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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 paramyxovirus 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

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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 isolation 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 DH-1, according to a standard procedure (35). The animals received 10 ml intratracheally and 2 ml intranasally of either viral inoculum containing $10^{6.9}$ 50% tissue culture infective doses of PI-3 virus per ml or pyrogen-free saline. Clinical signs and temperatures were observed and logged daily. Three animals from each group were euthanized on day 3, 6, or 17 postinoculation (p.i.) using 1 ml of euthanasia solution (Beuthanasia-D Special [pentobarbital sodium]; Schering-Plough Animal Health) per 10 kg of body weight injected into the external jugular vein. This protocol was approved by the Iowa State University Animal Care Review Committee.

Collection of samples. Lungs were evaluated for gross lesions, and tissue samples (with the most severe gross lesions from infected animals) from all lung lobes were collected into cryovials, snap-frozen in liquid nitrogen, and stored at −80°C until analysis, when a vial from each animal was used for RNA isolation by TRIZOL extraction, cDNA production, and subsequent real-time reverse transcriptase (RT) PCR analysis. The remaining lung tissue was fixed in 10% buffered formalin for histopathological evaluation of PI-3 virus-induced lesions and for immunohistochemical (IHC) staining of PI-3 viral antigen and SP-A. The sections used for histopathological evaluation were stained with hematoxylin and eosin.

Serum analysis. Five to 10 ml of whole blood was collected from each animal, once before the inoculation of pyrogen-free saline or PI-3 virus inoculum and once before euthanasia. A microtiter plate-based serum virus neutralization test was used to quantitate ovine PI-3 virus neutralizing antibodies as described previously (34).

IHC detection of PI-3 viral antigen. Sections of lung on silanated glass slides were stained with antibody to PI-3 virus antigen using a biotin-streptavidin-peroxidase method developed in our laboratory (48). Briefly, the slides were heated in an oven at 58°C for 30 min and deparaffinized using xylene and a series of graded alcohols until they were fully hydrated in ultrapure water. Next, a protease mixture, Protease XIV (Sigma, St. Louis, Mo.) was used for antigen retrieval in the following way. Section-containing slides were warmed to 37°C for 15 min in 50 mM Tris buffer, pH 7.6 (prewarmed to 37°C), and then placed into 0.1% Protease XIV solution (prewarmed to 37°C) for 12 min at room temperature. The slides were subsequently rinsed with 50 mM Tris, pH 7.6, twice for 5 min each time, rinsed once with BioGenex (San Ramon, Calif.) phosphate-buffered saline (BPBS) (standard PBS containing 0.1% Tween 20, pH 7.4–20× OptiMax wash buffer), and washed in BPBS for 5 min. The glass regions on each slide above and below the tissue-containing areas were wiped off and lined with a fast-drying liquid wax pen (PAP-Pen; BioGenex), creating hydrophobic boundaries to protect against reagent loss. The slides were then rinsed in BPBS for 5 min. In order to minimize nonspecific background staining, the slides were incubated for 20 min in 20% normal swine serum (NSS) (Invitrogen, Grand Island, N.Y.) diluted in BPBS. Without being rinsed, all of the slides were then placed in polypropylene five-slide mailers (5-Slide Plastic Mailer Containers; Evergreen Scientific, Los Angeles, Calif.), each containing 18 ml of either primary goat polyclonal anti-bovine PI-3 primary antibody (catalog no. 210-70-PI3; VMRD, Pullman, Wash.) diluted 1:1,000 with 5% NSS in BioGenex diluent (common reagent diluent) or colostrum-deprived non-pathogen-exposed fetal normal goat serum (fetal goat serum sample accession no. 966; obtained from H. D. Lehmkuhl, National Animal Disease Center, Ames, Iowa) for control slides, and incubated for 48 h in a cold room at 4°C. The slides were then warmed to room temperature for 30 min, rinsed with BPBS, treated with 3% hydrogen peroxide solution for 40 min (to quench endogenous tissue peroxidase activity), and rinsed again with BPBS for 5 min. The sections were preincubated with a biotinylated rabbit anti-goat secondary antibody (catalog no. 16-13-06; biotinylated rabbit anti-goat immunoglobulin G [IgG] [heavy and light chains]; Kirkegaard & Perry Laboratories, Gaithersburg, Md.) diluted 1:400 in BioGenex diluent containing 5% normal sheep serum (Sigma) for 30 s. The slides were then tilted to remove this solution from them, and without an intervening buffer rinse, incubated again with the same reagent for 45 min. The slides were then rinsed with BPBS and allowed to stand in BPBS for 5 min. Supersensitive streptavidin-conjugated peroxidase (BioGenex) was applied for 45 min, followed by thorough rinsing with BPBS, a 5-min incubation in BPBS, and a 4-min incubation with the peroxidase-sensitive chromogen Nova Red (Vector, Burlingame, Calif.). The slides were rinsed thoroughly with ultrapure water, counterstained for 2 min with one-quarter-strength acidified (pH 5.2) hematoxylin (Shandon Lipshaw/Lerner), rinsed three times with ultrapure water, immersed in Scott's tap water (an ~pH 8.0 tap water-like bluing agent for hematoxylin made by adding 83 mM magnesium sulfate and 24 mM sodium bicarbonate to ultrapure water) for 1 min, rinsed briefly in ultrapure water, and dehydrated through a series of graded alcohol and xylene baths. The slides were finally coverslipped using 3 drops of mounting medium (Permount; Fisher Scientific, Hanover, Ill.) in conjunction with either 24- by 40- or 24- by 50-mm glass coverslips (Richard Allen Scientific, Kalamazoo, Mich.). A reddish 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 deparaffinized 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 stopped, 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 with water and bathed in BPBS for 5 min. Using 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, protease-free BSA; Jackson Immunoresearch Laboratories Inc.) in BPBS. 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 BPBS and then subjected to 3% hydrogen peroxide (prepared in BPBS) treatment for 30 min. The slides were rinsed thoroughly with BPBS and then preincubated for 30 s with biotinylated rat anti-mouse IgM secondary antibody (catalog no. 553406 isotype rat [LOU] $IgG_{2a,\kappa}$; 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 super-sensitive streptavidin-conjugated peroxidase (BioGenex), rinsed thoroughly with BPBS, allowed to soak in BPBS 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 acidified hematoxylin, rinsed with ultrapure water three times, and finished up through coverslipping following the same series of procedures mentioned above (from this point on) for PI-3 IHC. Areas exhibiting a reddish precipitate were accepted as positive IHC reactions. Control sections, which instead of mouse IgM anti-SP-A primary antibody received either 1:50

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 from each animal were examined by light microscopy (4×/0.10 objective; Olympus) and scored for lesion severity using a predetermined scale: briefly, for lesion scores, 0, no inflammatory cells; 1, 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 and SP-A protein distribution and intensity within the lungs of control and infected animals by using a light microscope (40×/0.65 objective; Olympus). Scoring was based on a predetermined scale: for PI-3 viral-antigen staining, 0, no staining of cells; 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 rare 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 to 60% 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 fluorogenic real-time RT-PCR, total RNA was isolated using TRIZOL (TRIZOL Reagent Ultrapure; Invitrogen) in our own optimized procedure (10), and DNase treatment of 18 separate total RNA isolates was performed using Promega (Madison, Wis.) RQ1 RNase-free DNase reagents and guidelines immediately prior to cDNA synthesis using reagents (reverse-transcription reagents kit, 10× PCR buffer II, and 25 mM $MgCl_2$ solution) and guidelines from Applied Biosystems Inc. (ABI; Foster City, Calif.). The total cDNAs, corresponding to each total-RNA sample isolate, were then used as templates during fluorogenic real-time RT-PCR. 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 nuclease-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 μH electric homogenizer (Omni 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* and 4°C for 10 min, after which the aqueous (top) layers were transferred to a new nuclease-free 50-ml tube (recombining all partial sample volumes). Isopropanol (nuclease free 2-propanol [1.5 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 for 5 min 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 quantity and purity of the RNA isolates, spectrophotometer (model DU 640B; Beckman Instruments Inc., Fullerton, Calif.), readings of each total-RNA isolate (diluted 1:40 with RNA sample dilution buffer) were taken at 260 and 280 nm. The spectrophotometer was zeroed with RNA sample dilution buffer. Absorbances at 260 nm indicating RNA concentrations of ≥0.2 μg/μl were required in order for each RNA sample to be concentrated enough for the fluorogenic real-time RT-PCR procedure to proceed successfully. All RNA sample A_{260}/A_{280} ratios fell between 1.75 and 1.9, indicating very pure RNA in all cases. All total-RNA samples were stored at −80°C for no longer than 30 days prior to being used as templates for total-cDNA synthesis by reverse transcription.

DNase treatment of RNA isolates and cDNA synthesis by reverse transcription. Prior to cDNA syntheses, each total-RNA isolate was subjected to DNase treatment using a commercially available kit (RQ1 RNase-free DNase reagents; Promega) and a thermocycler (GeneAmp PCR System 2400; Perkin-Elmer LLC, Norwalk, Conn.). DNase treatments of total-RNA isolates were performed in 200-μl MicroAmp optical tubes (ABI) using GeneAmp 2400 thermocycling conditions of 30 min at 37°C, followed immediately by manually pausing the program in order to add RQ1 DNase stop solution (Promega); 1 μl of stop solution per μg of RNA present in each DNase reaction mixture was added to each tube, after which the mixture was gently vortexed and then quickly returned to the 2400 thermocycler to resume and finish the program with a 10-min hold at 65°C and a final safety hold at 4°C. Directly after DNase treatments, cDNAs were made from each of the RQ1 DNase-treated, TRIZOL-isolated total-RNA samples using the ABI reagents mentioned above for reverse transcription as follows: 2 μg of DNase-treated RNA was added to each 100-μl reverse-transcription reaction mixture volume, which was assembled to contain final concentrations of 1× TaqMan PCR buffer II, 5.5 mM $MgCl_2$, 2 mM deoxyribonucleoside triphosphate mixture (500 μM each deoxyribonucleoside triphosphate present), 2.5 μM random hexamers, 1.25 U of murine leukemia virus (also known as Multiscribe) RT/μl, and 0.4 to 0.8 U of RNase inhibitor (ABI)/μl. The reverse-transcription reaction thermocycling conditions were 10 min at 25°C, 30 min at 48°C, and 5 min at 95°C. A portion of each of the resulting cDNA samples was immediately diluted 1:5 with nuclease-free water and stored at −20°C. In addition, positive (TaqMan rRNA control reagents; ABI) and negative (nuclease-free water) RNA controls were prepared and subjected to the same procedures as each of the total-RNA sample isolates (the positive control human RNA was diluted to 0.001 μg/100 μl of reverse-transcription reaction volume, as suggested by the accompanying ABI product literature).

Primer and probe design. Sequence-specific oligonucleotide primers and fluorescent probe for detection and relative quantification of cDNA corresponding to each target mRNA of interest (ovine SP-A and SP-D and SBD-1) were designed using ABI Prism Primer Express version 2.0 software and were selected to be within the coding sequence of each mRNA. Final selected primer and probe sequences were additionally screened by comparing them for similarity to all available DNA and mRNA sequences via the Basic Local Alignment Search Tool (National Center for Biotechnology Information), and only unique sequences were used for primer and probe design. Ovine SP-A nucleic acid sequences included forward primer (5′-TGACCCTTATGCTCCTCTGGAT-3′), reverse primer (5′-GGGCTTCCAAGACAAACTTCCT-3′), and probe (5′-6FAM-TGGCTTCTGGCCTCGAGTGCG-TAMRA-3′). Ovine SP-D nucleic acid sequences included forward primer (5′-ACGTTCTGCAGCTGAGAAT-3′), reverse primer (5′-TCGGTCATGCTCAGGAAAGC-3′), and probe (5′-6FAM-TTGACTCAGCTGGCCACAGCCCAGAACA-TAMRA-3′). SBD-1 nucleic acid sequences included forward primer (5′-CCATAGGAATAAAGGCG TCTGTGT-3′), reverse primer (5′-CGCGACAGGTGCCAATCT-3′), and probe (5′-6FAM-CCGAGCAGGTGCCCTAGACACATGA-TAMRA-3′). (6FAM is 6-carboxyfluorescein, the fluorescent reporter dye, and TAMRA is 6-carboxytetramethylrhodamine, the fluorescent quencher dye.) The sequence-specific oligonucleotide primer-probe set we used to detect the cDNA that corresponded to our chosen endogenous reference (housekeeping) gene, 18S rRNA (to which we normalized all detected real-time signals for ovine SP-A and SP-D and SBD-1), was purchased commercially (TaqMan rRNA Control Reagents; ABI).

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 find the correct concentrations of primers and probes to use for each target, as well as the optimal useful dilution of cDNA (found to be 1:5 in previous studies) that would allow each PCR to proceed with optimum efficiency. To accomplish this, two separate optimization plates were set up for each target, one to optimize the primer concentration and another to optimize the probe concentration. For all optimization trials, each sample was analyzed in quadruplicate. The first plate was designed to enable the testing of various combinations and concentrations of the forward and reverse primers, ranging from 50 to 900 nM, while the probe amount remained fixed. In each well, the 50-μl PCR mixtures contained a constant concentration of target probe (200 nM), 5 μl of 1:5-diluted target-inclusive Stock I cDNA, 25 μl of a commercial master mix (TaqMan Universal PCR Master Mix 2X; ABI), and nuclease-free water (used to adjust each final volume to 50 μl). (Stock I cDNA is predetermined cDNA from Ewe [no. 265] that was shown by a preliminary, nonoptimized real-time test plate to express positively for SP-A, SP-D, and SBD-1. The 1:5 dilution refers to the dilution of full-strength Stock I cDNA [or any other sample cDNA whose original concentration is that which is obtained 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 50-cycle run lasted 2 h and 22 min, after which the GeneAmp 5700 sequence detection system software and Microsoft Excel were used in conjunction to analyze the resultant fluorogenic PCR amplification data. For the first optimization plate for each target, primer amounts that, upon analysis, provided the highest Rn (normalized reporter) value with the lowest primer concentration(s) were identified as the optimal concentrations for each primer pair for each of the respective targets of interest. ($Rn = Rn^+ - Rn^-$, where Rn^+ is the Rn value of a reaction containing all components and Rn^- is the Rn value of an unreacted sample [the baseline value or the value detected in the no-template control].) A second plate was designed for each target to enable the testing of various concentrations of each probe, ranging from 25 to 225 nM, in the presence of optimal primer concentrations (as established by plate 1 in each case). In each well, the 50-μl PCR mixture contained the identified optimal concentrations of the primer (which we found to be 300 and 300 nM for ovine SP-A, 300 and 300 nM for ovine SP-D, and 300 and 900 nM for SBD-1 forward and reverse primer concentrations), 5 μl of 1:5-diluted Stock I cDNA, 25 μl of the ABI commercial master mix (see above), and nuclease-free water. For the second plate for each target, upon analysis of the resultant data, the combination of reactants which yielded the lowest threshold cycle (C_T) (the cycle at which a significant increase in ΔRn is first detected) with the lowest probe concentration was chosen as the optimal fluorogenic-probe concentration in each case (we found these to be 50, 100, and 150 nM for ovine SP-A, SP-D, and SBD-1 probes, respectively). Next, as a validation test that the target and endogenous reference cDNA amplification reactions were all proceeding at equal efficiencies across a spectrum of Stock I cDNA concentrations, a third plate (the validation test plate) was designed to test various concentrations of cDNA ranging from full-strength Stock I cDNA to a 1:15,625 dilution of Stock I cDNA. In each well, constant (optimal) concentrations of forward and reverse primers and constant (optimal) concentrations of probes were used, along with 25 μl of the ABI master mix, 5 μl of sequentially diluted Stock I cDNA, and nuclease-free water. Also included on this plate were wells identical to the ones described above, but instead of ovine target primers and probe they contained the endogenous reference (human 18S rRNA) forward and reverse primers and probe at their ABI-established optimal real-time concentrations. This plate included all samples in triplicate and was run in the GeneAmp 5700 sequence detection system with conditions identical to those used in the optimization tests. Upon analysis of the resultant data for each cDNA concentration, the C_T of the endogenous reference was subtracted from the C_T of the target, and this value (ΔC_T) was plotted against the log concentration of input cDNA. A resultant line with a slope of $< \pm 0.1$ was considered to represent cDNA amplification reactions of target and endogenous references with equal efficiencies across the various cDNA concentrations tested. As the slope was not $< \pm 0.1$ in any case (for SP-A, SP-D, and SBD-1), the standard-curve method was used for data analysis of all three real-time PCR amplification reactions (if the slope had been $< \pm 0.1$, the C_T method for data analysis would have been used).

PCR. Separate microwell plates were designed to carry out fluorogenic real-time RT-PCR using the cDNA prepared previously from all 18 sheep samples, with three replicates run for each sample and three replicates of a negative control (nuclease-free water) on each plate. In addition, on each plate were three replicates each of five serial progressive 1:5 dilutions of the Stock I cDNA that served in the generation of a standard curve for both the target and the reference, as well as three replicates containing cDNA from the calibrator sheep lung (to which all samples could be compared but which we chose not to use in the present study). On each plate, both target and endogenous reference wells were run simultaneously for all samples represented on that plate. Each assay composition was as follows: in each well, the 50-μl PCR mixtures contained 25 μl of the commercially available ABI master mix, 5 μl of a 1:5 dilution of the cDNA to be tested (or nuclease-free water for the negative no-template control), the established optimal forward and reverse primers and probe concentrations in each case for the signal of interest in that well, and nuclease-free water. All such plates were run in the GeneAmp 5700 sequence detection system under conditions identical to those used with the optimization and validation test plates described above.

Using the GeneAmp 5700 software in conjunction with a departmentally designed Excel file, all resultant data were analyzed as follows. Each target signal was normalized to its corresponding reference signal (18S rRNA) by dividing the target input amount of cDNA by the reference input amount of cDNA for each replicate, followed by calculation of the average and standard deviation of all replicates from each sheep. At this point, the normalized target values from each animal could have been expressed relative to the calibrator animal's signal for each particular target (where the chosen calibrator cDNA target of interest would have been assigned a value of 1), but we decided against using the calibrator (control saline-treated lamb no. 8; the lowest SBD-1 expresser), since other real-time studies have demonstrated high interanimal variability of lung SBD-1 levels among control sheep (J. M. Caverly and M. R. Ackermann, unpublished data).

Quantitative-competitive ELISA for SP-A protein. A previously described quantitative-competitive enzyme-linked immunosorbent assay (ELISA) procedure (38) was used to assess lung samples for the presence of the SP-A protein. The procedure was performed in 96-well microtiter plates (Immulon I; 12.8- by 8.6-cm 96-well flat-bottom polystyrene plates; Dynatech Laboratories Inc., Chantilly, Va.). Briefly, 0.2 g of lung tissue and 0.8 ml of PBS (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4) were combined and homogenized with a TH tissue homogenizer (OMNI International Inc., Warrenton, Va.) for 30 s on ice. Samples were then sonicated on ice three times at 10-s intervals at 40% amplitude with a sonicator (model 550 Sonic Dismembrator, using the two-part microtip setup; Fisher Scientific). The resulting lung solutions were then clarified by microcentrifugation at 4°C for 5 min at 15,600 × *g* (Eppendorf model 5414; fixed speed), after which the supernatants were stored at −20°C. The lung homogenate supernatants were diluted 1:1,000 with high-performance liquid chromatography (HPLC)-grade water (Fisher Scientific) just before the assay. A standard curve was prepared using purified SP-A peptide and/or standard sheep bronchoalveolar lavage (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 $1{:}6 \times 10^6$ to $1{:}2.5 \times 10^5$ 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 microtitration plate. The wells were coated overnight with a 1:200 dilution of standard sheep BAL fluid in bicarbonate coating buffer (0.42 g of $NaHCO_3$ 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 mM 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 mM 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 of the SP-A standard-antibody, sheep BAL fluid standard-antibody, and sample-antibody overnight-incubated mixtures was added to appropriate wells to allow 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 to 2 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 Na_2HPO_4, 50 mM $NaH_2PO_4 \cdot H_2O$, 150 mM NaCl, and 0.05% Tween 20, pH 7.4) containing 5% NSS 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. Finally, percent inhibition (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 concentrations in lung samples. All dilution factors incurred 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

lung tissue 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 values, IHC values, and time effect within each group. Kendall's tau-b statistics were tested against zero. Nonparametric Wilcoxon tests (42) were used to determine if there was any statistically significant difference between histopathological lesions or IHC values in the two groups. Values were considered to be significant when P was <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 was <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 was <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 102.6 to 106.6°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 ± 0.28°F) and 5 (average temperature, 104.9 ± 0.40°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 −0.87 for the PI-3 virus-infected group ($P = 0.0048$). There was no statistically significant difference between the severity of histopathological lesions and IHC values for PI-3 viral antigen ($P = 0.4498$ for histopathological lesions; $P = 0.8965$ for IHC values) (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

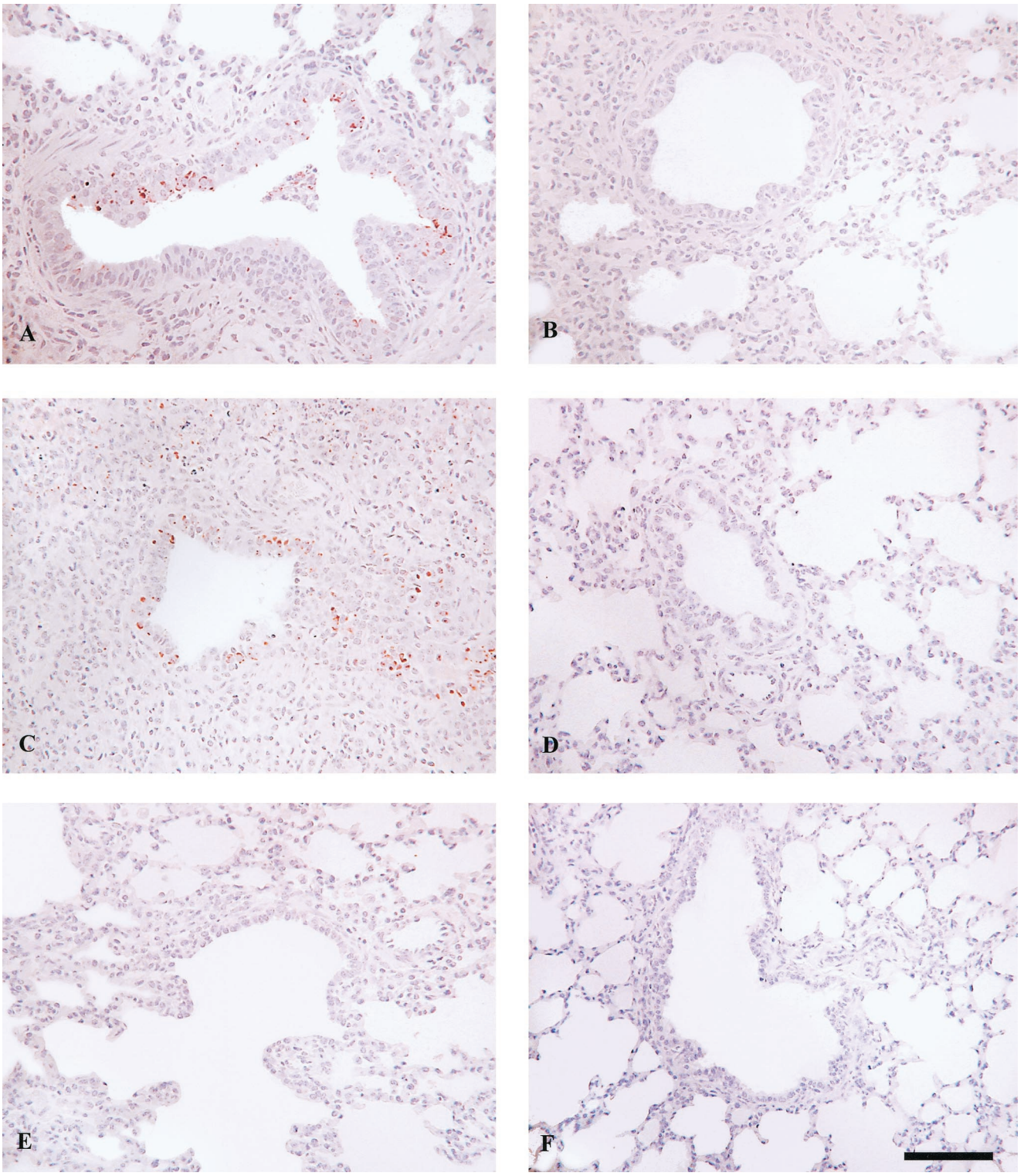

FIG. 1. IHC detection of PI-3 viral antigen in the lungs of lambs inoculated with PI-3 virus or sterile medium 3 (A and B), 6 (C and D), or 17 (E and F) days p.i. (A) PI-3 viral antigen is present within the cytoplasm of bronchiolar epithelial cells. (C) PI-3 viral antigen is present within the cytoplasm 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.

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

Day p.i.	Control		PI-3 inoculated	
	Lesion score[b]	IHC score[c]	Lesion score	IHC score
3	0	0	2.00 ± 0[d]	2.67 ± 0.33[d,e]
6	0	0	2.67 ± 0.33[d]	0.67 ± 0.33[e]
17	0	0	1.00 ± 0[d]	0

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

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

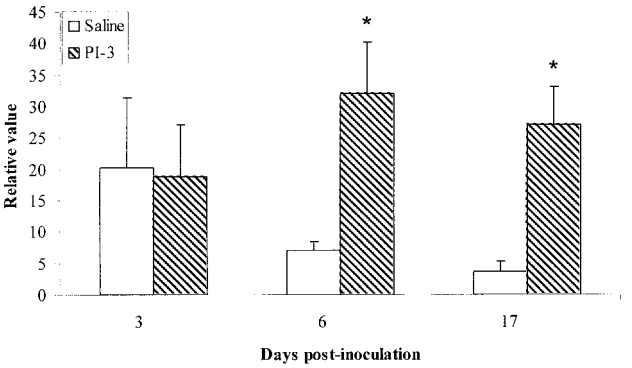

FIG. 3. SP-A mRNA levels detected in whole-lung homogenates of PI-3 virus-inoculated and control lambs by fluorogenic real-time RT-PCR. SP-A mRNA levels were significantly increased (*) in PI-3 virus-inoculated lambs 6 and 17 days p.i. compared to the control animals ($P = 0.09$ for day 6 p.i. and $P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

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-

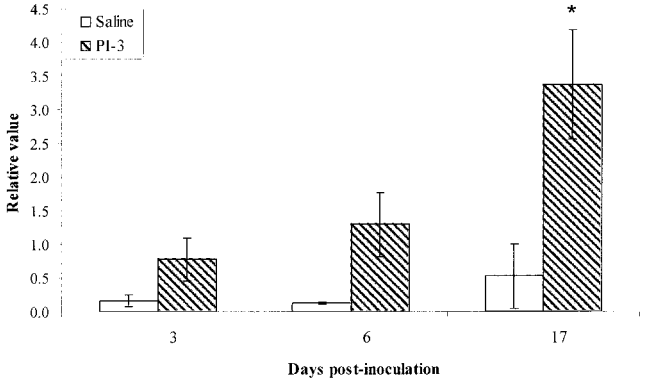

FIG. 2. SBD-1 mRNA levels detected in whole-lung homogenates of PI-3 virus-inoculated and control lambs by fluorogenic real-time RT-PCR. There was a trend toward increased SBD-1 mRNA levels in the PI-3 virus-infected group on days 3 and 6 and a significant increase (*) on day 17 compared to the control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

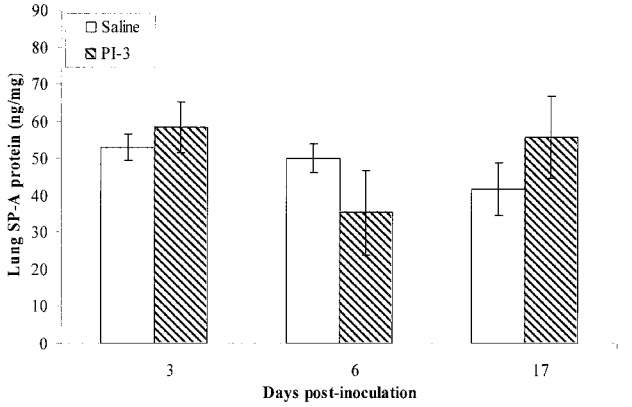

FIG. 4. SP-A protein levels assessed by quantitative-competitive ELISA in whole-lung homogenates of PI-3 virus-inoculated and control lambs. SP-A protein levels were not significantly changed by PI-3 virus inoculation compared to the control animals. A t test assuming unequal variances was used. The error bars indicate SEM.

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.[a]

Day p.i.	Control		PI-3 inoculated	
	Distribution	Intensity	Distribution	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

[a] 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).

fected with RSV (31). Although another study of RSV-infected children detected modest reduction of SP-A protein in the BAL fluid of infected patients, the SP-A protein was found to increase between the first and last day of sampling (31). Our IHC assessment of SP-A relied solely on mouse IgM anti-SP-A primary antibody, since all other commercial antibodies we tested (all of which were IgG) did not work in this application. In our study, protein expression was determined only for SP-A. We are in the process of developing reagents and techniques necessary to detect the expression of SP-D and SBD-1 proteins as well.

There are few spontaneous models of PI-3 virus respiratory tract infection, and we think that an ovine model may be a good additional candidate. The ovine model used in this study represents one of the few spontaneous models of PI-3 virus respiratory tract infections, and sheep are commonly used for the study of paramyxoviral pathogenesis and immunity (1, 4, 41). Ovine PI-3 virus is a member of the family *Paramyxoviridae* and is similar to human PI-3 virus in antigenic epitopes, epidemiology, and disease pathogenesis (15, 16, 36). PI-3 virus infection results in consistent lesions, and the pathogenesis and lesions (bronchiolar epithelial cell necrosis, inflammatory cell infiltrate, and epithelial cell proliferation) in sheep are similar to PI-3 virus infection in humans (7, 15, 23). Furthermore, human neonates have elevated risk for severe PI-3 virus infection (23), and similarly, neonatal sheep infected with PI-3 virus exhibit more severe lesions than older sheep (15, 36; B. Grubor and M. R. Ackermann, unpublished data). In addition, innate immunity in lambs can be readily studied without the influence of maternal antibody, since placental passage of maternal immunoglobulins does not occur in ruminants, including sheep. In this study, lambs were fed colostrum-containing milk in order for them to experience a more natural neonatal phase of life. The maternal antibodies may in some way influence each lamb's innate immune response; however, preinoculation serum antibody titers to PI-3 virus were found to be low in all animals. In addition, we found that the p.i. serum antibody titers to PI-3 virus were not significantly increased in infected versus control lambs, which was expected, since the p.i. time was not long enough to generate a significant response in naïve neonates. The consistency of our results, including clinical signs and gross and histopathological lesions, indicates that PI-3 virus in neonatal lambs reliably causes pneumonia and consistently incites bronchiolar and alveolar lesions, as seen previously in older lambs (>7 days of age) with the same PI-3 virus strain (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).

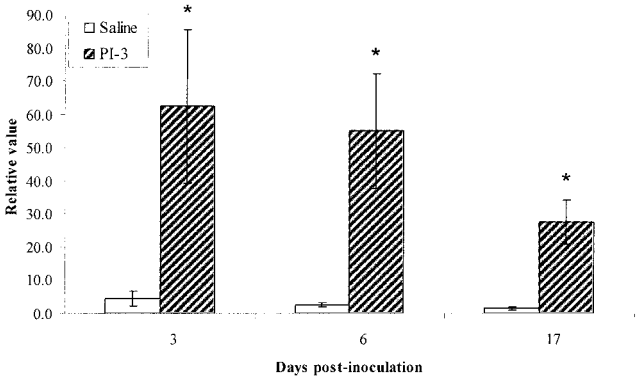

FIG. 5. SP-D mRNA levels detected in whole-lung homogenates of PI-3 virus-inoculated and control lambs by fluorogenic real-time RT-PCR. SP-D mRNA levels were significantly increased (*) in PI-3 virus-inoculated lambs 3, 6, and 17 days p.i. compared to the control animals ($P = 0.09$ for day 3 p.i., $P = 0.09$ 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.

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. corresponded 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 three important lung innate immune 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 bronchial epithelial cells (Ackermann and Gallup, unpublished), which generally lack PI-3 viral antigen and lesions during viral pneumonia.

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