compared with wild-type alveolar macrophages. In SP-D$^{-/-}$ mice, bacterial killing was associated with increased lung inflammation, increased oxidant production, and decreased macrophage phagocytosis. In contrast, in the absence of SP-A, bacterial killing was decreased and associated with increased lung inflammation, decreased oxidant production, and decreased macrophage phagocytosis. Increased oxidant production likely contributes to effective bacterial killing in the lungs of SP-D$^{-/-}$ mice. The collectins, SP-A and SP-D, play distinct roles during bacterial infection of the lung. ***The Journal of Immunology,*** **2000, 165: 3934–3940.**

Surfactant protein (SP)-A and SP-D are members of the collectin subgroup of the mammalian C-type lectins that also includes mannose-binding lectin and conglutinin (1, 2). The collectins are thought to be involved in innate host defense against various bacterial and viral pathogens. The collectins form multimeric structures resembling C1q (the first component of the complement cascade), consisting of multimeric collagenous amino-terminal domains and globular carboxy-terminal, carbohydrate binding domains (2). The C-type lectins bind carbohydrate surfaces of many microorganisms mediating phagocytosis and killing by phagocytic cells (3).

SP-A and SP-D are produced primarily by alveolar type II cells and nonciliated bronchiolar cells in the lung. SP-A binds to specific cell surface receptors on alveolar macrophages (4) and type II epithelial cells (5). In vitro, SP-A stimulates macrophage chemotaxis (6) and enhances the binding of bacteria and viruses to alveolar macrophages (3). SP-D binds to alveolar macrophages (7), binds and increases macrophage association with *Escherichia coli* (8), *Mycobacterium tuberculosis* (9), and *Pneumocystis carinii* (10), but does not enhance phagocytosis of these organisms in vitro. SP-D binds and increases phagocytosis of strains of *Pseudomonas aeruginosa* without causing bacterial aggregation (11).

Alveolar macrophages are thought to play a critical role in host defense of the lung. Alveolar macrophages bind, phagocytose, and kill bacteria in association with cellular activation, release of intracellular proteases, and reactive oxygen species. Reactive oxygen species are released by activated alveolar macrophages, directly killing bacteria. In vitro, both SP-A and SP-D can stimulate alveolar macrophages to generate oxygen radicals, measured as chemiluminescence (12, 13). Similarly, in vivo, alveolar macrophages from SP-A-deficient mice have impaired generation of reactive oxygen species (14).

Despite considerable in vitro evidence that SP-A is involved in host defense, its role in vivo has only recently been demonstrated. SP-A-deficient mice produced by targeted gene inactivation are susceptible to bacterial and viral pneumonia (15, 16). In vitro evidence supports a role of SP-D in pulmonary host defense, possibly mediated by different mechanisms than SP-A. In this study, to assess the role of SP-A and SP-D in vivo, SP-A- or SP-D-deficient mice were infected intratracheally with group B streptococcus (GBS) or *Haemophilus influenzae*. Microbial killing, inflammation, uptake of bacteria, and oxygen-radical generation by alveolar macrophages were compared in SP-A$^{-/-}$ and SP-D$^{-/-}$ mice in vivo.

Materials and Methods

Animal husbandry

Separate strains of mice lacking SP-A or SP-D were produced by targeted gene inactivation. Lungs of SP-A$^{-/-}$ or SP-D$^{-/-}$ mice do not contain detectable mRNA or protein (15, 17). In this study, wild-type, SP-D$^{-/-}$, and SP-A$^{-/-}$ mice with National Institutes of Health Swiss Black genetic background were studied. Mice were housed and studied under Institutional Animal Care and Use Committee-approved protocols in the animal facility of the Children's Hospital Research Foundation (Cincinnati, OH). Male and female mice of ~20–25 g (35–42 days old) were used.

Preparation of bacteria

A stock culture of GBS and *H. influenzae* were obtained from clinical isolates provided by Dr. J. R. Wright (Department of Cell Biology, Durham, NC). Bacteria were suspended in media containing 20% glycerol and frozen in aliquots at −70°C. Bacteria from the same passage were used to minimize variations in virulence related to culture conditions. Before each experiment, an aliquot was thawed and plated on tryptic soy-5% defibrinated sheep blood agar (GBS) or chocolate agar plates (*H. influenzae*),

*Children's Hospital Medical Center, Division of Pulmonary Biology and Critical Care Medicine, Cincinnati, OH 45229; and †Division of Pulmonary Biology/Critical Care Medicine, University of Colorado, Denver, CO 80262

Received for publication March 27, 2000. Accepted for publication July 14, 2000.

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[1] This work was supported by Cystic Fibrosis Foundation Grants HL03905 (to A.M.L.), HL58795 (to T.K.), HL61646 (to J.A.W.), and HL56387 (to J.A.W.).

[2] Address correspondence and reprint requests to Dr. Ann Marie LeVine, Children's Hospital Medical Center, Division of Pulmonary Biology, 3333 Burnet Avenue, Cincinnati, OH 45229-3039. E-mail address: levia0@chmcc.org

[3] Abbreviations used in this paper: SP, surfactant protein; GBS, group B streptococcus; MIP, macrophage inflammatory protein; BAL, bronchoalveolar lavage.

0022-1767/00/$02.00

inoculated into 4 ml of Todd-Hewitt (GBS) or trypticase soy (*H. influenzae*) broth (Difco Laboratories, Detroit, MI), and grown for 14–16 h at 37°C with continuous shaking. The broth was centrifuged, and the bacteria were washed in PBS at pH 7.2 and resuspended in 4 ml of the buffer. To facilitate studies, a growth curve was generated so the bacterial concentration could be determined spectrophotometrically and confirmed by quantitative culture of the intratracheal inoculum.

Purification of mouse SP-D

Mouse SP-D was obtained from bronchoalveolar lavage (BAL) from GM-CSF, SP-A double null mutant mice and purified by sequential affinity chromatography on maltosyl-agarose and gel filtration chromatography as described by Strong (18). Endotoxin contamination was not detected in SP-D preparations (<.06 endotoxin units/ml) using the *Limulus* Amoebocyte Lysate assay (Sigma, St. Louis, MO) according to manufacturer's directions.

Labeling of bacteria with FITC and agglutination of H. influenzae *and GBS with SP-D*

Bacteria were grown in broth overnight as described for preparation of bacteria. The OD at 600 nm of the resulting supernatant was measured to determine bacterial concentration. The suspension was then pelleted at maximum speed in a microfuge, and the pellet was resuspended in 0.9 ml PBS, pH 7.2, and heated to 95°C for 10 min to kill the bacteria. The heat-killed bacteria were then pelleted and resuspended in 1 ml 0.1 M sodium carbonate, pH 9.0. FITC (Molecular Probes, Eugene, OR) was added as a 10 mg/ml stock in DMSO to a final concentration of 0.01 mg/ml, and the suspension was incubated for 1 h in the dark at room temperature with gentle agitation. Labeled bacteria were washed four times for 5 min each time with PBS, pH 7.2, to remove unconjugated fluorophore, and finally diluted in PBS and stored in aliquots of 100 μl at −80°C.

To examine SP-D agglutination of bacteria, equal volumes of bacterial suspension (FITC-GBS 10^7 CFU/ml, FITC-*H. influenzae* 10^8 CFU/ml) and SP-D (10 μg/ml) with 2 mM $CaCl_2$ were mixed for 15 min at room temperature, centrifuged on glass slides, and examined by fluorescence microscopy. Control incubations were performed in calcium-free buffer.

Bacterial clearance

Administration of GBS (10^4 CFU) or *H. influenzae* (10^8 CFU) into the respiratory tract of the mice was performed by intratracheal inoculation as previously described (15). Quantitative cultures of lung homogenates were performed 6 and 24 h after inoculation of the animals with bacteria. Mice were exsanguinated after a lethal intraperitoneal injection of sodium pentobarbital. The lung was removed, weighed, and homogenized in 2 ml of sterile PBS. One hundred microliters of homogenate and further dilutions were plated on blood (GBS) or chocolate (*H. influenzae*) agar plates to quantitate bacteria.

Bronchoalveolar lavage

Lung cells were recovered by BAL. Animals were sacrificed as described for bacterial clearance, and lungs were lavaged three times with 1 ml of sterile PBS. The fluid was centrifuged at 800 × *g* for 10 min and resuspended in 1 ml of PBS. Differential cell counts were performed on cytospin preparations stained with Diff-Quick (Scientific Products, McGaw Park, IN).

Association of bacteria with alveolar macrophages

GBS and *H. influenzae* associated with alveolar macrophages in vivo were quantitated with light microscopy by counting the cell-associated organisms on cytospin preparations of lavage fluid 1 h after intratracheal inoculation. Organisms were scored as cell associated only if observed within the perimeter of the cells. In addition, bacterial binding and internalization by macrophages in vivo was measured by intratracheally inoculating mice with FITC-labeled GBS or *H. influenzae* followed by an evaluation of cell-associated fluorescence with a flow cytometer. One hour after infection, macrophages from BAL fluid were incubated in buffer (PBS, 0.2% BSA fraction V, 0.02% sodium azide) with PE-conjugated murine CD16/CD32 Abs (PharMingen, San Diego, CA) for 1 h on ice and washed two times in fresh buffer. Cell-associated fluorescence was measured on a FACScan flow cytometer, using CellQuest software (Becton Dickinson, San Jose, CA) without trypan blue. For each sample of macrophages, 20,000 cells were counted in duplicate, and the results were expressed as the percentage of macrophages with cell-associated bacteria. To discriminate between intra- and extracellular fluorescence, cells were divided into two equal aliquots, one of which was incubated in buffer containing 0.2 mg/ml of trypan blue for 3 min and the other in buffer. Trypan blue was added to quench fluorescence of extracellular FITC and eliminate fluorescence resulting from bacteria attached to the external surface of the cells.

Cytokine production

Lung homogenates were centrifuged at 800 × *g*, and the supernatants were stored at −20°C. TNF, IL-1β, IL-6, and macrophage inflammatory protein (MIP)-2 were quantitated using quantitative murine sandwich ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer's directions. All plates were read on a microplate reader (Molecular Devices, Menlo Park, CA) and analyzed with the use of a computer-assisted analysis program (Softmax; Molecular Devices). Only assays having standard curves with a calculated regression line value >0.95 were accepted for analysis.

BAL nitrite

Nitrite in BAL fluid was measured by the Griess reaction using a commercially available assay (Bioxytech NO Assay; OXIS International, Portland, OR). Methods followed the manufacturer's recommendations. The OD at 550 nm (OD_{550}) was measured using a Spectramax 250 microplate reader (Molecular Devices, Sunnyvale, CA). Nitrite concentrations were calculated by comparison with the OD_{550} of standard solutions of sodium nitrite.

Superoxide and hydrogen peroxide generation

Superoxide anion and hydrogen peroxide (H_2O_2) production by alveolar macrophages was determined as described (14). Eighteen hours after intratracheal inoculation of GBS (10^4 CFU), alveolar macrophages were collected by BAL with 1 ml of dye-free RPMI media (Life Technologies, Grand Island, NY) times three. BAL fluid from eight mice was pooled to provide sufficient numbers of macrophages for analysis. The lavage was centrifuged at 800 × *g* for 10 min, and the pellet was resuspended in 200 μl of PBS. Differential analysis of the cells revealed >95% macrophages. One hundred thousand cells were placed in wells of a 96-well plate with 1.2 mg/ml (~100 μmol/L) cytochrome *c*, with or without 20 μg/ml superoxide dismutase, in a final volume of 200 μl of HBSS. Superoxide anion production was determined after activation with 100 ng/ml PMA. OD at 550 nm was determined using a THERMOmax microplate reader (Molecular Devices) linked to a laboratory computer. Measurements were made initially, 5, 10, and 15 min, then every 15 min until 2 h at 37°C. OD was converted to nanomoles of cytochrome *c* reduced using a molar extinction coefficient of 21.1 $mM^{-1}cm^{-1}$. Each measurement was the mean of at least two replicates with eight determinations at each time. Data were expressed as nanomoles cytochrome *c* reduced per 1×10^5 cells. Superoxide production was assessed by subtracting activity in the presence of superoxide dismutase from total oxygen radical production. Hydrogen peroxide production by macrophages was measured using a commercially available assay (Bioxytech H_2O_2 -560 assay; OXIS International) based on the oxidation of ferrous ions (Fe^{2+}) to ferric ions (Fe^{3+}) by hydrogen peroxide. Ferric ions bind with the indicator dye, xylenol orange, which was measured at 560 nm. Sorbitol was added to the reaction to scavenge oxyl radicals and convert them to hydrogen peroxide and hydroperoxyl radicals, increasing the yield of ferric ions to ~15 moles per mole H_2O_2.

Western blot

Western blot analysis for SP-A and SP-D was performed on tissue homogenates. Lung tissue was homogenized in (500 μl) PBS to which was added 3.5 ml of 10 mM Tris-Cl (pH 7.4), 0.25 M sucrose, 2 mM EDTA, 1 mM PMSF, 10 μM leupeptin, and 10 μM pepstatin A. The homogenate was centrifuged at 250 × *g* for 10 min at 2°C, and the supernatant was centrifuged at 120,000 × *g* for 18 h. The pellet was resuspended in the above buffer (without sucrose) and subjected to SDS-PAGE on 10–27% gradient gels. Proteins were transblotted to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA) and blocked with TBST containing 5% BSA. SP-A and SP-D were detected with guinea pig anti-rat SP-A serum and rabbit anti-rat SP-D serum (the latter provided by Dr. E. Crouch, Department of Pathology, Barnes Jewish Hospital of St. Louis, Washington University, St. Louis, MO) using HRP-conjugated secondary Ab (Calbiochem, San Diego, CA). Membranes were rinsed and developed using enhanced chemiluminescence detection reagents (Amersham, Arlington Heights, IL). Immunoreactive bands were identified by exposing the membranes to XAR film (Kodak, Rochester, NY).

Statistical methods

Lung colony counts, total cell counts, cytokines, superoxide, and hydrogen peroxide were compared using the median scores nonparametric test. Findings were considered statistically significant at probability levels <0.05.

Results

Pulmonary pathology

The intratracheal dose of GBS for study (10^4 CFU) was determined based on previous studies (15). To determine an appropriate dose of *H. influenzae*, wild-type mice were inoculated intratracheally with *H. influenzae* at concentrations of 10^4-10^8 CFU (4 mice/group). The 10^8 CFU dose resulted in 50% mortality, deaths occurring after 48 h. Intratracheal administration of bacteria was well-tolerated and all animals survived the 24-h study period at these doses.

In SP-A$^{-/-}$ mice, increased numbers of cells were observed in BAL fluid 6 h after GBS and 6 and 24 h after *H. influenzae* infection (Fig. 1). Likewise, cell counts in BAL fluid were increased in SP-D$^{-/-}$ mice 6 h after GBS and 24 h after *H. influenzae* infection compared with wild-type mice. A significantly greater percentage of polymorphonuclear leukocytes was detected in BAL fluid from SP-A$^{-/-}$ compared with wild-type mice 24 h after *H. influenzae* infection (Fig. 2). Cell differentials were not different for SP-D$^{-/-}$ and wild-type mice infected with *H. influenzae* or among the groups with GBS infection.

SP-D agglutinates GBS and H. influenzae

SP-D (10 μg/ml) agglutinated FITC-labeled GBS and *H. influenzae* in a calcium-dependent manner (Fig. 3). No agglutination was observed in the absence of calcium or SP-D. Previous studies demonstrated that SP-A binds to GBS (14) and *H. influenzae* (19).

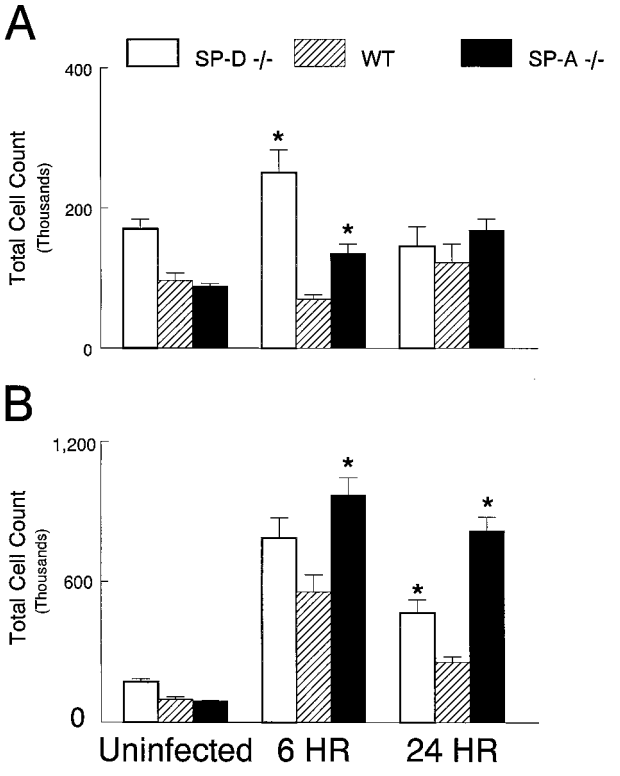

FIGURE 1. Increased total cell counts in BAL fluid from SP-A$^{-/-}$ and SP-D$^{-/-}$ mice. Lung cells were recovered by BAL, stained with trypan blue, and counted under light microscopy. SP-A$^{-/-}$ mice (■) had increased total cell counts in BAL fluid 6 h after GBS (*A*) and 6 and 24 h after *H. influenzae* (*B*) infection. SP-D$^{-/-}$ mice (□) had increased total cell counts in BAL fluid 6 h after GBS (*A*) and 24 h after *H. influenzae* (*B*) infection compared with wild-type mice (▨). Data are mean ± SEM with $n = 8$ mice per group; ∗, $p < 0.05$ compared with wild-type mice.

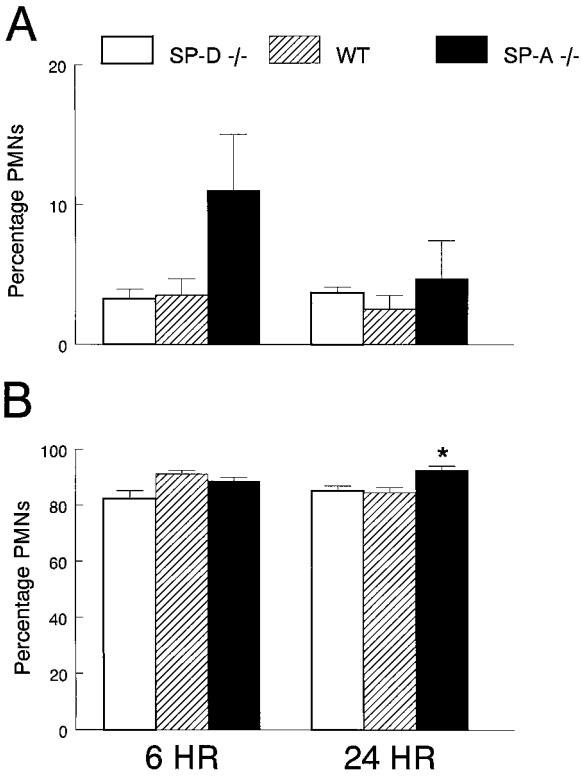

FIGURE 2. Increased neutrophils in BAL fluid from SP-A$^{-/-}$ mice following *H. influenzae* infection. Cytospin preparations of BAL fluid were stained with Diff-Quik to identify macrophages, lymphocytes, and polymorphonuclear leukocytes. The percentage of neutrophils in BAL fluid following GBS infection was similar for wild-type (▨), SP-D$^{-/-}$ (□), and SP-A$^{-/-}$ mice (■) (*A*). The percentage of neutrophils in BAL fluid was significantly greater 24 h after administration of *H. influenzae* to SP-A$^{-/-}$ (■) compared with wild-type (▨) mice (*B*). Data are mean ± SEM with $n = 8$ mice per group; ∗, $p < 0.05$ compared with wild-type mice.

Decreased bacterial clearance in SP-A$^{-/-}$ mice

Numbers of GBS and *H. influenzae* were increased in SP-A$^{-/-}$ compared with wild-type and SP-D$^{-/-}$ mice. The difference in bacterial counts between SP-A$^{-/-}$ and wild-type mice was most evident 6 h after infection, indicating that bacteria were killed in the lungs of SP-A$^{-/-}$ mice at a slower rate than from the lungs of wild-type mice. SP-D$^{-/-}$ mice killed GBS and *H. influenzae* as efficiently as wild-type mice (Fig. 4).

Decreased association of bacteria with alveolar macrophages in SP-D$^{-/-}$ and SP-A$^{-/-}$ mice

Numbers of bacteria associated with alveolar macrophages, assessed by light microscopy, were decreased in SP-A$^{-/-}$ and SP-D$^{-/-}$ mice 1 h after infection with GBS and *H. influenzae* compared with wild-type mice (Fig. 5). Similarly, the number of GBS and *H. influenzae* associated with alveolar macrophages, assessed by flow cytometry, were significantly less in SP-D$^{-/-}$ and SP-A$^{-/-}$ than in wild-type mice (Fig. 5). In the presence of trypan blue, which quenches extracellular fluorescence from surface-bound bacteria, the percentage of intracellular GBS was similar in SP-D$^{-/-}$ and wild-type macrophages (13.3 ± 1.7 vs 14.7 ± 1.3%, respectively, mean ± SEM). SP-D increased the association of GBS with alveolar macrophages but did not alter phagocytosis. In contrast, the number of *H. influenzae* internalized by alveolar macrophages was significantly less in SP-D$^{-/-}$ and SP-A$^{-/-}$ than in wild-type mice, suggesting that macrophage phagocytosis of *H. influenzae* was impaired in the absence of SP-D or SP-A (Fig. 5B).

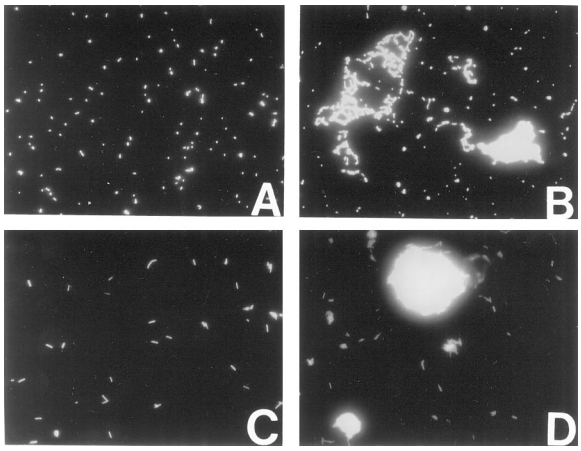

FIGURE 3. SP-D agglutinates GBS and *H. influenzae*. Fluorescence micrograph of FITC-labeled GBS (10^7 CFU) with SP-D treatment (10 μg/ml) without calcium (*A*). *B*, FITC-labeled GBS (10^7 CFU) incubated with SP-D (10 μg/ml) and 2 mM $CaCl_2$, demonstrating agglutination of the bacteria. *C*, FITC-labeled *H. influenzae* (10^8 CFU) with SP-D treatment (10 μg/ml) without calcium. *D*, FITC-labeled *H. influenzae* (10^8 CFU) incubated with SP-D (10 μg/ml) and 2 mM $CaCl_2$, demonstrating agglutination of the bacteria. No agglutination was observed in the absence of SP-D.

Cytokine levels in lung homogenates

Infection with GBS and *H. influenzae* significantly increased the proinflammatory cytokines, TNF, IL-1β, IL-6, and MIP-2 in lung homogenates from SP-A$^{-/-}$, SP-D$^{-/-}$, and wild-type mice. Six hours after infection with GBS and *H. influenzae*, levels of TNF and IL-6 were significantly greater in lung homogenates from SP-A$^{-/-}$ and SP-D$^{-/-}$ compared with wild-type mice (Fig. 6). IL-1β was increased after *H. influenzae* infection in lung homogenates from SP-A$^{-/-}$ and SP-D$^{-/-}$ mice. MIP-2, a neutrophil chemoattractant, was significantly greater in lung homogenates from SP-A$^{-/-}$ but not SP-D$^{-/-}$ mice after *H. influenzae* infection. Basal cytokine levels in the lungs of control mice inoculated with sterile PBS were low/absent and not different among SP-A$^{-/-}$, SP-D$^{-/-}$, and wild-type mice (data not shown).

Increased nitrite in BAL fluid from SP-A$^{-/-}$ and SP-D$^{-/-}$ mice

NO production after GBS and *H. influenzae* infection was estimated as nitrite in BAL fluid. NO reacts with superoxide to form peroxynitrite, which is a potent bactericidal radical. Compared with wild-type mice, BAL fluid from SP-D$^{-/-}$ mice had increased nitrite levels 6 and 24 h after GBS and *H. influenzae* infection (Fig. 7). Similarly, increased nitrite levels were observed in BAL fluid from SP-A$^{-/-}$ mice 24 h after GBS and 6 and 24 h after *H. influenzae* infection. Baseline nitrite levels in BAL fluid after PBS treatment were 2.4 $\pm$ 0.2, 2.8 $\pm$ 0.2, and 2.7 $\pm$ 0.1 μM for wild-type, SP-D$^{-/-}$, and SP-A$^{-/-}$ mice, respectively, mean $\pm$ SEM.

Superoxide and hydrogen peroxide production by alveolar macrophages

Superoxide and hydrogen peroxide production were assessed in macrophages isolated from BAL fluid 18 h after intratracheal administration of GBS (10^4 CFU). After stimulation with PMA, superoxide radical and hydrogen peroxide production by alveolar macrophages were significantly decreased in SP-A$^{-/-}$ and increased in SP-D$^{-/-}$ compared with wild-type mice (Fig. 8). Macrophage hydrogen peroxide production from PBS-treated controls was greater for SP-D$^{-/-}$ compared with wild-type mice (25.5 $\pm$ 5.0* and 3.4 $\pm$ 0.3 μM, respectively, mean $\pm$ SEM, with $n = 4$ determinants/group; *$p < 0.05$ compared with wild type mice).

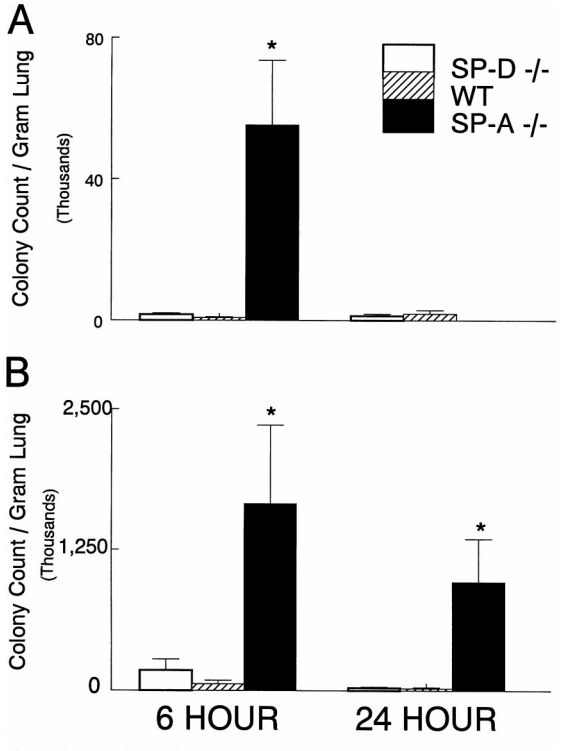

FIGURE 4. GBS and *H. influenzae* killing in lungs of SP-A$^{-/-}$ and SP-D$^{-/-}$ mice. Concentration of GBS and *H. influenzae* were determined by quantitative cultures of lung homogenates. Colony counts were significantly greater 6 h after administration of 10^4 CFU GBS (*A*) and 6 and 24 h after 10^8 CFU *H. influenzae* (*B*) in SP-A$^{-/-}$ (■) compared with wild-type (▨) mice. SP-D$^{-/-}$ mice (□) killed GBS and *H. influenzae* as efficiently as wild-type mice (▨). Clearance of GBS from the lung at 24 h is impaired in SP-A$^{-/-}$ mice as previously demonstrated (15) (data not presented in the graph). Data are mean $\pm$ SEM with $n = 10$ mice per group; *, $p < 0.05$ compared with wild-type mice.

SP-A and D levels following infection

Western blot analysis was used to assess whether SP-D or SP-A changed following GBS or *H. influenzae* infection. SP-D was not detected in SP-D$^{-/-}$ mice but was detected in lung homogenates from both wild-type and SP-A$^{-/-}$ mice; concentrations of SP-D did not change 24 h after GBS or *H. influenzae* infection in SP-A$^{-/-}$ mice (data not shown). As expected, SP-A was not detected in homogenates of the SP-A$^{-/-}$ mice. Similarly, SP-A was detected in lung homogenates from both wild-type and SP-D$^{-/-}$ mice and was not changed 24 h after GBS or *H. influenzae* infection in SP-D$^{-/-}$ mice (data not shown).

Discussion

Pulmonary killing of intratracheally administered GBS and *H. influenzae* was reduced in SP-A$^{-/-}$ mice compared with wild-type mice, whereas killing of either organism was not defective in SP-D$^{-/-}$ mice. Pulmonary inflammation was increased in both SP-A- and SP-D-deficient mice compared with wild-type controls as indicated by increased total cell counts, proinflammatory cytokines, and nitrites in the lung after bacterial infection. In the absence of SP-A or SP-D, association of bacteria with alveolar macrophages was decreased, reflecting a defect in opsonization and/or phagocytosis in both models. Superoxide and hydrogen peroxide production was decreased in alveolar macrophages isolated from SP-A$^{-/-}$ mice and increased from alveolar macrophages from SP-D$^{-/-}$ mice. These findings support the concept that both SP-A

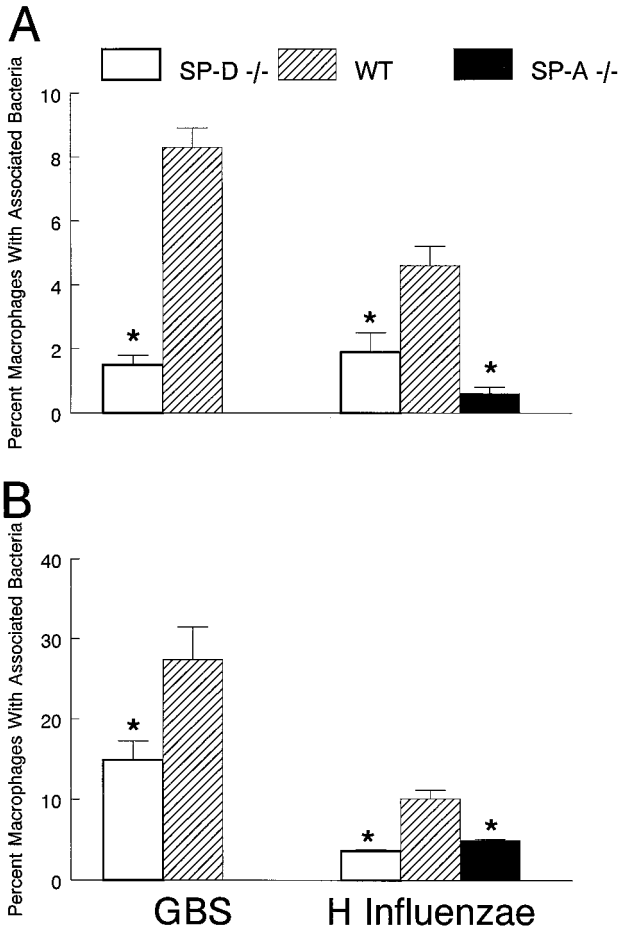

FIGURE 5. Decreased uptake/phagocytosis of bacteria in SP-A$^{-/-}$ and SP-D$^{-/-}$ mice. GBS and *H. influenzae* associated with alveolar macrophages in vivo were quantitated by counting cell-associated organisms in cytospin preparations of BAL fluid and by flow cytometry as described in *Materials and Methods*. One hour after intratracheal inoculation, significantly fewer alveolar macrophages contained *H. influenzae* from SP-D$^{-/-}$ (□) and SP-A$^{-/-}$ (■) compared with wild-type (▨) mice as evaluated by light microscopy (*A*). Similarly, fewer alveolar macrophages contained *H. influenzae* from SP-A$^{-/-}$ (■) and SP-D$^{-/-}$ (□) compared with wild-type (▨) mice as evaluated by flow cytometry (*B*). Numbers of GBS associated with alveolar macrophages were significantly less from SP-D$^{-/-}$ (□) compared with wild-type (▨) mice analyzed without trypan blue. Macrophages from SP-A$^{-/-}$ mice have impaired phagocytosis of GBS as previously described (14) (data not presented in the graph). Data represent mean ± SEM with $n = 8$ mice per group; ∗, $p < 0.05$ compared with wild-type mice.

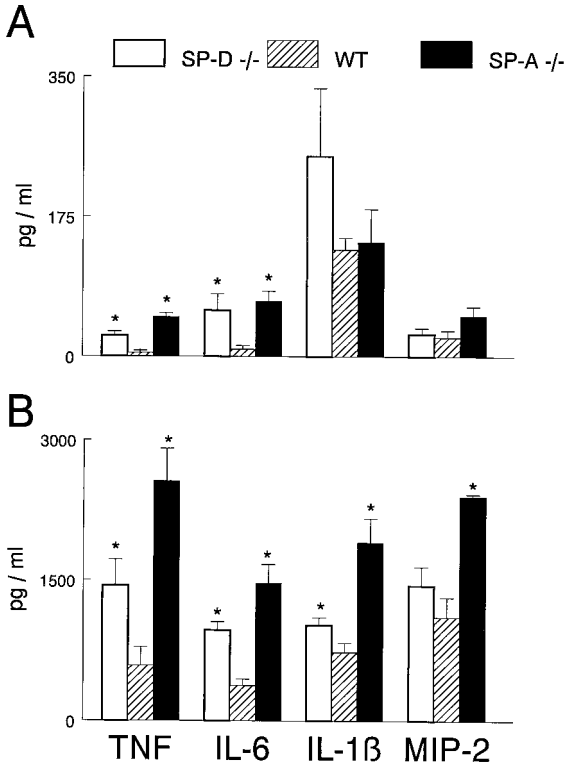

FIGURE 6. Increased cytokine concentrations in SP-A$^{-/-}$ and SP-D$^{-/-}$ mice after infection. TNF, IL-1β, IL-6, and MIP-2 were assessed in lung homogenates from SP-D$^{-/-}$ (□), SP-A$^{-/-}$ (■), and wild-type (▨) mice following GBS and *H. influenzae* infection. Increased concentrations of the proinflammatory cytokines TNF and IL-6 were found in lung homogenates from SP-D$^{-/-}$ and SP-A$^{-/-}$ mice 6 h after GBS infection (*A*). Similarly, TNF, IL-1β, and IL-6 concentrations were increased in SP-A$^{-/-}$ and SP-D$^{-/-}$ compared with wild-type mice after *H. influenzae* infection (*B*). MIP-2 was increased in the lungs of SP-A$^{-/-}$ mice after *H. influenzae* infection. Data are expressed as picograms per milliliter and represent mean ± SEM with $n = 10$ mice per group; ∗, $p < 0.05$ compared with wild type.

and SP-D play distinct and important roles in the initial pulmonary host defense against these bacterial pathogens.

SP-A and SP-D are members of the C-type lectin family of polypeptides that includes mannose binding lectin and conglutinin. C-type lectins share structural features including collagenous amino-terminal and "globular" carboxy-terminal domains, the latter serving as a carbohydrate recognition domain that functions in opsonization. In the presence of calcium, SP-A binds to a variety of monosaccharides including mannose, fucose, glucose, and galactose. Likewise, SP-D binds complex carbohydrates but with affinities that are distinct from SP-A; SP-D binding maltose, glucose, and mannose (2). The polysaccharide capsule of GBS and *H. influenzae* consists of repeating monosaccharides that are likely recognized by the carbohydrate recognition domain of SP-A or SP-D. In this study, SP-D agglutinated both GBS and *H. influenzae* in the presence of calcium, and previous studies demonstrated SP-A binding to GBS (14).

Binding and uptake of *H. influenzae* by alveolar macrophages was decreased in SP-D$^{-/-}$ mice. However, SP-D did not enhance macrophage phagocytosis of *H. influenzae* in vitro (11). Macrophages from SP-D$^{-/-}$ mice had less cell-associated (bound and internalized) GBS, however, phagocytosis of the GBS was similar to wild-type macrophages. Macrophages from SP-D$^{-/-}$ mice are lipid laden, which may affect the ability to phagocytose bacteria (17). SP-D may agglutinate and bind various bacteria but may be more selective in functioning as an opsonin to enhance phagocytosis. In vitro, SP-D enhanced macrophage association with *E. coli* (8), *Mycobacterium tuberculosis* (9), and *Pneumocystis carinii* (10) but did not enhance phagocytosis. In contrast, SP-D enhanced phagocytosis of three of six strains of *P. aeruginosa* by alveolar macrophages, suggesting that SP-D-mediated phagocytosis is bacterial strain-specific. Interestingly, SP-D did not enhance aggregation of *P. aeruginosa* despite enhancing phagocytosis (11). SP-D aggregates bacteria, perhaps facilitating mucociliary clearance and preventing microbial adherence, invasion, and colonization of the airway/alveolar epithelium, thus enhancing host defense independent of phagocytosis.

After bacterial infection, neutrophil accumulation was similar in the lungs of the SP-D$^{-/-}$ and wild-type mice. In vitro, SP-D is chemotactic for neutrophils (20), and enhanced uptake of bacteria,

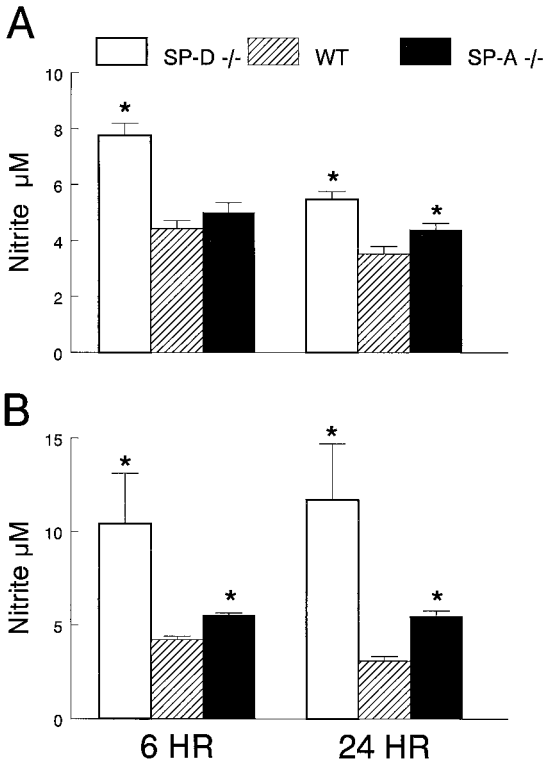

FIGURE 7. Increased nitrite concentrations in BAL fluid from SP-D$^{-/-}$ and SP-A$^{-/-}$ mice following infection. Nitrite in BAL fluid was measured by the Griess reaction as described in *Materials and Methods*. BAL fluid from SP-D$^{-/-}$ mice (□) contained increased nitrite concentrations 6 and 24 h after GBS (*A*) and *H. influenzae* (*B*) infection compared with wild-type mice (▨). Similarly, SP-A$^{-/-}$ mice (■) had increased nitrite levels in lavage fluid 24 h after GBS (*A*) and 6 and 24 h after *H. influenzae* (*B*) infection. Data represent mean ± SEM with $n = 8$ mice per group; ∗, $p < 0.05$ compared with wild type.

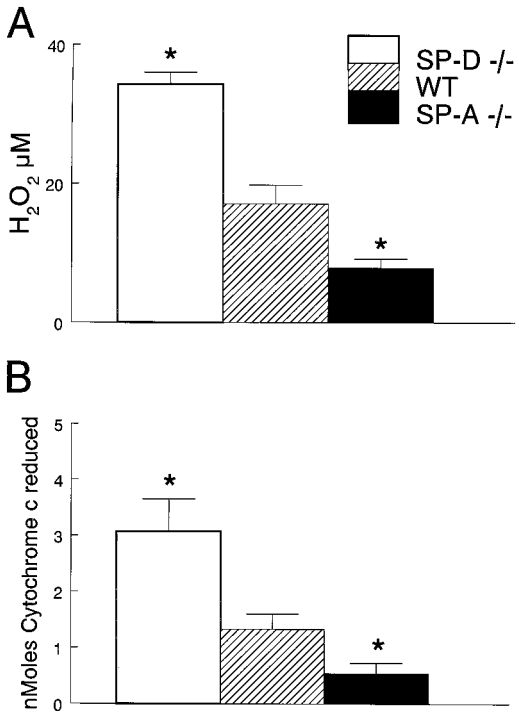

FIGURE 8. Superoxide and hydrogen peroxide production by alveolar macrophages. Superoxide and hydrogen peroxide production were assessed in cells isolated from BAL fluid 18 h after intratracheal administration of GBS. Cells in BAL fluid from SP-D$^{-/-}$, SP-A$^{-/-}$, and wild-type mice consisted of >95% macrophages. Superoxide production by alveolar macrophages was measured by the reduction of cytochrome *c* in the presence and absence of superoxide dismutase as described in *Materials and Methods*. Data are expressed as nanomoles cytochrome *c* reduced per 1×10^5 cells. Hydrogen peroxide production by macrophages (1×10^6) was measured as described in *Materials and Methods*. After stimulation with PMA, hydrogen peroxide (*A*) and superoxide radical (*B*) production by alveolar macrophages were significantly decreased in SP-A$^{-/-}$ (■) and increased in SP-D$^{-/-}$ (□) compared with wild-type mice (▨). Data are mean ± SEM for eight experiments; ∗, $p < 0.05$ compared with wild-type mice.

including *E. coli*, *S. pneumoniae*, and *S. aureus* by neutrophils (21). However, this study demonstrates that SP-D is not a critical determinant of neutrophil chemotaxis or killing because bacterial clearance was not impaired in the absence of SP-D. These data suggest fundamental differences in SP-D effects on macrophages and neutrophils but these effects may be bacterial strain-dependent.

After bacterial infection, markers of inflammation, including inflammatory cells, cytokines, and nitrite, were increased in the lungs of SP-D$^{-/-}$ mice. SP-D$^{-/-}$ mice are able to mount an immune response to bacterial infection; however, the response is greatly increased compared with wild-type controls. NO reacts with superoxide to form peroxynitrite, which is a potent bactericidal radical. This study demonstrated increased nitrite concentration in BAL fluid from SP-D$^{-/-}$ mice after GBS and *H. influenzae* infection, which may contribute to microbial killing in combination with elevated superoxide and hydrogen peroxide in the lung. Increased cytokine production may reflect increased cells in BAL fluid after bacterial infection. Uninfected SP-D$^{-/-}$ mice have increased numbers of alveolar macrophages in the lung; however, proinflammatory cytokine concentrations are not increased. The results of this study demonstrate that despite efficient bacterial killing in SP-D$^{-/-}$ mice, intratracheal inoculation of bacteria still stimulates an inflammatory response. Thus, effects of SP-D on inflammatory responses are not dependent on bacterial proliferation.

Oxygen radical production by alveolar macrophages was increased in SP-D$^{-/-}$ mice. However, SP-D enhanced lucigenin-dependent chemiluminescence of rat alveolar macrophages in vitro, and this response was not inhibited by surfactant lipids (13). Because phospholipids are increased in the lungs of SP-D$^{-/-}$ mice, the lipid excess may inhibit the neutrophil respiratory burst as demonstrated in vitro (22). However, in this study, oxygen radical production by macrophages was increased in the absence of SP-D in vivo with and without bacterial stimulation. SP-D$^{-/-}$ mice have increased numbers of enlarged, foamy macrophages in the alveolar space, develop emphysema, and have abnormalities in phospholipid metabolism (17). Thus, it is difficult to determine from these studies whether the increased oxygen radical production by the macrophages from the SP-D$^{-/-}$ mice is a direct effect of the lack of SP-D or a result of abnormalities in surfactant metabolism that may activate alveolar macrophages.

Phagocytosis of *H. influenzae* by alveolar macrophages was decreased in the absence of SP-A, findings similar to previous in vivo studies with GBS (14). In vitro, SP-A bound GBS (14) and *H. influenzae* (19) in a calcium-dependent manner, suggesting that SP-A acts as an opsonin for these organisms. SP-A bound to *S. aureus* and *S. pneumoniae* in vitro and increased adherence of *S. aureus* to alveolar macrophages (23). Thus binding of SP-A to carbohydrate recognition sites on the surface of bacteria may play an important role in the early clearance of bacteria from the lungs.

After bacterial infection, markers of inflammation, including inflammatory cells, cytokines, and nitrite were increased in the lung

of SP-A$^{-/-}$ mice, supporting previous in vivo studies with *P. aeruginosa* (24) respiratory syncytial viral (16) and adenoviral infection (25). McIntosh (26) reported that SP-A blunted TNF release from LPS-stimulated macrophages. This finding, that cytokine production was more robust in SP-A$^{-/-}$ than in wild-type mice, in vivo, supports the McIntosh study, suggesting that SP-A decreases the release of cytokines in response to bacterial infection. It is unclear from this study whether these differences are directly related to the absence of SP-A or to the increased severity of infection and failure of early bacterial clearance in the SP-A$^{-/-}$ mice.

Oxidant production was distinct in SP-A$^{-/-}$ vs SP-D$^{-/-}$ mice. Following bacterial infection, oxygen radical production by alveolar macrophages was decreased in SP-A$^{-/-}$ mice and increased in SP-D$^{-/-}$ mice compared with controls. Previous studies demonstrated that oxygen radical production by macrophages is impaired in the absence of SP-A in vivo (14) and SP-A enhanced lucigenin-dependent chemiluminescence of rat alveolar macrophages in vitro (12). Bacterial burden of *H. influenzae* was greater in the lung of the SP-A$^{-/-}$ mice; however, SP-D$^{-/-}$ mice were able to efficiently kill the bacteria. SP-A and SP-D bind and agglutinate GBS and *H. influenzae* (14, 19); however, clearance was impaired only in the absence of SP-A. Differences in bacterial clearance in SP-A$^{-/-}$ mice may be related to the impaired oxygen radical production by macrophages in the absence of SP-A. The finding that nitrite was increased following infection in both SP-A$^{-/-}$ and SP-D$^{-/-}$ mice suggests that nitrite alone is not sufficient for bacterial killing. The finding that bacterial killing was similar for SP-D$^{-/-}$ and wild-type mice was surprising because binding and opsonization of the bacteria were deficient in the SP-D$^{-/-}$ mice. However, increased numbers of macrophages and reactive oxygen species in SP-D$^{-/-}$ mice may compensate for the defect in opsonization.

In summary, in the absence of SP-D, bacterial killing in vivo was unchanged; however, lung inflammation was more severe in SP-D$^{-/-}$ and SP-A$^{-/-}$ mice, suggesting that SP-D and SP-A play roles in modulating cytokine production and inflammatory responses during bacterial pneumonia. In addition, SP-D and SP-A bind and agglutinate bacteria, which may, in part, enhance bacterial removal from the lung through mucociliary and macrophage clearance. Because the airway is the usual portal of entry for GBS, *H. influenzae*, and other respiratory pathogens, the local production of SP-A and SP-D is likely to play a role in innate defense responses to inhaled bacteria.

Acknowledgments

We thank Dr. Gary Ross for assistance with Western analysis and William Hull for isolation and purification of SP-D.

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