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Porcine Pulmonary Collectins Show Distinct Interactions with Influenza A Viruses: Role of the N-Linked Oligosaccharides in the Carbohydrate Recognition Domain

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Influenza virus (IAV) infections are a major cause of respiratory disease in humans and animals. Pigs can serve as important intermediate hosts for transmission of avian IAV strains to humans, and for the generation of reassortant strains; this may result in the appearance of new pandemic IAV strains in humans. We have studied the role of the porcine lung collectins surfactant proteins D and A (pSP-D and pSP-A), two important components of the innate immune response against IAV. Hemagglutination inhibition assays revealed that both pSP-D and pSP-A display substantially greater inhibitory activity against IAV strains isolated from human, swine, and horse, than lung collectins from other animal species. The more potent activity of pSP-D results from interactions mediated by the asparagine-linked oligosaccharide located in the carbohydrate recognition domain of pSP-D, which is absent in SP-Ds from other species characterized to date. Presence of this sialylated oligosaccharide moiety enhances the anti-influenza activity of pSP-D, as demonstrated by assays of viral aggregation, inhibition of infectivity, and neutrophil response to IAV. The greater hemagglutination inhibitory activity of pSP-A is due to porcine-specific structural features of the conserved asparagine-linked oligosaccharide in the carbohydrate recognition domain of SP-A. A more efficient lung collectin-mediated immune response against IAV in pigs may play a role in providing conditions by which pigs can act as “mixing vessel” hosts that can lead to the production of reassortant, pandemic strains of IAV. The Journal of Immunology, 2003, 171: 1431–1440.

Influenza virus (IAV)³ is a widespread pathogen that causes outbreaks of acute respiratory disease in several animal species including humans, pigs, and birds (1). It is a major cause of mortality in the human population, resulting in as many as 50,000–70,000 annual influenza deaths in the United States (2). Infection and replication of IAV occurs primarily in the bronchial and bronchiolar epithelial cells (3) and can result in a viral pneumonia or secondary bacterial infection. In pigs, influenza is regarded as one of the most prevalent respiratory diseases (4). Furthermore, accumulating evidence suggests a key role for pigs by acting as mixing vessel hosts after coinfection by human and avian IAVs. This can lead to the production of reassortant IAVs that can have pandemic potential in humans (5–7). Although specific host immune responses against IAV are needed for the ultimate elimination of IAV from the lungs (8, 9), it has been shown that non-specific innate immune responses play an essential role in containment of IAV in the airway (10–12). This first line defense system might be especially important during early stages of IAV infection.

Surfactant protein A (SP-A) and D (SP-D) are important components of the innate immune system of the lung (13, 14). They are members of the collectin family, a group of collagenous carbohydrate binding proteins that are present as large, multimeric glycoproteins in alveolar lining fluid or serum. Other members of the family include the serum mannose-binding lectin (MBL), and the bovine serum proteins conglutinin and collectin-43 (CL-43) (15–17). SP-A and SP-D can mediate defense against inhaled infectious agents like bacteria, yeasts, fungi, and viruses due to interaction with polysaccharides and glycoproteins present on these microorganisms. Binding to pulmonary pathogens can lead to the formation of large microbial aggregates, which results in direct inhibition of infectivity. Furthermore, by acting as opsonins, SP-A or SP-D can modulate function and activity of alveolar macrophages and neutrophils which contributes to a more efficient clearance and killing of these microorganisms (13).

The structure of the collectins is characterized by the presence of four distinct polypeptide domains: an N-terminal cysteine-rich region, a collagenous region, an α-helical coiled-coil “neck” region, and a C-terminal Ca²⁺-dependent carbohydrate recognition domain (CRD). The basic structural unit of the collectins is a trimer. The trimeric building blocks can be organized into higher order multimers. SP-A, like MBL, is found as an octadecamer (hexamer of trimers), whereas SP-D is assembled into dodecamers, composed of four trimers, or higher order oligomeric complexes (18). CL-43 is isolated from serum as a trimer (19). Apart from

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3 Abbreviations used in this paper: IAV, influenza A virus; SP-A, surfactant protein A; SP-D, surfactant protein D; MBL, mannose-binding lectin; CL-43, collectin-43; CRD, carbohydrate recognition domain; HA, hemagglutination; p, porcine; h, human; deg, N-deglycosylated; con, sham-treated; CRF, collagenase-resistant fragment; DIG, digoxigenin; Rr, recombinant rat; Rh, recombinant human; MDCK, Madin-Darby canine kidney.
differences in the degree of multimerization, the collectins also show variations in the size of their collagen domains, posttranslational modifications and carbohydrate specificity of the CRD. All these differences appear to have an effect on the recognition of pathogens by collectins and may also affect their interaction with phagocytic cells (20).

Both SP-D and SP-A express anti-influenza activity in vitro and in vivo (11, 12, 21, 22). Neutralization is obtained through direct inhibition of viral infectivity, formation of viral aggregates, and enhanced uptake of IAV by phagocytic cells. However, mechanisms of IAV inhibition by SP-A and SP-D are different. SP-A inhibits IAV through a Ca\(^{2+}\)-independent manner against between sialic acid residues present on a conserved N-linked oligosaccharide moiety located in its CRD, and the hemagglutinin, an envelope glycoprotein of IAV. It is likely that this interaction involves the sialic acid receptor present on the globular domain of the hemagglutinin (22). In contrast, SP-D binds in a Ca\(^{2+}\)-dependent manner via its CRD to N-linked high-mannose carbohydrates present on the hemagglutinin, and the other IAV envelope glycoprotein, neuraminidase (23). SP-D also contains a conserved asparagine-linked oligosaccharide which is located in its collagen domain (Asn70). However, it was previously demonstrated that deletion of this carbohydrate by site-directed mutagenesis does not inhibit the anti-IAV activity of SP-D (24). This might be due to the poor accessibility of this carbohydrate moiety, located in the hub of the SP-D multimer. In general, SP-D exhibits much greater hemagglutination (HA) inhibition activity compared with SP-A (24) and is more potent in aggregating IAV, especially higher order multimeric forms of SP-D (25). However, IAV strains as A/Puerto Rico/8/34 (PR-8), which lack high-mannose oligosaccharides on their hemagglutinin molecules, are inhibited by SP-A while they are highly resistant to inhibition by SP-D (24). The distinct modes of interaction of SP-A and SP-D suggest complementary roles for both lung collectins in vivo, which might enhance the potential of innate immune defense toward a wider range of IAV strains.

Characterization of porcine SP-D (pSP-D) by cDNA cloning revealed that pSP-D has structurally unique features that have not been found in SP-D from other species examined to date (26). These include a cysteine within the collagen domain, an insertion of 3 aa in the CRD and the presence of a terminally sialylated N-linked sugar within the CRD (27). Considering that this Asn323-linked sialylated oligosaccharide is located in saccharide-binding loop 1 of the CRD, it may contribute to IAV recognition and neutralization by pSP-D, and function as an extra mode of interaction in addition to the usual, Ca\(^{2+}\)-dependent interactions between IAV and the sugar binding site of SP-D.

This study addresses innate immune defense against IAV in the porcine lung by analysis of the HA inhibitory activity of the porcine lung collectins pSP-D and porcine SP-A (pSP-A) against a wide spectrum of different IAV strains. It was shown that pSP-D and pSP-A exhibit significantly greater activity against IAV compared with SP-D and SP-A, respectively, from other animal species. The porcine proteins also showed a broader range of interaction with various strains of IAV. The greater anti-IAV activity of pSP-D was found to result in large part from the presence of the sialylated N-linked oligosaccharide located in the CRD of pSP-D.

### Materials and Methods

#### Reagents

PBS and Dulbecco’s PBS\(^{\text{TM}}\) (PBS with 1 mM calcium and 0.5 mM magnesium) were purchased from Life Technologies (Grand Island, NY). BSA, mannan-agarose and mono- and disaccharides used for the assessment of saccharide selectivity were supplied by Sigma-Aldrich (St. Louis, MO). Chicken erythrocytes were purchased from Rockland (Gilbertsville, PA).

pSP-D was purified from bronchoalveolar lavage fluid as described previously (25). Absorption of pSP-D by Western blotting and immunostaining with polyclonal rabbit anti-porine SP-A Ab, N-deglycosylated pSP-D (pSP-Ddeg) was obtained by treatment of pSP-D with recombinant N-glycanase (from Flavobacterium meningosepticum; Glyko, Novato, CA). Purified pSP-D (50 µg/100 µl), dissolved in 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 5 mM EDTA, was mixed with 15 mM N-glycanase and incubated at 37°C for 3 h. Sham-treated pSP-D (pSP-Dcon) was obtained by incubation in the absence of enzyme. After incubation, pSP-Ddeg and pSP-Dcon were purified and separated from any N-glycanase by adding mannan-Sepharose (0.5-ml bed volume per 0.5-ml incubation mixture) and a final concentration of 10 mM CaCl\(_2\) to the incubation mixture. Mannan-bound pSP-Ddeg and pSP-Dcon were isolated as described for the isolation of pSP-D (27), with the exception of gel filtration chromatography. The state of multimerization of pSP-Ddeg was determined by gel filtration fractionation using a Sephacryl-300 High Resolution column (Amersham Pharmacia Biotech, Freiburg, Germany) and compared with pSP-D. To verify whether the N-linked oligosaccharide present in the CRD of pSP-D was efficiently removed, an aliquot of pSP-Ddeg was treated with collagenase as described previously (27). The collagenase-resistant fragment (CRF) of pSP-Ddeg was analyzed by SDS-PAGE and, after transfer to nitrocellulose, immunostained with polyclonal rabbit anti-pSP-D. A second blot was used to test for the presence of glycoconjugates by digoxigenin (DIG) glycan staining (Roche Diagnostics, Mannheim, Germany).

pSP-A was purified from the surfactant fraction using the procedure described for the isolation of rat SP-A (28) with the addition of a gel filtration purification step using a Sephacryl-300 High Resolution column (Amersham Pharmacia Biotech, Freiburg, Germany). Fractions were analyzed by SDS-PAGE and pSP-A positive fractions were pooled and stored in aliquots at -20°C. pSP-A preparations were analyzed for the presence of pSP-D contamination which could not be detected by Western blotting and immunostaining with polyclonal rabbit anti-pSP-D Ab. N-deglycosylation of pSP-A (60 µg/50 µl) was performed in 5 mM Tris-HCl (pH 7.4) and after addition of 15 mM N-glycanase, the mixture was incubated at 37°C for 16 h. After incubation, N-glycanase (molecular mass 35 kDa) was removed by repetition of the gel filtration purification step. N-deglycosylation of pSP-A was verified by Western blot analysis. In this analysis, polyclonal rabbit anti-pSP-A Ab were used to detect SP-A while the presence or absence of glycoconjugates was determined by DIG glycan staining.

Recombinant rat SP-D (RrSP-D) and recombinant human SP-D (RhSP-D) assembled as dodecamers, were both produced in CHO-K1 cells as described (25, 29). Human SP-A (hSP-A), isolated from bronchoalveolar lavage fluid of patients with alveolar proteinosis as described previously (28), was generously provided by Dr. J. van Iwaarden (Laboratory of Pediatrics, Erasmus University Rotterdam, Rotterdam, The Netherlands).

#### Assessment of saccharide selectivity

The affinity of pSP-D, pSP-Dcon, pSP-Ddeg, and RhSP-D for various saccharides was tested by measuring the competition of SP-D binding to BSA-mannan-coated 96-well plates with the following mono- and disaccharides: N-acetylglucosamine, N-acetylmannosamine, t-fucose, t-galactose, t-glucose, myo-inositol, maltose, and D-mannose. Volumes indicated are volumes added per well. Coating of BSA-mannan (100 ng/50 µl) was performed in 0.1 M NaHCO\(_3\) buffer (pH 9.6) for 16 h at 4°C. After removal of the coating solution, the wells were blocked with 2% (w/v) BSA in washing buffer (5 mM Tris-HCl (pH 7.4), 0.9% (w/v) NaCl, 0.05% (v/v) Tween 80 and 5 mM CaCl\(_2\)) for 1 h. Plates were washed four times with washing buffer, and collectin preparations (25 ng/50 µl, diluted in 0.1% BSA in washing buffer) were mixed with 50 µl of the various saccharides (final concentrations: 0.025–100 mM) and incubated for 1 h at 37°C. After subsequent washing, mannan-bound collectin was detected using rabbit polyclonal Ab against pSP-D or hSP-D. After washing, the primary Ab were detected by HRP-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA); both primary and conjugated Ab were diluted in washing buffer and incubated for 1 h. After a final wash, the plates were stained using a mixture of 400 µM tetramethylbenzidine reagent (Merck, Darmstadt, Germany) and 1 mM H\(_2\)O\(_2\) in 0.1 M citric acid buffer (pH 4.0). The reaction was stopped by adding 50 µl of 2 M H\(_2\)SO\(_4\) and the absorption was measured at 450 nm.

Results were expressed as IC\(_{50}\) values, the concentrations of the various saccharides required for 50% inhibition of collectin binding to BSA-mannan.
Virus preparations

The IAV preparations used in this study are outlined in Table I. IAV was grown in the chorioallantoic fluid of 10-day-old chicken eggs and purified on a discontinuous sucrose gradient as described previously (30). Virus stocks were dialyzed against PBS, aliquoted, and stored at −70°C. A/Philippines/82(H3N2) (Phil), A/Memphis/71p_Bel/H3N1 (Mem), and their bovine serum β-inhibitor-resistant variants, PhilBS and MemBS, respectively, were provided by Dr. E. M. Anders (Department of Microbiology, University of Melbourne, Melbourne, Australia). The PR-8 strain was provided by Dr. J. Abramson (Department of Pediatrics, Bowman Gray School of Medicine, Wake Forest University, Winston Salem, NC). Both swine IAV strains, A/swine/iowa/3421/90 “classical” H1N1 (Swine Cl) and A/swine/iowa/8548−1/98 triple reassortant H3N2 (Swine Tr), were provided by Drs. R. G. Webster and S. Krauss (St. Jude’s Hospital, Memphis, TN) and were inactivated with 0.025% (v/v) formalin for 3 days at 4°C (31). A2/Aichi/268 (Aichi) and A/equine2/Miami/1/63 (Equine) were purchased from the American Type Culture Collection (Manassas, VA).

HA titers of each strain were determined by titration of virus samples in PBS+ with thoroughly PBS-washed human type O, Rh− RBCs as described (32). Titration of swine IAV strains was conducted using PBS-washed chicken erythrocytes.

**HA inhibition assay**

HA inhibition was measured by serially diluting collectin preparations in round-bottom 96-well plates (Nuncell U-Vinyl plates; Costar, Cambridge, MA) using PBS++ as diluent (25 μl/well). The highest collectin concentrations tested for HA inhibitory activity were 280 ng/ml for SP-D and 2800 ng/ml for MemBS and MemHS, in which 2800 ng/ml was the highest concentration for all collectin preparations tested. After adding 25 μl of IAV solution, giving a final concentration of 40 HA U/ml or 4 HA U/well, the IAV/collectin mixture was preincubated for 15 min, followed by the addition of 50 μl of human erythrocyte suspension in PBS++; chicken erythrocytes were used for analysis of both swine IAVs. The entire procedure was performed at room temperature. The minimal concentration of a collectin required to fully inhibit the HA caused by the virus, was determined by reading the plates after 2 h. HA inhibition was detected as the formation of a pellet of RBCs. To enable graphical comparisons of HA inhibition results, data were mathematically converted and expressed as the number of HA units inhibited by 100 μg of collectin.

If no HA inhibition could be measured when the highest dose of collectin tested was mixed with 40 HA units/ml of IAV, the inhibitory activity of that collectin against the analyzed IAV was assigned “not detectable.”

**Viral aggregation**

Aggregation of IAV particles (Phil 82) was assessed as previously described (33). At t = 0, suspensions of virus were mixed with 800 ng/ml pSP-Dcon or pSP-Ddeg in PBS++ in a final volume of 1.0 ml. Under continuous stirring, the light transmission was monitored during 12 min using a highly sensitive SLM/Aminco 8000 C (SLM Instrument, Urbana, IL) spectrophotometer. The excitation and emission wavelengths were 350 nm. Due to viral aggregation, a decline in light transmission was observed. Results are expressed as percent of control light transmission (virus without pSP-D).

**Neutralization of infectivity**

Madin-Darby canine kidney (MDCK) cell monolayers were prepared in 96-well plates and grown to confluence. These layers were then infected with an IAV preparation (Phil strain) diluted in PBS++ which was preincubated for 30 min at 37°C in the presence or absence (control) of increasing amounts of collectin. After exposure of the MDCK cells to the IAV or IAV/collectin mixture for 30 min at 37°C, the cells were washed three times in serum-free DMEM containing 1% (w/v) penicillin-streptomycin. The monolayers were then incubated for 7 h at 37°C, whereafter they were washed, fixed, and FITC-labeled for IAV nucleoprotein as described (34). Fluorescent foci, which appeared to be single-infected cells in general, were counted, and results were expressed as percentage of control foci present after infection with collectin-treated virus as compared with untreated virus.

**Uptake of IAV by neutrophils**

IAV (Phil) was FITC-labeled as described previously (33) and was preincubated with control buffer or various concentrations of SP-D for 30 min at 37°C. These mixtures were added to freshly isolated human neutrophils and incubated for 30 min at 37°C. After quenching of extracellular fluorescence by addition of trypan blue (0.2 mg/ml) and subsequent washing and fixation of cells, neutrophil-associated fluorescence was measured using flow cytometry.

**Measurement of neutrophil H2O2 production**

Viral aggregates, obtained from the viral aggregation assay which is described elsewhere, were mixed with freshly isolated human neutrophils. H2O2 production was measured continuously using the fluorescent scopoletin method as previously described (35).

**Statistical analysis**

Where multiple comparisons were made with a control condition, statistical analysis was conducted by repeated-measures ANOVA. In those cases in which the F test indicated that there was a significant difference (p < 0.05) among groups, comparisons with the control condition were made with Dunnett’s test. In those cases where only two means were compared, statistical analysis was conducted by the Student t test.

**Results**

**Isolation and N-deglycosylation of collectins**

For the purification of pSP-D, containing both dodecamers and higher order oligomers, and pSP-A from porcine lung lavage fluid, we used techniques as previously described (27, 28). Both collectin preparations were analyzed by reducing SDS-PAGE and migrated as monomers of expected size (Fig. 1, lanes 1 and 7). Presence of the N-linked oligosaccharide in the CRD of pSP-D was confirmed by treatment with collagenase to remove sugars linked to the collagen domain. The resulting CRF was strongly stained after DIG glycan detection (Fig. 1, lane 5). The N-deglycosylation of pSP-D was conducted using a more physiological incubation buffer containing 150 mM NaCl and shorter incubation times (3 h) than described previously (27). Analysis by gel filtration chromatography, using a Sephacryl S-300 High Resolution column, showed that pSP-Ddeg eluted at the void volume of the column, identical to pSP-D, indicating that N-glycanase treatment did not affect the degree of multimORIZATION of pSP-D (>1500 kDa; data not shown). Removal of the N-linked carbohydrate moiety present in...
in the CRD of pSP-D was efficient, as judged by Western blotting and immunostaining of the CRF of pSP-Ddeg which appeared to be ~5 kDa smaller compared with the CRF of pSP-D. Furthermore, DIG glycan staining of pSP-Ddeg-CRF revealed that no glycoconjugates were present (Fig. 1, lane 6). Immunoblot analysis of pSP-A showed that the major band of pSP-A (30–35 kDa, reduced) had almost disappeared after N-glycanase treatment, with an accompanying increase in the staining of a 25-kDa band. DIG glycan staining showed that glycoconjugates were only present in the main band of pSP-A (30–35 kDa), while no staining was observed in the N-deglycosylated pSP-A preparation (pSP-Adeg).

To determine whether removal of the N-linked carbohydrate from the CRD of pSP-D affects the carbohydrate binding properties of pSP-D, saccharide selectivity of pSP-D, pSP-Dcon, and pSP-Ddeg was determined, using a variety of mono- and disaccharides as competitors for pSP-D binding to BSA-mannan-coated plates (Table II). No significant change in carbohydrate-binding profile was observed after removal of the N-linked carbohydrates of pSP-D although the IC_{50} values were slightly increased for both pSP-Dcon and pSP-Ddeg preparations compared with untreated pSP-D. The order of preference for pSP-D was identical to that of published data on natural rat and human SP-D when BSA-maltose was used as solid phase instead of BSA-mannan (36) but differed from that of CL-43, a bovine trimeric collectin with a unique carbohydrate-binding profile (16). The saccharide binding profile of the RhSP-D preparation used in this study was also determined and proved to be similar to that of amniotic fluid derived hSP-D (36). The IC_{50} values for pSP-D and RhSP-D were in the same range except for the IC_{50} value measured for N-acetylmannosamine which was considerably lower for RhSP-D compared with that of pSP-D.

**Inhibitory activity of pSP-D and pSP-A on HA by IAV**

The HA inhibitory activity of both porcine lung collectins was tested against IAV strains of varying subtype and with different host specificity, including human, swine, and equine strains (Table I). Two mutant strains, PhilBS and MemBS, were included which are resistant to HA inhibition by MBLs present in serum, known as β inhibitors, which inhibit the infectivity and HA activity of IAVs of the H1 and H3 subtypes. These strains differ from the parent Phil and Mem strains in that the high-mannose oligosaccharide overlying the sialic acid receptor-binding site of the hemagglutinin molecule is absent (37). It was found that pSP-D was in general much more potent in inhibiting HA by IAV than pSP-A, except toward the Mem and mutant MemBS strains, for which, interestingly, the opposite was observed (Fig. 2). Highest inhibition by pSP-D was measured against the common wild-type human Phil strain. Its variant PhilBS, the β inhibitor-resistant mutant strain, was more resistant to inhibition by pSP-D. Despite this, pSP-D displayed considerable inhibitory activity against this strain. HA inhibition by pSP-D of IAV strains isolated from human, horse, or swine did not reveal any major host related differences in activity. Furthermore, the antiviral activity of pSP-D was not restricted to a certain subtype of IAV because all four different subtypes tested were inhibited by pSP-D. Of all the strains tested, PR-8, a mouse-adapted strain that lacks high-mannose oligosaccharides on its hemagglutinin (38), was by far the most resistant to inhibition by pSP-D. However, pSP-D was still more effective (6.3 ± 1.3 HA units inhibited per 100 ng) compared with pSP-A (4.6 ± 1.6 HA U/100 ng).

**HA inhibition of IAV by pSP-D: comparison with RhSP-D and RrSP-D and effect of N-deglycosylation on pSP-D**

To compare HA inhibitory activity of pSP-D with SP-Ds from other species, two recombinant SP-D preparations, RhSP-D and RrSP-D, were tested in parallel with pSP-D (Fig. 3A). To better illustrate the differences in activity between the different mammalian SP-Ds tested against the various IAV strains, the inhibitory

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**Table II.** Saccharide selectivity of pSP-D and the effect of N-glycanase treatment, as compared to saccharide selectivity of RhSP-D and CL-43

<table>
<thead>
<tr>
<th>Sugar Inhibitor</th>
<th>pSP-D</th>
<th>pSP-Dcon</th>
<th>pSP-Ddeg</th>
<th>RhSP-D</th>
<th>CL-43</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myo-inositol</td>
<td>1.7 (0.1)</td>
<td>3.5 (0.6)</td>
<td>4.8 (0.1)</td>
<td>3.4 (0.4)</td>
<td></td>
</tr>
<tr>
<td>Maltose</td>
<td>4.8 (0.9)</td>
<td>6.6 (1.2)</td>
<td>8.9 (0.2)</td>
<td>4.1 (0.9)</td>
<td>1.9</td>
</tr>
<tr>
<td>L-Fucose</td>
<td>5.1 (0.6)</td>
<td>8.2 (0.6)</td>
<td>10.2 (0.5)</td>
<td>4.4 (0.1)</td>
<td>0.8</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>6.4 (0.4)</td>
<td>9.8 (1.6)</td>
<td>17.3 (1.1)</td>
<td>4.0 (1.0)</td>
<td>1.5</td>
</tr>
<tr>
<td>D-Mannose</td>
<td>6.5 (1.4)</td>
<td>13.2 (2.2)</td>
<td>19.8 (0.9)</td>
<td>2.9 (0.4)</td>
<td>0.2</td>
</tr>
<tr>
<td>D-Galactose</td>
<td>14.0 (0.3)</td>
<td>26.2 (3.1)</td>
<td>36.5 (0.9)</td>
<td>7.9 (3.8)</td>
<td>9.0</td>
</tr>
<tr>
<td>ManNAc</td>
<td>15.1 (0.8)</td>
<td>24.2 (4.9)</td>
<td>39.5 (1.4)</td>
<td>1.8 (0.3)</td>
<td>0.4</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>25.7 (3.0)</td>
<td>64.7 (4.2)</td>
<td>65.2 (2.8)</td>
<td>13.5 (4.5)</td>
<td>1.0</td>
</tr>
</tbody>
</table>

*Microtiter plate wells were coated with BSA-mannan and were incubated with pSP-D, pSP-Dcon, pSP-Ddeg, or RhSP-D in the presence of increasing concentrations of sugars listed. Binding of collectin to BSA-mannan was measured by immunodetection as described in Materials and Methods. Results are expressed as concentrations (millimolar) of saccharide required for 50% inhibition of collectin binding (IC_{50}) to BSA-mannan. Data are averages of three experiments; SEM values in parentheses.

*Values for CL-43 are taken from Ref. 16.*
activities of both recombinant SP-Ds were expressed as percent of pSP-D inhibitory activity, which was set at 100% for each individual strain. For all IAV strains analyzed, pSP-D caused greater inhibition than RhSP-D or RrSP-D. As regards the human IAV strains analyzed, enhanced activity of pSP-D compared with other SP-Ds was most prominent against the PhilBS and PR-8 strains, which were resistant to RhSP-D and RrSP-D. Furthermore, the activity of pSP-D against the wild-type human strains Phil and Aichi and the equine strain was markedly higher compared with that of RhSP-D. pSP-D was most potent against the triple reassortant swine IAV, and classic swine strain was only susceptible to inhibition by pSP-D.

Among the SP-D species characterized to date, only pSP-D is N-glycosylated in its CRD. To study the contribution of this N-linked oligosaccharide to the HA inhibitory activity of pSP-D against IAV, N-glycanase-treated pSP-D (pSP-Ddeg) was tested. As a control, pSP-D was incubated under identical conditions but in the absence of N-glycanase. There was no significant difference in HA inhibitory activity of pSP-Dcon compared with untreated pSP-D against any of the strains tested (data not shown). The effect of N-deglycosylation was documented by expressing the activity of pSP-Ddeg as the percentage of the inhibitory activity of pSP-Dcon (Fig. 3B). Treatment of pSP-D with N-glycanase reduced the activity significantly against all strains tested although the percentage of the decrease in activity differed among the various strains (Fig. 3B). Remarkably, the contribution of the N-linked sugar on HA inhibition of PR-8 by pSP-D was moderate as compared with most other strains tested. The activity of pSP-Ddeg against the wild-type strains Phil, Mem, Aichi, and Swine Tr was similar to that of RhSP-D and, except for the Phil strain, to that of RrSP-D.
Previous studies have shown that the HA inhibition activity of SP-D is calcium-dependent due to interactions of its CRD with \(N\)-linked sugars present on IAV (21, 23). In contrast, interactions of SP-A with IAV are \(Ca^{2+}\)-independent (24) and binding is mediated via the sialic acids present on the \(N\)-linked oligosaccharide in the CRD of SP-A (22). To determine whether the sialylated \(N\)-linked oligosaccharide present in the CRD of pSP-D can interact with IAV in a similar, \(Ca^{2+}\)-independent fashion, HA inhibition assays with pSP-Dcon and pSP-Ddeg were performed in the absence or presence of 5 mM EDTA. Table III shows that the HA inhibitory activity of both pSP-D preparations against Phil, PhilBS, and PR-8 was no longer detectable after addition of EDTA.

**Table III. Effect of EDTA addition on HA inhibitory activity of pSP-D on various IAV strains**

<table>
<thead>
<tr>
<th></th>
<th>Phil</th>
<th>PhilBS</th>
<th>PR-8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Ca^{2+})</td>
<td>EDTA</td>
<td>(Ca^{2+})</td>
</tr>
<tr>
<td>pSP-Dcon</td>
<td>85 (21)</td>
<td>0</td>
<td>57 (16)</td>
</tr>
<tr>
<td>pSP-Ddeg</td>
<td>42 (11)</td>
<td>0</td>
<td>15 (0.6)</td>
</tr>
</tbody>
</table>

*Inhibition by pSP-D of HA by Phil, PhilBS, and PR-8 was determined after preincubation of the IAV/pSP-D mixture in the presence of either 1 mM \(Ca^{2+}\) or 5 mM EDTA. HA inhibition was determined for pSP-Dcon and pSP-Ddeg. Data were analyzed as described in Materials and Methods. Values are means of at least three experiments; SEM values in parentheses.

Anti-IAV activity of pSP-D assessed by functional assays

The role of the carbohydrate moiety was further studied by performing assays on viral aggregation, inhibition of infectivity and neutrophil response to IAV. The activity of pSP-Dcon was not significantly different from that of untreated pSP-D in any of these assays (data not shown).

Fig. 4 shows that the addition of pSP-D caused aggregation of IAV as assessed by a time-dependent decrease in light transmission through a suspension of the Phil strain. The Phil IAV-aggregation curve for pSP-Dcon was similar to that of RrSP-D and RhSP-D when equal amounts of collectins were used (800 ng/ml) (34). Removal of the \(N\)-glycosylation in the CRD significantly reduced the viral aggregation activity of pSP-D. The maximal change in light transmission by pSP-Ddeg was \(-30\%\) lower compared with pSP-Dcon.

Inhibition of IAV infection (Phil strain) was determined using MDCK cell monolayers in a fluorescent focus forming assay (Fig. 5). Preincubation of Phil with 5 ng/ml pSP-Dcon reduced the number of infectious foci by \(-70\%\). However, \(N\)-deglycosylation of pSP-D resulted in a 2-fold decrease in neutralizing activity.

FITC-labeled IAV (Phil strain) was used to measure the effect of IAV preincubation with pSP-Dcon or pSP-Ddeg on IAV uptake by human neutrophils. IAV uptake was stimulated by both pSP-D preparations in a concentration-dependent manner (Fig. 6A). After removal of the \(N\)-linked carbohydrate moiety, the uptake of IAV was reduced compared with addition of pSP-Dcon.

Precipitation of IAV with SP-D enhances neutrophil respiratory burst responses to IAV and also increases respiratory burst responses of IAV-treated neutrophils to other stimuli (e.g., bacterial peptides or phorbol ester) (21, 32, 33). As shown in Fig. 6B, pSP-Dcon also caused significantly greater increases in IAV-stimulated \(H_2O_2\) production than pSP-Ddeg.

**HA inhibition of IAV by pSP-A: comparison with hSP-A and effect of N-deglycosylation**

Given that pSP-D exhibits unique anti-IAV properties, we also compared the antiviral activity of the other collectin present in...
porcine lung, pSP-A, with SP-A isolated from human alveolar proteinosis lavage (hSP-A). HA inhibition by pSP-A was only detectable in three human IAV strains: PR-8, Mem, and MemBS (Fig. 2) and the activity of pSP-A against these strains was compared with that of hSP-A (Fig. 7A). Results showed that, although the activity against PR-8 was similar for SP-A from both species analyzed, there was much higher activity of pSP-A than of hSP-A against both the Mem and the MemBS strain. In line with the Ca\(^{2+}\)-independent interactions of SP-A with IAV, EDTA did not reduce the HA inhibitory activity of pSP-A (data not shown).

Analysis of the activity of pSP-A after N-deglycosylation (pSP-Adeg) revealed that the HA inhibitory activity of pSP-A was largely dependent on the presence of the N-linked oligosaccharide (Fig. 7B), consistent with previous studies on the interaction of hSP-A with IAV (22). No significant difference was found for HA inhibition of PR-8 between pSP-A and pSP-A treated in the absence of N-glycanase (data not shown). N-deglycosylation of pSP-A resulted in a reduction of inhibitory activity against Mem and MemBS approximately to the level of that of hSP-A.

**Discussion**

This report describes the anti-IAV properties of pSP-D and pSP-A, compares the activity with that of SP-D and SP-A, respectively, from other species, and highlights the role of the N-linked carbohydrate moiety present in the CRD of pSP-D and pSP-A in interaction with IAV.

The two porcine collectins pSP-D and pSP-A used in this study, were isolated from porcine bronchoalveolar lavage and purified as previously described (27, 28). Both proteins are N-glycosylated in their CRDs and the protocols used to enzymatically remove the N-linked carbohydrates from pSP-D and pSP-A proved efficient. Because many interactions of SP-D with IAV are affected by the degree of multimerization of SP-D (25, 39, 40), the size of pSP-D was determined and data were analyzed as described in Materials and Methods. Results are expressed as percentage of HA inhibitory activity for hSP-A as compared with pSP-A (100%) for each individual IAV strain tested. After incubation of pSP-A in the presence of N-glycanase (pSP-Adeg) for 16 h at 37°C and subsequent purification as described in Materials and Methods, HA inhibitory activity of pSP-A and pSP-Adeg on various IAV strains was determined. Results are expressed as percentage of HA inhibitory activity for pSP-Adeg as compared with pSP-A (100%) for each individual IAV strain tested. Treatment of pSP-A in the absence of N-glycanase (pSP-Acon) did not significantly change the HA inhibitory activity compared with that of untreated pSP-A (data not shown). Values are mean ± SEM of at least five experiments. A value of 0 indicates nondetectable HA inhibitory activity, *, p < 0.05; **, p < 0.01; ***, p < 0.001 compared with pSP-A. Statistical analysis was conducted by the unpaired Student t test.
before and after N-glycanase treatment was analyzed by gel filtration chromatography and revealed no detectable differences between both preparations (>1500 kDa). Therefore, it can be concluded that N-glycanase treatment of pSP-D did not seriously alter its multimer size. Another key feature of SP-D in terms of interactions with IAV, is its ability to bind via its lectin domain, in a Ca\(^{2+}\)-dependent manner, to oligosaccharides present on the hemagglutinin of IAV (23). Treatment of pSP-D in the presence or absence of N-glycanase did not alter the carbohydrate binding properties of pSP-D; only a slight increase in the IC\(_{50}\) value of the various saccharides tested was observed (Table II). Therefore, it was concluded that differences found in activity between pSP-D and pSP-Deg with the various IAV assays performed are caused by the contribution of the N-linked carbohydrate.

**pSP-D exhibits HA inhibition activity against all IAV strains tested, irrespective of host specificity and IAV subtype**

In accordance with earlier findings, based upon comparison studies between SP-A and SP-D from human and rat (24, 41), pSP-D was generally much more potent in inhibiting HA activity of IAV than pSP-A (Fig. 2). Of all the IAV strains tested, PR-8 showed highest resistance to inhibition by pSP-D. This is attributed to the absence of high-mannose oligosaccharides on the hemagglutinin of PR-8 which results in weak CRD-mediated interactions with SP-D (23, 38). Studies on collectin/IAV interactions to date have shown that only SP-A exhibits HA inhibitory activity against PR-8, consistent with Ca\(^{2+}\)-independent binding of the hemagglutinin of IAV to sialic acid moieties present on the conserved N-linked carbohydrate in the CRD of SP-A (24). It is notable that pSP-D, in contrast to SP-Ds from other species, showed HA inhibitory activity against PR-8, with levels similar to that of SP-A.

The N-linked oligosaccharide present in the CRD of pSP-D contributes to the HA inhibitory activity of pSP-D

Recombinant dodecameric human and rat SP-D have been extensively characterized in terms of their interactions with IAV, including HA inhibition studies, and appear functionally indistinguishable from their natural counterparts (23, 25, 34). Accordingly, these proteins were compared with pSP-D in a variety of functional assays. In this study, it was shown that pSP-D has substantially higher HA inhibitory activity against the entire spectrum of IAV strains investigated. In all cases, the N-glycosylation contributed to the enhanced activity observed for pSP-D, although this contribution was strain-dependent (Fig. 3). This contribution is likely to be the result of the unique N-linked glycan present in the CRD of pSP-D. As mentioned in the Introduction, removal of the N-linked oligosaccharide from the collagen domain of SP-D has no inhibitory effect on the anti-IAV activity of this protein. With regard to the human IAV strains, inhibition by pSP-D was most distinctive from RhSP-D and RrSP-D in its HA inhibitory activity toward PhilBS and PR-8, both IAV strains that are resistant to neutralization by the two recombinant SP-Ds, as well as natural rat and human SP-D. The large effect of deglycosylation (Fig. 3B) demonstrates that the relatively high activity of pSP-D against PhilBS is largely dependent on the presence of the N-linked carbohydrate on pSP-D. The resistance of this mutant strain to inhibition by SP-D is due to loss of an important high-mannose oligosaccharide chain present on the hemagglutinin. In the case of pSP-D, this resistance is markedly reduced due to interaction of the N-linked carbohydrate of pSP-D with this strain. This implies that pSP-D not only enhances its activity against IAV, but also broadens the spectrum of IAV strains that can be inhibited by pSP-D by virtue of the N-linked oligosaccharide present in the CRD of pSP-D. The interaction of pSP-D with PR-8 was more complex. Inhibition of the HA activity of the PR-8 strain was only slightly different between pSP-Dcon and pSP-Ddeg, indicating that structural features other than the N-linked sugar are involved. pSP-D has an insertion of 3 aa in sialic acid-binding loop 4 of the CRD when compared with the aligned sequences of rodent and human SP-D. An insertion of 3 aa in CL-43, also present in loop 4, appears to affect sialic acid binding and interactions with IAVs (16, 34). However, sialic acid binding selectivity of pSP-D was similar to that of human or rat SP-D (and distinct from CL-43; Table II). Therefore, it is unlikely that sugar binding properties of the CRD account for the differences in HA inhibitory activity observed against PR-8 between pSP-Ddeg (Fig. 3B) and other SP-Ds (Fig. 3A). Because the recombinant SP-Ds exclusively consist of dodecamers, it might be possible that the additional presence of higher order multimers in the pSP-D preparation, also contribute, to a limited extent, to the higher HA inhibitory activity observed for pSP-D. Further studies will be needed to elucidate why pSP-Ddeg has greater HA inhibitory activity against PR-8 than human or rat SP-D.

**HA by swine IAVs is preferentially inhibited by SP-D but depends on the host origin of SP-D and the hemagglutinin**

Given the important role of pigs as intermediate hosts in which new viruses can emerge that are capable of infecting humans, we included two swine influenza virus strains, “classical” swine H1N1 (Swine Cl) and a triple reassortant H3N2 strain (Swine Tr) to test for HA inhibition by pSP-D and pSP-A. The Swine Cl belongs to the H1N1 subtype of viruses that was first isolated in 1930 (42). This virus is antigenically conserved, genetically closely related to the 1918 “Spanish flu” strain, and prevalent throughout the major pig populations of the world. Zoonotic transmission of H1N1 viruses from pigs has been described (43) and in some cases these infections proved to be fatal (44). The other swine strain, Swine Tr, is an isolate that arose from reassortment events among recent human H3N2, classical swine H1N1, and avian influenza A viruses; the hemagglutinin gene was derived from a human H3N2 virus that was circulating in 1995 (45). We found that both swine strains were susceptible to HA inhibition by SP-D and not by SP-A, with the highest activity of SP-D against the reassortant strain. Of the SP-Ds tested, inhibition of HA by both swine strains was strongest for pSP-D and significantly dependent on the presence of its N-linked carbohydrate moiety. It is of note that HA inhibitory activity against the Swine Cl strain was only measurable for pSP-D but not detectable for RhSP-D and RrSP-D. One may speculate that this specificity observed for pSP-D is due to swine-specific features of the IAV H1 hemagglutinin. In agreement with this idea, the human-derived H3 hemagglutinin from the Swine Tr strain is not only inhibited by pSP-D, but also by RhSP-D. This further emphasizes that the host origin of the hemagglutinin may be decisive for its interaction with SP-D from that particular host, and that adaptation of IAV after interspecies transmission may affect recognition by SP-D. Furthermore, preliminary HA inhibition data that we obtained with an avian-like swine IAV (A/swine/Italy/1390-2/95), of which all the genes are of avian origin, showed that this strain was resistant to HA inhibition by all collectins tested, including pSP-D (data not shown). Avian IAV may adapt in pigs to mammalian hosts and may be subsequently transmitted from pigs to humans (46). Whether resistance of this strain against neutralization by both porcine lung collectins reflects poor inhibition against avian-like swine IAV, or avian IAV in general, requires further study.
The N-linked oligosaccharide in the CRD of pSP-D enhances viral aggregation and the neutrophil response to IAV, and decreases viral infectivity

Overall, HA inhibition studies on various IAV strains showed that the N-linked carbohydrate in the CRD of pSP-D significantly increases the inhibitory activity of pSP-D. This finding was reflected by several functional assays in which the Phil strain was used, because HA inhibition by pSP-D was highest against this strain. Viral aggregation, IAV uptake by neutrophils, and IAV-induced H2O2 production by neutrophils were stimulated by pSP-D, while pSP-D inhibited IAV infectivity. All the effects were severely diminished by N-glycanase treatment of pSP-D. Because the degree of multimerization of SP-D affects its activity in the functional assays mentioned, it is of importance to note that no differences were observed in the gel filtration elution profiles before and after N-glycanase treatment of pSP-D. Previous studies showed that aggregation of IAV modulates IAV-neutrophil interactions (47).

Therefore, it is likely that the increased uptake of IAV and the enhanced respiratory burst response by neutrophils is caused by viral aggregation induced by virtue of the N-linked oligosaccharide. The assay of the neutralization of IAV infectivity, in which MDCK monolayers were infected with Phil preincubated with pSP-D (Fig. 5), appears to show an even more dramatic effect of N-deglycosylation on the activity of pSP-D. We assume that the sialic acids present on the N-linked carbohydrate of pSP-D are involved in binding to the sialic acid receptor on the hemagglutinin of IAV. This interaction might facilitate a more efficient blocking of the hemagglutinin of IAV than that brought about by deglycosylated pSP-D, with the result that binding of IAV to sialic acid-containing proteins present on MDCK cells is less likely to occur.

The HA inhibitory activity of pSP-A is markedly greater than hSP-A and is determined by the N-linked oligosaccharide in the CRD of pSP-A

The present study demonstrates that pSP-D is functionally distinct as compared with SP-Ds from other animal species. To determine whether the unique properties of pulmonary defense in pigs are reflected by other components of the innate immune systems, we also studied pSP-A by measuring the antiviral activity by HA inhibition analysis, and compared its activity with that of hSP-A (Fig. 7). Although SP-A is much less potent than SP-D in neutralizing IAV (21, 24), it should be noted that pulmonary levels of SP-A are ~10-fold higher (48) and therefore may contribute significantly in pulmonary defense against IAV. Although the inhibitory activity of pSP-A and hSP-A against PR-8 was similar, we found a dramatically increased inhibitory activity for pSP-A as compared with hSP-A against the Mem and MemBS strains. Furthermore, like for other SP-As tested in earlier studies, the activity of pSP-A against IAV was calcium-independent and entirely dependent on the presence of the N-linked carbohydrate on the pSP-A (Fig. 7). Taken together, these findings suggest that in terms of HA inhibition of Mem and MemBS, the N-linked carbohydrate present on pSP-A has structural properties that makes it functionally superior compared with the moiety present in hSP-A.

The mechanisms by which pSP-D and pSP-A interact with IAV are different

Although in accordance with earlier findings (22, 24), pSP-A interacts with IAV in a Ca2+-independent fashion via its sialic acid residues, it was found for pSP-D that removal of calcium via addition of EDTA results in complete loss of activity against either Phil, PhilBS, or PR-8 (Table III). In the case of pSP-D, the Ca2+-dependent CRD interactions with high-mannose oligosaccharides present on the hemagglutinin of IAV appears to be decisive, and the N-linked oligosaccharide in its CRD provides in an additional, secondary mode of interaction which cannot take place in the absence of CRD-dependent interactions. It is worth mentioning that differences in the multimeric organization of both collectins might play a role as well. SP-A is organized as an octadecamer, having six trimeric CRDs, and therefore 18 N-linked oligosaccharides, clustered comparatively close to each other. Therefore, one pSP-A molecule may simultaneously interact with several sialic acid receptors present on a single IAV particle. In contrast, the trimeric CRDs of SP-D are spatially more distributed and this may imply that an initial, Ca2+-dependent interaction of the CRD with glycoconjugates present on IAV is required before sialic acid-mediated interactions of pSP-D with the sialic acid receptor on the hemagglutinin of IAV can be established.

In conclusion, these data suggest that in pigs, pulmonary innate immune defense against IAV is more efficient due to a unique distribution of N-linked sugars present on pSP-D and a functionally distinct N-linked glycan present on pSP-A. A more effective non-specific immune response could inhibit the induction of specific acquired immune responses which are elemental for the ultimate elimination of IAV. Evasion of IAV-induced immunity could give rise to conditions where IAV infection can persist. Therefore, it could contribute to cocirculation of IAV and genetic reassortment, processes that occur frequently in pigs. Further studies on the interactions between the sialic acid receptor binding site on the hemagglutinin of IAV and the sialic acid moieties present on pSP-D and pSP-A are currently in progress, and could provide a more detailed understanding of the molecular mechanisms that underlie the generation of new pathogenic strains of IAV in humans.

References