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Enhanced Surfactant Protein and Defensin mRNA Levels and Reduced Viral Replication during Parainfluenza Virus Type 3 Pneumonia in Neonatal Lambs

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Defensins and surfactant protein A (SP-A) and SP-D are antimicrobial components of the pulmonary innate immune system. The purpose of this study was to determine the extent to which parainfluenza type 3 virus infection in neonatal lambs alters expression of sheep beta-defensin 1 (SBD-1), SP-A, and SP-D, all of which are constitutively transcribed by respiratory epithelia. Parainfluenza type 3 viral antigen was detected by immunohistochemistry (IHC) in the bronchioles of all infected lambs 3 days postinoculation and at diminished levels 6 days postinoculation, but it was absent 17 days postinoculation. At all times postinoculation, lung homogenates from parainfluenza type 3 virus-inoculated animals had increased SBD-1, SP-A, and SP-D mRNA levels as detected by fluorogenic real-time reverse transcriptase PCR. Protein levels of SP-A in lung homogenates detected by quantitative-competitive enzyme-linked immunosorbent assay and protein antigen of SP-A detected by IHC were not altered. These studies demonstrate that parainfluenza type 3 virus infection results in enhanced expression of constitutively transcribed innate immune factors expressed by respiratory epithelia and that this increased expression occurs concurrently with decreased viral replication.

Paramyxovirus infections by respiratory syncytial virus (RSV) and parainfluenza type 1 (PI-1), PI-2, PI-3, and PI-4 viruses are major causes of respiratory disease in young children. Although RSV is the cause of 50 to 90% of hospitalizations for bronchiolitis, PI-3 virus causes a spectrum of diseases similar to RSV diseases (23). These include respiratory tract infections that are complicated in 30 to 50% of cases by otitis media. Most children are infected with PI-3 virus by 2 years of age and with PI-1 and PI-2 viruses by 5 years of age (33, 44). Ovine PI-3 virus infection is a spontaneous disease of sheep that can cause respiratory infections in growing lambs (>7 days of age) experimentally that are similar to those seen in children (36). Immunity to RSV and PI-3 virus are often not long lasting or protective, and traditional therapies (bronchodilators, steroids, and ribavirin) for severe parainfluenza virus infections generally have no overall significant benefit (28, 47). In contrast, innate immune factors, such as defensins and surfactant proteins, are increasingly appreciated for their direct and indirect activities against viral infections.

Defensins are cationic peptides produced by a wide range of species (8) that have activities against bacterial, viral, and fungal pathogens (8, 17, 24). Human beta-defensin 1 (HBD-1) and HBD-2 are thought to exert their antimicrobial activities by forming pores and causing membrane disruption (37). Other activities include healing of epithelium; monocytic, dendritic and T-cell chemotaxis (50); synergism with other antimicrobial factors, such as lysozyme and lactoferrin (46); and complement activation (46). HBD-1 also participates in cell regulation by promotion of cell differentiation and maturation in vitro (19) and inactivates enveloped viruses (20, 46). In addition, alpha-defensins have been shown to induce protection against human immunodeficiency virus type 1 (HIV-1) (52). Sheep beta-defensin 1 (SBD-1) is a member of the beta-defensin family with constitutive expression and tissue distribution similar to those of HBD-1 (29, 30). SBD-1 expression is developmentally regulated in late gestation through the neonatal period, with maximal expression in some tissues reached weeks after birth (29). This suggests a window of immature SBD-1 expression in the neonate that provides an environment conducive to more severe PI-3 virus infection.

Surfactant protein A (SP-A) and SP-D are calcium-dependent lectins and members of the collectin family (12, 13, 40). In the lung, SP-A and SP-D are secreted by type II pneumocytes and Clara cells and have important roles in immunomodulation, surfactant homeostasis, and pulmonary defense (12, 13, 14, 39, 40, 43). SP-A and SP-D interact with bacterial, fungal, and viral pathogens by binding and, in some cases, forming aggregates (12, 13, 27, 40, 43, 45), which can inactivate the pathogen, stimulate phagocytosis, enhance antigen presentation, potentiate oxidant responses of neutrophils (12, 13, 14, 27, 32, 39, 43, 51), and activate macrophages via Toll-like
receptor 4 (21). Deficiency of SP-A and SP-D in vivo is associated with increased risk of infection (3) and may contribute to enhanced inflammation and inflammatory-cell recruitment during infection (39).

The susceptibility of neonatal lambs (<5 days of age) to PI-3 virus and the effect of PI-3 virus infection on the expression of beta-defensins and surfactant proteins have not been determined. Potential decreases in expression may leave the lung predisposed to viral reinfection or secondary bacterial infection. The purpose of this study was to test the hypothesis that PI-3 virus infection alters the expression of the constitutively transcribed innate immune factors SBD-1, SP-A, and SP-D in the lungs of neonatal lambs.

MATERIALS AND METHODS

Experimental design. Eighteen colostrum-fed neonatal lambs (3 to 5 days old), of both sexes and mixed breed, were obtained from Laboratory Animal Resources, Iowa State University. The lambs were randomly assigned to two groups, and each group was maintained in a separate climate-controlled isolated room until sacrifice. After a 24-h period of acclimation, one group (n = 9 animals) received saline while the other (n = 9 animals) received the ovine PI-3 virus. The viral inoculum consisted of infectious supernatant prepared from a culture of ovine fetal turbinate (OFTu) cells previously infected with ovine PI-3 virus strain viral inoculum consisted of infectious supernatant prepared from a culture of ovine PIV-3 virus strain until sacrifice. At the end of the 72-h incubation, and after the slides were allowed to achieve the 1:20 dilution containing 5% normal sheep serum (Sigma) for 30 min, the sections were then tilted to remove this solution from them, and without an intervening buffer rinse, reincubated with the same secondary antibody reagent for 1:50 dilution, they were rinsed with ultrapure water, dehydrated through a series of graded alcohol and xylene baths. The slides were then coveredslipped using 3 drops of mounting medium (Permount; Fisher Scientific, Hanover, Ill.) in conjunction with either 24- or 48- or 24- by 50-mm glass coverslips (Richard Allen Scientific, Kalamazoo, Mich.). A red color (the oxidized, peroxidase-developed Nova Red chromogen precipitate) observed by light microscopy within the tissue sections on sample slides was interpreted as a positive IHC reaction. Control sections incubated with fetal normal goat serum instead of primary polyclonal goat anti-PI-3 antibody were found to lack IHC staining. Lung sections from all 18 animals were evaluated.

IHC detection of SP-A protein. Sections of lung on silanated glass slides were first heated in an oven at 58°C for 30 min and deparafinized and rehydrated using the same solvent series described above. Antigen retrieval was achieved using a power-adjustable commercial microwave oven (Panasonic, Danville, Ky.) and Citra Plus pH 6.2 antigen retrieval solution (Citra Plus buffer 10× concentrate; BioGenex) by putting the slides in plastic (eight-slide) Coplin containers with the Citra Plus pH 6.2 solution (already diluted 1:10 with ultrapure water to achieve the 1× working solution). The retrieval solutions containing the slides were brought just to boiling in the microwave oven at an initial power setting of 1,000 W, the microwave oven was switched off, and then the slides in Citra Plus were heated in the microwave oven for an additional 10 min at a reduced power setting of 300 W. Following antigen retrieval, the containers with slides were placed at 20°C for ~20 min to speed cooling back to room temperature, after which the slides were rinsed twice in BPBS and then incubated for 5 min with a fast-drying liquid wax pen (PAP-pen). Reagent barrier lines were applied to each slide at this point (as described above for PI-3 IHC). Next, the slides were incubated for 20 min in a blocking solution of 1% bovine serum albumin (BSA) (IgG-free, peroxidase-free BSA; Jackson Immunoresearch Laboratories Inc.) in PBBS. The sections were then placed on metal slide racks in a humidified, sealed container and incubated with anti-SP-A primary antibody or control IgG- or IgM-containing serum for 3 days in a cold room at 4°C. The primary antibody was mouse IgM anti-human SP-A (catalog no. MAB3270; Chemicon International Inc., Temecula, Calif.) diluted 1:50 in BioGenex diluent containing 2% NSS and 1% BSA. At the end of the 72-h incubation, and after the slides were allowed to warm to room temperature (~30 min), they were rinsed with PBBS and then subjected to 3% hydrogen peroxide (prepared in PBBS) treatment for 30 min. The slides were rinsed thoroughly with PBBS and then preincubated for 30 min with biotinylated rat anti-mouse IgM secondary antibody (catalog no. 553406 isotype rat [LOU] IgG2a;a; BD Pharmingen, San Diego, Calif.) diluted 1:200 in BioGenex diluent. The reagent was then dumped off the slides with no intervening buffer rinse and subjected to the same biotinylated secondary antibody reagent for another 40 min. Subsequently, sections were incubated for 35 min with supersensitive streptavidin-conjugated peroxidase (BioGenex), rinsed thoroughly with PBBS, allowed to soak in PBBS for 5 min, and then subjected to a 5-min exposure to Nova Red chromogen. The sections were counterstained for 3 min in one-quarter-strength acidic hematoxylin, rinsed with ultrapure water three times, and then finished up by coverslipping following the same series of steps as described above (see section A). Pointing (this point) for PI-3 IHC, a BioGenex chromogenic reagent precipitate was observed by light microscopy within the tissue sections on sample slides was interpreted as a positive IHC reaction. Control sections, which instead of mouse IgM anti-SP-A primary antibody received either 1:50 dilution of a mouse IgG2a anti-SP-A primary antibody, received saline, an identical power-adjustable commercial microwave oven protocol, and citra plus pH 6.2 antigen retrieval solution. The sections were then subjected to the same sequence of steps as described above. As a positive control for staining, slides from a mouse lung were also incubated with the same SP-A antibodies, followed by sequential incubation with biotinylated goat anti-rat IgM and streptavidin-peroxidase (BioGenex).
normal mouse IgG or 1:50 normal mouse serum (mouse IgM-containing serum; Sigma) in BioGenex diluent (also containing 2% NSS and 1% BSA), lacked staining. Lung tissues from all 18 animals were evaluated.

**Scoring system.** At least five fields from two hematoxylin and eosin lung sections were examined by light microscopy (40× objective) and scored for lesion severity using a predetermined scale: briefly, for lesion scores, 0, no inflammatory cells; 1, to 30% of lung sections affected (mild pneumonia); 2, 30 to 60% of lung sections affected (moderate pneumonia); 3, >60% of lung sections affected (severe pneumonia).

For IHC scoring, a minimum of five fields were assessed for viral-antigen distribution. SP-A protein distribution and intensity within the lung were evaluated using a Zeiss Axioskop II fluorescence microscope (Olympus) and scored for lesion severity using a predetermined scale: briefly, for lesion scores, 0, no staining; 1, <30% of bronchioles had detectable staining in <5 cells (type II cells)/bronchiole; in which macrophages stained; 2, <30% of bronchioles had detectable staining in >10 cells (type II cells)/bronchiole in which occasional macrophages stained; 3, >30% of bronchioles had detectable staining in >10 cells (type II cells)/bronchiole in which macrophages stained; for the distribution and intensity of SP-A protein staining, 0, no staining of cells; 1, <30% of epithelial cells/bronchiole stained with minimal detectable intracytoplasmic staining; 2, 30% of epithelial cells/bronchiole stained with >50% of the cell cytoplasm stained; 3, >60% of epithelial cells/bronchiole stained with >50% of the cell cytoplasm stained. Mean values and standard errors of the mean (SEM) were calculated for each group.

**Fluorogenic real-time RT-PCR.** For two-step, real-time, fluorogenic real-time RT-PCR, total RNA was isolated using TRIZOL (TRIZOL Reagent; Invitrogen). The total cDNAs, corresponding to each total-RNA sample isolate, were then used as templates for total-cDNA synthesis by reverse transcription. Random hexamers were used to prime each of our reverse-transcription reactions, and human 18S rRNA was chosen as the housekeeping-reference gene.

**Total-RNA isolation from lung by TRIZOL.** In a nuclelease-free 50 ml conical centrifuge tube, 0.3 g of lung tissue (previously stored at −80°C) from each of the 18 animals was combined with 3 ml of TRIZOL reagent and homogenized for 30 s using a μl electric homogenizer (Omi International Inc., Gainesville, Va.). The homogenate was allowed to sit for 5 min, after which 0.6 ml of chloroform (nuclease free; Fisher Scientific) was added and the mixture was shaken vigorously for 15 s. The sample was allowed to sit for 3 min at room temperature and then was split into three nuclease-free 1.5 ml tubes and microcentrifuged at 15,600 × g at 4°C for 10 min, after which the aqueous (top) layers were transferred to a new nuclease-free 50-ml tube (recombining all partial volume samples). Isopropanol (nuclease free 2-propanol [1 ml]; Fisher Scientific) was then added to the aqueous layer, and the solution was mixed and allowed to stand for 10 min at room temperature. The mixture was then split into two nuclease-free 1.5-ml vials and microcentrifuged for 10 min at 15,600 × g and 4°C. The isopropanol layer was poured off of each vial; large white RNA pellets were visible at this point. Each pellet was washed three times with 0.5 to 1 ml of precooled (−20°C) 75% ethanol. The pellets were washed twice with 75% ethanol, followed by the third addition of 0.5 to 1 ml of 75% ethanol and vortexing until the pellets were dislodged. The samples were then microcentrifuged at 15,600 × g and 4°C, and the ethanol was carefully poured off. The pellets were allowed to air dry for 20 to 40 min at room temperature under a laminar flow hood with the vial caps open. The dried pellets were resolubilized in a total of 500 μl of RNA sample dilution buffer (Ambion nuclease-free water containing 0.1 mM EDTA). RNA sample pellets that were difficult to resolubilize were warmed to 65°C in a heating block for 5 min and gently agitated in solution, and then like samples were pooled in a single nuclease-free 1.5-ml vial. To assess the quality and purity of the RNA isolates, spectrophotometer (model DU 640B; Beckman Instruments Inc., Fullerton, Calif.) readings of each total-RNA isolate were performed as suggested by ABI in order to determine the concentration of target cDNA that corresponded to our chosen endogenous reference (housekeeping) gene, 18S rRNA (to which we normalized all cDNA samples) using the GeneAmp 5700 sequence detection system. Optimizations and validation tests were performed as detailed in the experimental section.

**Optimization and validation tests.** The GeneAmp 5700 sequence detection system allowed dual amplification and analysis of cDNAs corresponding to both a target gene of interest and the endogenous reference gene concurrently on the same plate but within separate wells. Optimization and validation experiments were performed as suggested by ABI in order to determine the efficiency of primers and probes and to test the PCR system using appropriate controls. The GeneAmp 5700 sequence detection system allows the control and normalization of different signals relative to a single RNA sample. The asymmetric amplification of the target gene can be detected directly from each reverse-transcription reaction, which is theoretically 0.02 μg of
cDNA/μl, assuming 100% efficiency of each reverse transcription.) All plates were run in the GeneAmp 5700 sequence detection system real-time PCR machine (ABI) using the following thermocycler conditions: hold for 2 min at 50°C, hold for 10 min at 95°C, and 50 cycles of 15 s at 95°C, followed by 1 min at 60°C. Each plate contained 384 wells of equal volumes (2 μl) containing a 1:1,000 dilution of Stock I cDNA, along with standard stock reference cDNA samples. Each plate was coated overnight with a 1:200 dilution of standard sheep BAL fluid (courtesy of Jeffrey Whitsett) (38), which allowed the comparison of lung samples to known amounts of either SP-A peptide (0.05 to 10 ng/ml or mg) or equivalent amounts of BAL fluid (which we found to be in the 1.6 × 106 to 1.25 × 106 dilution range of standard sheep lung BAL fluid). Serially diluted 2% SP-A peptide standard solutions were prepared in the range of 0.1 to 20 ng/ml from a 5,400-ng/ml stock solution of purified SP-A. All standards and samples were administered in triplicate to wells of a standard 96-well microwell plate. The wells were coated overnight with a 1:200 dilution of standard sheep BAL fluid in bicarbonate coating buffer (0.42 g of NaHCO3 in 50 ml of HPLC-grade water). During the same period, 300 μl of the 2% SP-A peptide standard solutions, equivalent standard sheep BAL fluid dilutions, and appropriately diluted sample unknowns were added to 300 μl of a 2% primary antibody solution made by combining 2 μl of a 50% rabbit (R436) anti-SP-A IgG antibody (courtesy of Jeffrey Whitsett) with 1 ml of 100% normal goat serum (Sigma), 4.998 ml of HPLC-grade water, and 4 ml of a 5× diluent (50 ml Tris-HCl; 750 mM NaCl; 2.5% IgG-free, protease-free BSA [Jackson Immunoresearch Laboratories]; pH 7.4) and incubated overnight at 37°C. The next day, the wells and plates were washed three times with a wash buffer (5 mM Tris-HCl, 0.05% Tween 20 [catalog no. P-9416; Sigma] at pH 8.0), the plate was tapped thoroughly of excess fluid into a Terri cloth, and 200 μl of 5% normal goat serum in diluent (10 ml Tris-HCl; 150 mM NaCl; 0.5% IgG-free, protease-free BSA; pH 7.4) was added to each well and allowed to incubate for 15 min at room temperature. This solution was subsequently shaken from the wells, and the plate was blotted dry. Next, 100 μl of each 1:1,000 dilution of standard BAL fluid standard dilution-antibody overnight-incubated mixtures was added to appropriate wells to unbound primary antibody in each solution to bind (BAL-SP-A) antigen. Blank wells received unchallenged 1:20,000-diluted primary antibody (initially diluted 1:10,000; used at a final concentration of 1:20,000 in each well) solution at this time. The plate was allowed to incubate for 1 h at 37°C; the fluid was shaken from the wells, and the plate was washed three times. Immediately after this, 100 μl of a 1:1,000 dilution of a goat anti-rabbit-horseradish peroxidase conjugate (catalog no. 474-1506; goat anti-rabbit-horseradish peroxidase IgG; Kirkegaard & Perry Laboratories) in PBS-Tween buffer (50 mM anhydrous NaH2PO4, 50 mM NaH2PO4·H2O, 150 mM NaCl; and 0.05% Tween 20, pH 7.4) containing 5% NBS was added to each well and allowed to incubate for 1 h at 37°C. The fluid was again shaken from the wells, rinsed three times with wash buffer, and washed once with PBS, pH 7.4 (PBS without Tween 20), followed by the addition of 100 μl of ABTS [2,2'-azinobis(3-ethylbenzthiazolinesulfonic acid)] peroxidase substrate solution (Kirkegaard & Perry Laboratories) to each well. The substrate reaction was allowed to proceed for 12 min at room temperature and was stopped by the addition of 100 μl of 0.75% sodium dodecyl sulfate solution (made from 10% lauryl sulfate solution; Sigma) to each well. The different color intensities in the wells were measured in an MR700 microplate reader (Dynatech Laboratories Inc.) at 410 nm. This color intensity (calculated as a function of average sample absorbances divided by the average blank-well absorbance) versus concentration of SP-A standards (and/or equivalent dilutions of standard sheep BAL fluid) was plotted, and the resultant standard curve was used to estimate unknown SP-A protein concentrations in lung samples. Each well was coated with the primary antibody overnight, which may have biased the results in lung samples, as proteins were incubated by samples throughout the course of the procedure were taken into account so that the final numbers of nanograms of SP-A per milligram of sheep
lungs were reflective of the actual lung SP-A levels in each lamb. Nasal septa, trachea, and other upper respiratory tissues were not evaluated for surfactant proteins in this study; only lung tissues from all 18 animals were assessed.

**Statistical analysis.** Nonparametric Kendall’s tau-b correlation tests (42) were used to determine if there was any correlation among histopathological lesions and IHC values (Table 1). Kendall’s tau-b statistics were tested against zero. Nonparametric Wilcoxon tests (43) were used to determine if there was any statistically significant difference between histopathological lesions or IHC values for PI-3 viral antigen in the two groups. Values were considered to be significant when \( P < 0.05 \). For fluorogenic real-time RT-PCR, statistical analysis was performed using the means of SBD-1, SP-A, or SP-D mRNA levels from three replicate wells per sheep normalized to the reference. To determine if there were significant treatment and time effects, a \( t \) test assuming unequal variances was used (42). Values were considered to be significant when \( P < 0.1 \). For quantitative-competitive ELISA, the means of SP-A protein levels from three replicate wells per sheep were assessed. The final numbers of nanograms of SP-A per milligram of sheep lung tissue for all sheep were analyzed by using a \( t \) test assuming unequal variances in order to determine if there were significant treatment and time effects. Values were considered to be significant when \( P < 0.05 \). The software used was JMP release 5.0 (SAS Institute Inc. Cary, N.C.).

**RESULTS**

**Clinical signs.** Lambs inoculated with the PI-3 virus, but not control lambs, developed clinical signs of respiratory infection as previously described (15, 36). Briefly, the infected animals were reluctant to move, had reduced activity and expiratory dyspnea (“thumping”), showed intermittent coughing, had reduced feed intake, and developed a sustained, mild increase in body temperature ranging from 103.6 to 106.5°F during the first 7 days p.i. The febrile response appeared to be biphasic, and it peaked on days 1 (average temperature, 104.6 ± 0.2°F) and 5 (average temperature, 104.9 ± 0.4°F) p.i. in PI-3 virus-infected lambs.

**Serology.** Postinoculation serum antibody titers to PI-3 virus varied from 1:8 to 1:64 among animals but were not significantly increased in infected lambs compared to preinoculation values and control animals.

**Gross pathology.** The predominant gross lesions included extensive multifocal consolidation in all infected lobes with slight predominance of ventral to cranioventral distribution involving 20 to 90% of the lobes. Frequently, there was also mild interlobular edema and multifocal hyperinflation of the lobules. The control animals lacked lesions.

**Histopathology.** The lesions were similar to those reported previously in slightly older (7-day-old) lambs (15). On day 3 p.i., lesions were characterized by mild to moderate multifocal necrotizing acute bronchiolitis and bronchointerstitial pneumonia. On day 6 p.i., there was histiocytic and suppurative interstitial pneumonia accompanied by type II pneumocyte hypertrophy and hyperplasia, and bronchiolitis with epithelial cell hyperplasia. On day 17 p.i., there was mild fibrous interstitial pneumonia with lymphohistiocytic peribronchitis, peribronchiolitis, and perivasculitis in the PI-3 virus-infected group. The control animals lacked lesions.

**IHC for PI-3 viral antigen.** PI-3 viral antigen was present in all three PI-3 virus-infected lambs at 3 days p.i. and was detected in >30% of bronchioles with microscopic lesions in these lambs. PI-3 viral antigen was present within the cytoplasm of the bronchiolar epithelial cells and only in rare macrophages and type II pneumocytes and also very rarely in the bronchial epithelial cells (Fig. 1A). On day 6 p.i., PI-3 viral antigen was present in two-thirds of infected animals and in macrophages and type II pneumocytes and was only rarely present in bronchiolar epithelial cells (Fig. 1C). PI-3 viral antigen was absent on day 17 p.i. (Fig. 1E). The control animals lacked PI-3 viral antigen (Fig. 1B, D, and F).

There was a high correlation between the day p.i. and the IHC values for PI-3 viral antigen. That is, the IHC values for PI-3 viral antigen decreased with time. The correlation was \( r^2 = 0.87 \) for the PI-3 virus-infected group (42). There was no statistically significant difference between the severity of histopathological lesions and IHC values for PI-3 viral antigen (Fig. 1F) (Table 1).

**Expression of SBD-1.** SBD-1 in the lung was assessed by fluorogenic real-time RT-PCR of cDNA prepared from homogenized lung. When normalized to 18S rRNA levels, there was a trend of increased SBD-1 mRNA expression on all days p.i. (3, 6, and 17 days) compared to the control animals. The increase was statistically significant on day 17 p.i. (Fig. 2).

**Expression of SP-A.** SP-A was assessed by fluorogenic real-time RT-PCR, quantitative-competitive ELISA procedures on lung homogenates, and IHC on lung sections. SP-A mRNA levels increased significantly 6 and 17 days following PI-3 virus infection compared to levels in the control animals (Fig. 3). SP-A protein levels in lung homogenates assessed by quantitative-competitive ELISA were not significantly altered by PI-3 virus infection (Fig. 4). The intensity of staining for SP-A protein assessed by IHC (Table 2) agreed with quantitative-competitive ELISA results for SP-A protein in lung homogenates. The IHC staining distributions for SP-A in both control and infected animals were predominantly present within the cytoplasm of nonciliated bronchiolar cells (most intense in the apical portion) and less often in type II pneumocytes and macrophages. In the infected animals on day 3 p.i., there was a mild decrease in staining distribution and intensity which was most obvious within the consolidated areas, where there was a loss (necrosis) of bronchiolar epithelial cells.

**Expression of SP-D.** SP-D in the lung was assessed by fluorogenic real-time RT-PCR. SP-D mRNA levels were significantly increased in the lung during PI-3 virus infection on all days p.i. compared to those in the control animals (Fig. 5).

**DISCUSSION**

SBD-1 (like HBD-1), SP-A, and SP-D, are constitutively expressed in the normal lung (12, 13, 29). Constitutive expression of SBD-1, SP-A, and SP-D allows a constant presence of these factors in airways to defend against infection and to help prevent the initial attachment of microbes to the respiratory mucosa. Despite reported constitutive expression, our results suggest that PI-3 virus infection enhances levels of SBD-1, SP-A, and SP-D mRNAs. The mechanism by which PI-3 virus enhances expression was not determined; however, SP-A and SP-D expression can be increased in response to a variety of stimuli, including glucocorticoids (18) and vascular endothelial cell growth factor (9). In contrast, tumor necrosis factor alpha reduces SP-A expression (49). It could also be that PI-3 virus infection enhances the expression of constitutive genes globally in proliferative type II cells that replace virus-infected cells, or PI-3 virus infection may enhance the stability of certain
FIG. 1. IHC detection of PI-3 viral antigen in the lungs of lambs inoculated with PI-3 virus or sterile medium 3 (A and B), 6 (C and D), or 17 (E and F) days p.i. (A) PI-3 viral antigen is present within the cytoplasm of bronchiolar epithelial cells. (C) PI-3 viral antigen is present within the cytoplasm of macrophages and bronchiolar epithelial cells. (E) Bronchioles in lungs from lambs 17 days p.i. lack PI-3 viral antigen. (B, D, and F) Control animals lack PI-3 viral antigen. Bar = 100 μm.
mRNAs, including those of SBD-1, SP-A, and SP-D, in infected or proliferative cells.

The increase in SBD-1, SP-A, and SP-D mRNA levels and the simultaneous decrease in PI-3 virus replication may suggest that these factors are synthesized in order to bind to PI-3 virus, as collectins bind to other viruses, and to neutralize it directly or indirectly. Indirect activity may be accomplished via several mechanisms. First, SP-A and SP-D can trigger macrophage activity (40), causing the clearance of RSV (39) and influenza virus (12, 43). Secondly, studies demonstrate that beta-defensins may cause chemotaxis of dendritic cells and lymphocytes to the site of infection in order to promote adaptive immunity (50). Furthermore, there is strong evidence that Toll-like receptor 4 is involved in the innate response to other paramyxoviruses (RSV) (26) and that it can be activated by murine beta-defensin 2 (5). In addition, SBD-1, SP-A, and SP-D may also affect interferon activity in order to enhance antiviral activity (11). The mechanism of direct antiviral activity by SBD-1 may be through its ability to induce pore formation in the PI-3 viral envelope; however, this remains to be determined. Recently, alpha-defensins have been shown to induce anti-HIV-1 activity (52). Although increased expression of the mRNAs of these innate immune factors and decreased viral replication may be unrelated phenomena, our present data suggest that this response of the respiratory tract is likely virus specific. Bacterial (Mannheimia haemolytica) infection, when introduced in particularly high concentrations, significantly reduced SBD-1 mRNA expression in the sheep lung compared to that in uninfected controls (M. R. Ackermann and J. M. Gallup, unpublished data).

Our present observation of increased lung SP-A mRNA levels in the absence of significant changes in SP-A protein expression in lung homogenates and histological sections may be due to several reasons. We suspect that there may be increased production of SP-A mRNA (and perhaps protein), but this increase could also be accompanied by lymphatic drainage or direct uptake of SP-A protein by the pulmonary capillaries due to damage to the epithelium-endothelium barrier (25). Alternatively, utilization of the SP-A protein may be increased due to its binding and aggregation of PI-3 virions. Both possibilities support steady levels of SP-A protein in the lung, which has previously been shown in the BAL fluid of children in-

**TABLE 1.** Lesion and IHC scores of PI-3 virus-infected and control neonatal lambs 3, 6, and 17 days p.i.*

| Day p.i. | Control | PI-3 inoculated |
|---------|---------|-----------------|
|         | Lesion score | IHC score | Lesion score | IHC score |
| 3       | 0 0 2.00 ± 0ட | 2.67 ± 0.33d* | 0.00 ± 0d  | 0 0 0 |
| 6       | 0 0 2.67 ± 0.33d* | 0.67 ± 0.33d* | 0 0 0 |
| 17      | 0 0 1.00 ± 0d  | 0 0 0 |

* PI-3 virus-infected lambs had lesions and viral antigen that were not present in controls.
* Values are means for lesion score ± SEM (three samples per group).
* Values are means for PI-3 viral-antigen IHC score ± SEM (three samples per group).
* Significantly increased compared to controls.
* Significantly increased compared to day 17 p.i.

FIG. 2. SBD-1 mRNA levels detected in whole-lung homogenates of PI-3 virus-inoculated and control lambs by fluorogenic real-time RT-PCR. There was a trend toward increased SBD-1 mRNA levels in the PI-3 virus-infected group on days 3 and 6 and a significant increase (*) on day 17 compared to the control animals (P = 0.06). A t test assuming unequal variances was used. The error bars indicate SEM.

FIG. 3. SP-A mRNA levels detected in whole-lung homogenates of PI-3 virus-inoculated and control lambs by fluorogenic real-time RT-PCR. SP-A mRNA levels were significantly increased (*) in PI-3 virus-inoculated lambs 6 and 17 days p.i. compared to the control animals (P = 0.09 for day 6 p.i. and P = 0.05 for day 17 p.i.). A t test assuming unequal variances was used. The error bars indicate SEM.

FIG. 4. SP-A protein levels assessed by quantitative-competitive ELISA in whole-lung homogenates of PI-3 virus-inoculated and control lambs. SP-A protein levels were not significantly changed by PI-3 virus inoculation compared to the control animals. A t test assuming unequal variances was used. The error bars indicate SEM.
demonstrated in previous reports (15, 36). In sheep, the normal body temperature is 102°F, while critical temperature is considered to be 104°F, above which hyperthermia (fever) is said to be present (6). The mild but long-lasting increases in the rectal temperatures of the lambs infected with PI-3 virus in this study could be attributed to higher susceptibility of neonatal animals to the virus in light of previous reports that older lambs do not have such prolonged increases in temperature (36).

The IHC procedure we developed for the detection of PI-3 viral antigen more precisely defined viral-antigen distribution than a previous immunofluorescence technique (2, 15). The persistence and distribution of the PI-3 viral antigen as assessed by IHC correlated well with the time effect. On day 3 p.i., viral antigen was widely distributed in >30% of the damaged bronchioles, affecting smaller airways in particular. Only rare macrophages and type II pneumocytes contained antigen. On day 6 p.i., the virus persisted only in some animals (two-thirds of the PI-3 group) and was present in macrophages and type II pneumocytes, while hyperplastic repaired bronchioles generally lacked the antigen. Complete clearance of virus by day 17 p.i. corresponds with the resolution of lung lesions.

This work has determined the extent to which an important paramyxoviral pathogen, PI-3 virus, alters the expression of innate immune-related factors in neonatal lambs. In future studies, we will use laser capture microdissection to retrieve epithelium in order to localize SBD-1, SP-A, and SP-D mRNA expression within various regions of lung epithelia (bronchi, bronchioles, and alveoli) and to determine the alterations that occur in these regions in PI-3 virus-infected animals. Early results obtained with laser capture microdissection one-step fluorogenic RT-PCR (22) indicate that the highest level of SBD-1 mRNA expression is present within the bronchiolar epithelial cells (Ackermann and Gallup, unpublished), which generally lack PI-3 viral antigen and lesions during viral pneumonia.

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**TABLE 2.** IHC scores for SP-A protein expression (distribution and intensity) in the lungs of PI-3 virus-infected and control neonatal lambs 3, 6, and 17 days p.i.*

| Day p.i. | Control Distribution | Control Intensity | PI-3 inoculated Distribution | PI-3 inoculated Intensity |
|---------|---------------------|------------------|-------------------------------|--------------------------|
| 3       | 3 ± 0               | 3 ± 0            | 1.5 ± 0.41                    | 2 ± 0                    |
| 6       | 2.33 ± 0.33         | 2.33 ± 0.33      | 2 ± 0.58                     | 2 ± 0                    |
| 17      | 3 ± 0               | 2.33 ± 0.33      | 2 ± 0.58                     | 2.33 ± 0.33              |

* SP-A protein distribution was not significantly altered by PI-3 virus infection. Values are means for SP-A IHC score ± SEM (three samples per group).

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FIG. 5. SP-D mRNA levels detected in whole-lung homogenates of PI-3 virus-inoculated and control lambs by fluorogenic real-time RT-PCR. SP-D mRNA levels were significantly increased (*) in PI-3 virus-inoculated lambs 3, 6, and 17 days p.i. compared to the control animals (P = 0.09 for day 3 p.i., P = 0.09 for day 6 p.i., and P = 0.06 for day 17 p.i.). A t test assuming unequal variances was used. The error bars indicate SEM.
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REFERENCES

1. Al-Darraji, A. M., R. C. Cutlip, and H. D. Lehmkuhl. 1982. Experimental infection of lambs with bovine respiratory syncytial virus and Pasteurella haemolytica: immunofluorescent and electron microscopic studies. Am. J. Vet. Res. 43:230–235.

2. Allkan, F., A. Ozkul, S. Bilge-Dagalp, K. Yesilbag, T. C. Oguzoglu, Y. Akca, and I. Burgu. 2000. Virological and serological studies on the role of PI-3 virus. BRIV, BVDV and BIV-1 on respiratory infections of cattle. I. The detection of etiological agents by direct immunofluorescent technique. Dtsch. Tierarztl. Wochenschr. 107:193–195.

3. Awasthi, S., J. J. Coalson, B. A. Yoder, E. Crouch, and R. J. King. 2001. Deficiencies in lung surfactant proteins A and D are associated with lung infection in premature neonatal baboons. Am. J. Respir. Crit. Care Med. 163:389–397.

4. Belknap, E. B., D. K. Ciszewski, and J. C. Baker. 1995. Experimental respiratory syncytial virus infection in calves and lambs. J. Vet. Diagn. Invest. 7:296–298.

5. Biragyn, A., P. A. Rufini, C. A. Leifer, E. Klyushnenkova, A. Shakhov, O. Chertov, A. K. Shirakawa, J. M. Farber, D. M. Segal, J. J. Oppenheim, and M. A. D'Alessio. 2001. VEGF induces airway epithelial cell proliferation in human fetal lung in vitro. Am. J. Physiol. Lung Cell. Mol. Physiol. 281:L1001–L1010.

6. Blood, D. C., and O. M. Radostits. 1989. Veterinary medicine: a textbook of the diseases of cattle, sheep, pigs, goats, and horses, 7th ed. W. B. Saunders, Philadelphia, Pa.

7. Bouljihad, M., and H. W. Leipold. 1994. An ultrastructural study of pulmonary-bronchiolar and alveolar epithelium in sheep. Zentbl. Veterinarmed. A 41:52–55.

8. Brogden, K. A., R. C. Cutlip, B. P. McCray, Jr., and R. F. Tack. 2003. A new in-vitro airway epithelial model. Respir. Res. 4:138–144.

9. Brown, K. R., K. M. England, K. L. Goss, J. M. Snyder, and M. J. Acarregui. 2003. Surfactant-associated proteins (SP-A, SP-B) are increased proportionally to alveolar phospholipids in sheep silicosis. Lung 171:63–74.

10. Chertov, A. K. Shirakawa, J. M. Farber, D. M. Segal, J. J. Oppenheim, and M. A. D'Alessio. 2001. Expression of human beta-defensin 2. Science 298:1118–1121.

11. Cutlip, R. C., H. D. Lehmkuhl, S. R. Bolin, and K. A. Brodgen. 1985. Seropneumoniaserologic survey for antibodies to selected viruses in the respiratory tract of sheep. Am. J. Vet. Res. 46:2621–2630.

12. Lehmkuhl, H. D., R. C. Cutlip, S. R. Bolin, and K. A. Brodgen. 1985. Characterization of a porcine parainfluenza type 3 virus isolated from the lung of a lamb with pneumonia. Am. J. Vet. Res. 46:626–628.

13. Lehmkuhl, H. D., and R. C. Cutlip. 1983. Experimental parainfluenza type 3 infection in young lambs: clinical, microbiological, and serological response. Vet. Microbiol. 8:437–442.

14. Lehrer, R. I., A. Barton, K. A. Daher, S. S. Harwig, T. Ganz, and M. E. Selsted. 1989. Interaction of human defensins with Escherichia coli. Mechanisms of bactericidal activity. J. Clin. Invest. 84:553–561.

15. Lesur, O., R. A. Veldhuizen, J. A. Whitsett, W. M. Hull, F. Possmayer, A. Cantin, and R. Begin. 1993. Surfactant-associated proteins (SP-A, SP-B) are increased proportionally to alveolar phospholipids in sheep silicosis. Lung 171:63–74.

16. LeVine, A. M., J. A. Gwоздz, J. Stark, M. Bruno, J. Whitsett, and T. R. Korhagen. 1999. Surfactant-A protein enhances respiratory syncytial virus clearance in vivo. J. Clin. Invest. 103:1015–1021.

17. McCormack, F. X., and J. A. Whitsett. 2002. The pulmonary collectins, SP-A and SP-D, orchestrate innate immunity in the lung. J. Clin. Invest. 109:719–725.

18. Meehan, J. T., R. C. Cutlip, H. D. Lehmkuhl, J. P. Kluge, and M. R. Ackermann. 1994. Defected cell types in ovine lung following exposure to bovine respiratory syncytial virus. Vet. Pathol. 31:229–236.

19. Petrie, A., and P. Watson. 1999. Statistics for veterinary and animal science. Blackwell Science, Oxford, United Kingdom.

20. Reading, P. C., L. S. Morye, E. C. Crouch, and E. M. Anders. 1997. Collectin-mediated antiviral host defense of the lung: evidence from influenza virus infection in mice. J. Virol. 71:821–825.

21. Reed, G., P. H. Jewett, J. Thompson, S. Tollefson, and P. F. Wright. 1997. Epidemiology and clinical impact of parainfluenza virus infections in otherwise healthy infants and young children <5 years old. J. Infect. Dis. 175:807–813.

22. Restrepo, C. A., L. Dang, J. Savov, W. I. Mariencheck, and J. R. Wright. 1999. Surfactant protein D stimulates phagocytosis of Pseudomonas aeruginosa by alveolar macrophages. Am. J. Respir. Cell. Biol. 21:576–585.

23. Schutte, B. C., and P. B. McCray, Jr. 2002. Beta-defensins in lung host defense. Annu. Rev. Physiol. 64:709–748.

24. Sharland, M., and A. Bedford-Russell. 2001. Preventing respiratory syncytial virus bronchiolitis. BMJ 322:62–63.

25. Shu, S., G. J. Xu, and L. Fan. 1995. The glucose oxidase-DAB-nickel method in peroxidase histochemistry of the nervous system. Neurosci. Lett. 85:169–171.

26. Whitsett, J. A., J. C. Clark, J. R. Wispe, and G. S. Pruhner. 1992. Effects of TNF-a and phosphor ester on human surfactant protein and MxSOD gene transcription in vivo. Am. J. Physiol. 262:L1093–L1097.

27. Yang, D., O. Chertov, S. N. Bykovskaiia, Q. Chen, M. J. Buffo, J. Shogam, M. Anderson, J. M. Schroeder, J. M. Wang, O. M. Howard, and J. J. Oppenheim. 1999. Beta-defensins: linking innate and adaptive immunity through den- tritic and T cell CR3beta. Science 286:525–528.

28. Young, D. F., L. Didcock, S. Goodbourn, and R. E. Randall. 2000. Paramyxoviridae use distinct virus specific mechanisms to circumvent the interferon response. Virology 269:430–490.

29. Zhao, L., W. Yu, T. He, J. Yu, R. E. Cafrery, A. E. Dalmasso, S. Fu, T. Pham, J. Mei, and J. J. Ho. 2002. Contribution of human alpha-defensin-1, -2, -3 to the anti-HIV-1 activity of CD8 antiviral factor. Science 298:995–1000.