JOURNAL OF VIROLOGY, Nov. 1997, p. 8204–8212 Vol. 71, No. 11
0022-538X/97/$04.00+0

Collectin-Mediated Antiviral Host Defense of the Lung: Evidence from Influenza Virus Infection of Mice

PATRICK C. READING,[1] LEIANA S. MOREY,[1] ERIKA C. CROUCH,[2] AND E. MARGOT ANDERS[1]*

Department of Microbiology and Immunology, University of Melbourne, Parkville, Victoria 3052, Australia,[1] and Department of Pathology, Washington University School of Medicine, St. Louis, Missouri 63110[2]

Received 31 March 1997/Accepted 29 July 1997

Collagenous lectins (collectins) present in mammalian serum and pulmonary fluids bind to influenza virus and display antiviral activity in vitro, but their role in vivo has yet to be determined. We have used early and late isolates of H3N2 subtype influenza viruses that differ in their degree of glycosylation to examine the relationship between sensitivity to murine serum and pulmonary lectins in vitro and the ability of a virus to replicate in the respiratory tract of mice. A marked inverse correlation was found between these two parameters. Early H3 isolates (1968 to 1972) bear 7 potential glycosylation sites on hemagglutinin (HA), whereas later strains carry 9 or 10. Late isolates were shown to be much more sensitive than early strains to neutralization by the mouse serum mannose-binding lectin (MBL) and rat lung surfactant protein D (SP-D) and bound greater levels of these lectins in enzyme-linked immunosorbent assays and Western blot analyses. They also replicated very poorly in mouse lungs compared to the earlier strains. Growth in the lungs was greatly enhanced, however, if saccharide inhibitors of the collectins were included in the virus inoculum. The level of SP-D in bronchoalveolar lavage fluids increased on influenza virus infection. MBL was absent from lavage fluids of normal mice but could be detected in fluids from mice 3 days after infection with the virulent strain A/PR/8/34 (H1N1). The results implicate SP-D and possibly MBL as important components of the innate defense of the respiratory tract against influenza virus and indicate that the degree or pattern of glycosylation of a virus can be an important factor in its virulence.

Collectins are a group of soluble mammalian proteins that contain collagenous regions linked to Ca^{2+}-dependent (C-type) lectin domains (49). The members of this family, which include mannose-binding protein (MBP) and conglutinin in plasma and the pulmonary surfactant proteins A and D (SP-A and SP-D), bind to polysaccharides on a wide variety of microorganisms and are thought to function in innate immune defense through their ability to act as opsonins and, in the case of MBP, to initiate complement activation (for reviews, see references 21, 24, and 50). A genetic deficiency in MBP is associated with an opsonic defect and recurrent bacterial and fungal infection in infants (46, 48), and a similar association has recently been reported in adults (47).

Collectins also bind to the glycoproteins of a number of enveloped viruses including human immunodeficiency virus (HIV), herpes simplex virus and influenza virus, but their role in vivo in antiviral host defense is not yet clear. Binding of human MBP to HIV-1 and HIV-2 initiated complement activation (20), and in the absence of complement, MBP inhibited HIV-1 infectivity in vitro (12). However, binding of human MBP or bovine conglutinin to herpes simplex virus type 2 did not neutralize virus infectivity, and when administered to mice before the virus, these collectins enhanced rather than inhibited virus replication in the liver (13). In this situation, the bound collectin (with or without complement) may provide the virus with an alternative route of entry through interaction with a complement receptor or putative collectin receptor (31) on susceptible host cells.

We and others have shown that collectins display potent activity against influenza A virus in vitro. As well as causing hemagglutination inhibition (1, 14, 16, 18, 19, 30), MBP, conglutinin, SP-A, and SP-D all neutralize influenza virus infectivity (1, 2, 5, 14, 18, 19), although with SP-A, neutralization occurs through binding of the viral hemagglutinin (HA) to sialic acid on the SP-A molecule rather than through the carbohydrate-binding activity of SP-A (5). Neutralization of influenza virus by the MBP-like lectin in guinea pig serum was shown to be mediated through activation of the classical complement pathway and resulted in irreversible inactivation of the virus (2). The guinea pig lectin also promotes lysis of influenza virus-infected cells (38). Furthermore, MBP, SP-D, and conglutinin all act as opsonins promoting activation of neutrophils by influenza virus and protecting against deactivating effects of the virus (16, 18, 19), and SP-A was shown to opsonize influenza virus for uptake by rat alveolar macrophages (4).

While these activities point strongly to collectins playing an important role in innate host defense against influenza virus, all evidence to date has come from in vitro studies. In an attempt to address the role of collectins in vivo, we have used strains of the H3N2 subtype of influenza virus that differ in their degree of glycosylation and have examined the relationship between the sensitivity of a virus to serum and lung collectins in vitro and its ability to establish infection in the respiratory tract of mice.

MATERIALS AND METHODS

Viruses and viral glycoproteins. HKx31 (H3N2) and BJx109 (H3N2) influenza viruses are laboratory-derived, high-yielding reassortants of A/PR/8/34 (H1N1) with A/Aichi/2/68 (H3N2) (25) and A/Beijing/353/89 (H3N2), respectively. Other viruses used were the Mt. Sinai strain of A/PR/8/34 (H1N1); a further panel of H3N2 subtype viruses, i.e., A/Northern Territory/60/68 (NT/68), A/Memphis/1/71 (Mem/71), A/Udorn/307/72 (Udorn/72) A/Port Chalmers/1/73 (Pt. Chalmers/73), A/Bangkok/1/79 (Bangkok/79), A/Beijing/353/89 (Beijing/89), and A/Beijing/32/92 (Beijing/92); and additional H3N2 reassortants, i.e., A/Texas/1/77 × A/PR/8 (Texas/77-X), A/Bangkok/1/79 × A/PR/8 (Bangkok/79-X), A/Philippines/2/82 × A/PR/8 (Phil/82-X) and A/Beijing/32/92 × A/PR/8 (Beijing/92-X). The number and location of potential glycosylation sites on HA of the H3 subtype viruses are listed in Table 1. Viruses were obtained from Alan Hampson, World

* Corresponding author. Phone: 61 3 9344 5702. Fax: 61 3 9347 1540.

TABLE 1. Locations of potential glycosylation sites on the HA of H3N2 influenza A virus strains

Virus	Glycosylation site at amino acid residue:											Total no. of sites	Reference
	Stalk					Head							
	8 (HA1)	22 (HA1)	38 (HA1)	285 (HA1)	154 (HA2)	63 (HA1)	81 (HA1)	126 (HA1)	144 (HA1)	165 (HA1)	246 (HA1)		
NT/68	+	+	+	+	+		+			+		7	6
Aichi/68, HKx31[a]	+	+	+	+	+		+			+		7	51
Memphis/71	+	+	+	+	+		+			+		7	35
Udorn/72	+	+	+	+	+		+			+		7	33
Pt. Chalmers/73	+	+	+	+	+	+	+			+		8	6
Texas/77-X[a]	+	+	+	+	+	+		+		+		8	6
Bangkok/79, Bangkok/79-X[a]	+	+	+	+	+	+		+		+		8	6
Philippines/82-X[a]	+	+	+	+	+	+		+	+	+	+	10	34
Beijing/89, BJx109[a]	+	+	+	+	+	+		+		+	+	9	41[b]
Beijing/92, Beijing/92-X[a]	+	+	+	+	+	+		+		+	+	9	32

[a] H3N2 high-growth reassortant of the virus indicated with A/PR/8/34.
[b] Sequence published for HA1 only; HA2 sequence from unpublished data of P. C. Reading and A. Sahasrabudhe (GenBank accession no. U97740).

Health Organization Collaborating Centre for Influenza, Melbourne, Australia, with the exception of Mem/71, which was provided by Graeme Laver, Australian National University, Canberra, Australia, and Udorn/72, which was obtained from David Boyle, Australian Animal Health Laboratories, Geelong, Victoria, Australia.

Viruses were grown in eggs and purified from allantoic fluid as described previously (1). The viral glycoproteins HA and neuraminidase (NA) (hereafter denoted HANA) were prepared by solubilization of 3 mg of purified virus with 60 mM *n*-octyl-β-D-glucoside (Sigma) in TBS (0.05 M Tris-HCl, 0.15 M NaCl [pH 7.2]) at 37°C for 60 min. Nucleocapsid was pelleted by centrifugation in a Beckman air-driven centrifuge (100,000 × *g* for 30 min at 4°C), and the supernatant was dialyzed extensively against TBS with 0.1% NaN_3 ($TBSN_3$).

Mice. C57BL/10 female mice were used at 6 to 8 weeks of age.

Collectins and antibodies. Mouse serum was collected from freshly clotted blood, stored at −70°C, and used as the source of serum mannan-binding lectin (MBL). Recombinant rat SP-D and rabbit antiserum to rat SP-D were prepared as described previously (9, 28). The influenza virus-specific monoclonal antibodies (MAbs) 244/2 and CY3/3, specific for HKx31 HA and BJx109 HA, respectively, and MAb 165, specific for the host carbohydrate antigen common to all egg-grown influenza viruses, were prepared in the Department of Microbiology and Immunology, University of Melbourne. MAb A-3, specific for the nucleoprotein (NP) of type A influenza viruses, was provided by Nancy Cox, Influenza Branch, Centers for Disease Control and Prevention, Atlanta, Ga. Rabbit antiserum against NA of the N2 subtype, prepared against baculovirus-expressed NA of A/NT/60/68 (H3N2), was the gift of Jennifer McKimm-Breschkin, Biomolecular Research Institute, Parkville, Victoria, Australia. Rabbit antiserum against human MBP was provided by Alan Ezekowitz, Children's Hospital, Boston, Mass.

Virus neutralization assay. Neutralization of virus infectivity was measured by fluorescent-focus reduction in monolayers of MDCK cells cultured in 96-well plates (Nunc, Glostrup, Denmark). Dilutions of mouse serum or rat SP-D were prepared in complement fixation test (CFT) buffer (Oxoid; barbitone-buffered saline [pH 7.2], 0.25 mM $CaCl_2$, 1.8 mM $MgCl_2$) and mixed with virus in a total volume of 100 μl. The dose of influenza virus used was that which gave 50 to 100 fluorescent foci per low-power field (see below) after incubation in CFT diluent alone. The mixtures were incubated at 37°C for 30 min, and then 50 μl was inoculated onto MDCK monolayers that had been washed with serum-free RPMI 1640 supplemented with 30 μg of gentamicin per ml. After adsorption of the virus for 45 min at 37°C under 5% CO_2, the inoculum was removed and 100 μl of the latter medium was added to each well. The plates were incubated at 37°C under 5% CO_2 for a further 7 to 8 h, the medium was removed, and the wells were fixed with 80% (vol/vol) acetone. The wells were stained for fluorescent foci by incubation for 30 min with 50 μl of a 1/1,000 dilution of the anti-NP MAb A-3 in phosphate-buffered saline (PBS), followed by a 1/60 dilution of fluorescein-conjugated rabbit anti-mouse immunoglobulin (Silenus, Melbourne, Australia). The plates were viewed under ×128 magnification, and the total number of fluorescent foci in four representative fields was counted and expressed as a percentage of the number of foci in the corresponding area of duplicate control wells infected with virus alone.

To test for the inhibition of neutralization by sugars, the diluted serum and SP-D were incubated with the sugar for 20 min at 20°C before addition of virus. To test for reversal of neutralization, the sugar was added to virus-serum or virus–SP-D mixtures after these had been incubated at 37°C for 30 min, and the resulting mixtures were incubated for a further 20 min at 20°C before plating. The appropriate concentration of sugar was also included in the virus control.

Detection of collectin binding to influenza virus by enzyme-linked immunosorbent assay (ELISA). To compare the binding of collectins to different influenza viruses, wells of a flexible polyvinyl microtiter tray were coated with a series of concentrations of purified influenza virus in 50 μl of $TBSN_3$, blocked for 1 h with 10 mg of bovine serum albumin (BSA) per ml, and washed with TBS containing 0.05% Tween 20 (TBS-T). The wells were incubated for 2 h with 50 μl of either rat SP-D (0.5 μg/ml) or a 1/100 dilution of normal mouse serum in TBS-T containing 5 mg of BSA per ml and either 50 mM $CaCl_2$ (BSA_5–TBS-T–Ca^{2+}) or 5 mM EDTA (BSA_5–TBS-T–EDTA) and then washed. Binding of SP-D was detected by the addition of rabbit antiserum against rat SP-D (1/400 dilution in BSA_5–TBS-T–Ca^{2+}) for 3 h, followed by the addition of horseradish peroxidase (HRP)-conjugated swine anti-rabbit immunoglobulin (Dako) and substrate [2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)] (0.2 mM in 50 mM citrate buffer [pH 4.0] containing 0.004% H_2O_2). Binding of mouse serum MBL was detected in a similar manner by using rabbit antiserum against human MBP (1/600 dilution), which cross-reacts with the mouse lectin.

Assay for collectins in mouse respiratory secretions. The presence of MBL and SP-D in mouse bronchoalveolar lavage (BAL) fluids and nasopharyngeal washings was assayed by ELISA on wells coated with yeast mannan (Sigma) at 50 μg/ml. Samples were subjected to titer determination in BSA_5–TBS-T–Ca^{2+}, and bound collectins were detected as described above, mouse SP-D being readily detected with the rabbit anti-rat SP-D antiserum. For the collection of BAL fluids, the mice were killed by cervical dislocation and the lungs were flushed in situ with 1 ml of CFT buffer through a blunted 23-gauge needle inserted into the trachea. To prepare nasal washings, the mice were lightly anesthetized with Penthrane (Abbott Australasia), 0.5 ml of CFT buffer was injected into the external nares with a 26-gauge needle, and the outflow was collected from the nasopharynx with a Pasteur pipette.

Western blot analysis of lectin binding to influenza virus glycoproteins. HANA glycoprotein preparations were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (5 to 10% polyacrylamide gradient gels) under nonreducing conditions and transferred to a polyvinylidene difluoride membrane. The membrane was blocked with TBS containing 5 mg of BSA per ml and 20 mM $CaCl_2$ (BSA_5-TBS-Ca^{2+}) and washed with TBS-T containing 20 mM $CaCl_2$ (TBS-T–Ca^{2+}). Individual tracks were probed with anti-HA MAb 244/2 or CY3/3 (1/500), rabbit anti-N2 antiserum (1/50), mouse serum (1/100), or rat SP-D (1 μg/ml) diluted in BSA_5-TBS-Ca^{2+}. After overnight incubation, the membranes were washed five times in TBS-T–Ca^{2+}. To detect the bound lectins, rabbit anti-human MBP (1/600) or rabbit anti-rat SP-D (1/400) antiserum, in BSA_5-TBS-Ca^{2+}, was applied to the membranes for 3 h. After the membranes were washed, the binding of these antibodies, the rabbit anti-NA antibodies, and the mouse anti-HA MAbs was detected with HRP-conjugated swine anti-rabbit immunoglobulin or rabbit anti-mouse immunoglobulin (Dako), as appropriate, followed by substrate (3,3′ diaminobenzidine tetrahydrochloride) (0.69 mM in TBS containing 0.0075% H_2O_2). SeeBlue prestained standards (Novex, San Diego, Calif.) were used to estimate molecular weights.

NA and NA inhibition assays. Influenza virus NA activity and its inhibition by collectins were measured by an enzyme-linked microplate assay in which *Arachis hypogaea* (peanut) lectin was used to detect β-D-galactose-*N*-acetylglucosamine sequences exposed after the removal of sialic acid from fetuin (29). Wells of a microtiter plate were coated with 50 μl of fetuin (Sigma F-2379; 20 μg/ml in TBS) overnight at 4°C and washed with TBS-T. In the wells of a second plate, dilutions of rat SP-D and mouse serum were prepared in BSA_5-TBS-Ca^{2+} and mixed with influenza virus in a total volume of 100 μl, in the presence or absence of 100 mM D-mannose. After 15 min at room temperature, 50-μl volumes of the mixtures were transferred to wells of the fetuin-coated plate and incubated at 37°C for 45 min. After the wells were washed, 50 μl of biotin-labelled peanut lectin (Sigma L-6135; 20 μg/ml in BSA_5-TBS-Ca^{2+}) was added to each well, and after 30 min at room temperature, the wells were washed and incubated with 50

μl of HRP-conjugated streptavidin (Silenus; 1/1,000 in BSA_5-TBS-Ca^{2+}) for 20 min. Finally, the wells were developed with substrate as described above for ELISA.

Replication of influenza viruses in the respiratory tracts of mice. Mice were lightly anesthetized with Penthrane and inoculated intranasally with 10^4 or 10^5 PFU of influenza virus in 50 μl of PBS. After 3 days (unless stated otherwise), the mice were killed by cervical dislocation and the lungs were removed, rinsed, and disrupted in 1.5 ml of Hanks' balanced salt solution containing 100 U of penicillin per ml and 100 μg of streptomycin per ml (HBSS) by being pressed through a sieve with the plunger of a disposable 2-ml syringe. In some experiments, tracheas were also placed individually in 1.5 ml of HBSS and homogenized by grinding with sterile sand. The resulting lung and tracheal extracts were clarified by centrifugation, and the supernatants were frozen at −70°C. Nasopharyngeal washings were prepared from a separate group of mice from those providing trachea and lungs. The mice were anesthetized with Penthrane, 1 ml of sterile PBS containing 1 mg of BSA per ml was injected slowly into the external nares, and the outflow was collected from the nasopharynx and frozen at −70°C. The samples were assayed for infectious virus by measuring plaque formation on MDCK cells in the presence of trypsin (2).

Preparation of mouse lung-grown stocks of influenza virus. A lung extract prepared from mice, as described above, 3 days after infection with 10^4 PFU of HKx31 virus was used as the mouse lung-grown stock of this virus. Mouse-lung grown BJx109 virus was prepared similarly from lungs taken 1 day after infection with 10^5 PFU of virus. For reasons described in Results, BJx109 was administered in diluent containing 10 mg of mannan (Sigma) per ml. To remove mannan from the resulting virus stock, the lung extract was clarified by centrifugation in an Eppendorf microcentrifuge and then centrifuged at 75,000 × *g*. The virus pellet was resuspended in serum-free RPMI 1640 medium with antibiotics and stored at −70°C.

Statistics. Statistical analysis was performed by Student's *t* test.

RESULTS

We have established previously that mouse serum contains a Ca^{2+}-dependent MBL that binds to influenza virus and represents the β inhibitor (14); this lectin can be detected with rabbit antiserum raised against recombinant human MBP. Mouse serum contains two related mannose-binding proteins, MBP-A and MBP-C (22, 42). In this study, we have not differentiated between these two forms of mouse MBP and will use the term mannose-binding lectin (MBL) to denote this fact.

Neutralization of HKx31 and BJx109 viruses by mouse MBL and rat SP-D. Strains of H3N2 subtype influenza viruses that circulated from 1968 to 1972 carry a total of 7 potential glycosylation sites on their HA molecules, whereas strains isolated after 1982 carry 9 or 10 (Table 1). To determine whether the level of glycosylation affects the sensitivity of influenza viruses to murine collectins, we compared strains HKx31 and BJx109, which bear the HA molecules of Aichi/68 and Beijing/89, respectively, for their ability to be neutralized by mouse serum MBL and by recombinant rat SP-D.

Both viruses were neutralized by mouse serum, with BJx109 being markedly more sensitive than HKx31 (Fig. 1A and B). Neutralization was abolished by depletion of MBL from the serum by absorption with mannan-Sepharose (data not shown) or by the addition of D-mannose to the serum before the addition of virus (Fig. 1A and B). The addition of L-rhamnose, which is not a ligand for the mouse lectin, had no effect on neutralization titers (data not shown).

We have shown previously that neutralization of $A/Mem71_H$-Bel_N (H3N1) virus by the MBL in guinea pig serum was mediated by activation of complement and could not be reversed by the addition of mannose after the virus-serum mixture had been incubated at 37°C (2). Neutralization of HKx31 virus by mouse serum was similarly irreversible after incubation at 37°C, as was neutralization of BJx109 at the higher serum concentrations (Fig. 1A and B), indicating the involvement of complement in neutralization by the mouse serum lectin also. In contrast, the neutralization of BJx109 at low serum concentrations was completely reversed by the late addition of mannose, indicating that when complement is limiting, neutralization of this very sensitive virus can occur through complex formation with lectin alone.

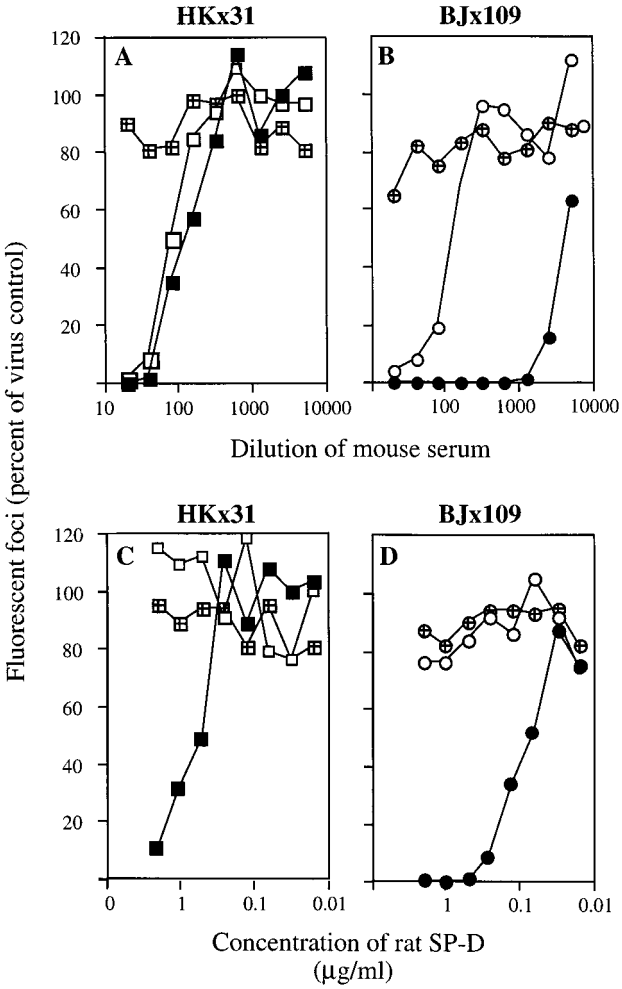

FIG. 1. Neutralization of HKx31 and BJx109 influenza viruses by mouse serum MBL and rat SP-D. HKx31 (■) and BJx109 (●) were added to dilutions of mouse serum (A and B) or rat SP-D (C and D) and incubated for 30 min at 37°C, and the amount of infectious virus remaining was determined by the fluorescent-focus assay. Inhibition of neutralization by D-mannose was examined by adding the sugar to serum or SP-D dilutions 20 min before the addition of virus (⊞, ⊕) or by adding the sugar to virus-serum or virus–SP-D mixtures after the 37°C step (□, ○). In either case, the final concentration of mannose in the virus-lectin-sugar mixes was 50 mM.

Neutralization of HKx31 and BJx109 viruses by recombinant rat SP-D is shown in Fig. 1C and D; again, BJx109 was considerably more sensitive than HKx31. For both viruses, neutralization was abolished by the addition of mannose before or after the 37°C incubation step, consistent with the formation of reversible complexes of virus and SP-D.

Binding of mouse MBL and rat SP-D to HKx31 and BJx109 viruses. To determine whether the difference in sensitivity of HKx31 and BJx109 viruses reflected a difference in binding of the lectins, wells of a microtiter plate were coated with increasing concentrations of the purified viruses and the binding of mouse serum MBL and recombinant rat SP-D to each virus was examined by ELISA. For comparison, a parallel set of wells was developed by using a carbohydrate-specific MAb (MAb 165) which recognizes the cross-reactive host antigen common to all egg-grown influenza viruses.

As shown in Fig. 2, BJx109 bound significantly higher levels

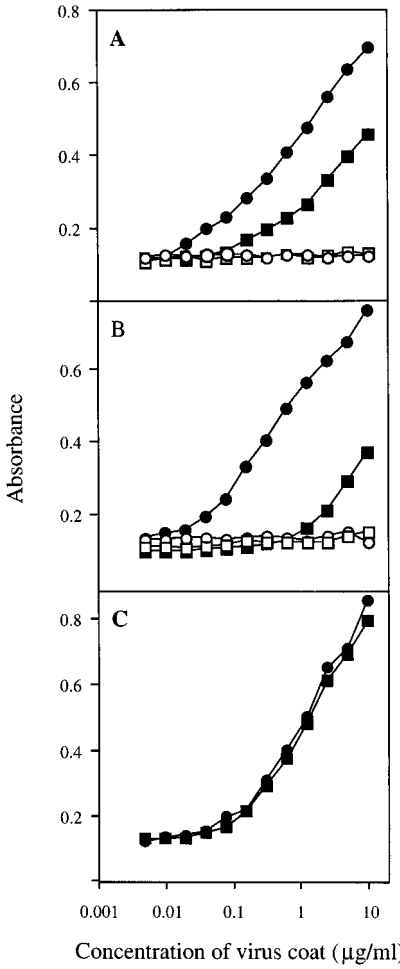

FIG. 2. (A and B) Binding of mouse serum MBL (A) and rat SP-D (B) to purified HKx31 (■, □) and BJx109 (●, ○). Mouse serum (1/100) or rat SP-D (0.5 μg/ml) in 50 mM $CaCl_2$ (solid symbols) or 5 mM EDTA (open symbols) were applied to virus-coated wells, and lectin binding was detected by ELISA. (C) Equivalent coating levels of the two viruses was confirmed by using MAb 165, which binds to the host-derived carbohydrate antigen characteristic of egg-grown influenza viruses.

of both lectins than did HKx31; as expected from the Ca^{2+} dependence of these lectins, binding was abolished in the presence of EDTA. Binding of MAb 165 to the two viruses was identical and served to confirm that there was equal coating of the two viruses on the wells. The oligosaccharide side chains that carry host antigen specificity on HA are located at residues 8 and 22 of HA1 and residue 154 of HA2 (7, 52), and these glycosylation sites are common to all H3 subtype viruses.

To determine to which of the viral glycoproteins the lectins bind, HANA preparations of the two viruses were resolved by SDS-PAGE under nonreducing conditions and probed in a Western blot with mouse serum MBL, rat SP-D, or antibodies to HA or NA (Fig. 3). Under these conditions, the HA runs as a series of bands corresponding to monomers, dimers, trimers and higher molecular forms of the 80-kDa molecule and the NA runs as a single major band of approximately 240 kDa, corresponding to the tetramer. The two murine lectins, MBL and SP-D, gave similar patterns of binding on each HANA preparation, binding to both the HA and NA molecules of each virus; binding was abolished in the presence of 5 mM EDTA or 100 mM D-mannose (data not shown). With equal

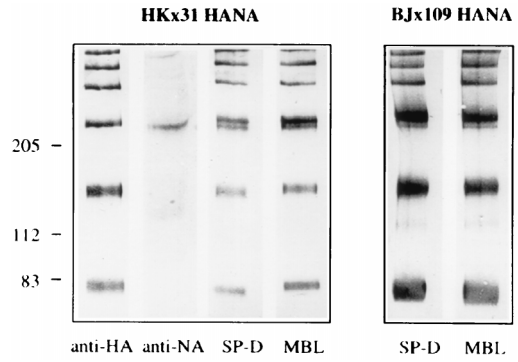

FIG. 3. Western blot showing binding of collectins to the HA and NA glycoproteins of influenza virus. HANA glycoprotein preparations from HKx31 and BJx109 viruses were resolved by SDS-PAGE under nonreducing conditions and probed with specific antibodies or collectins as described in Materials and Methods. The two HANA preparations were examined in the same experiment and under identical conditions of electrophoresis, transfer, and development of the blots; 0.8 μg of HANA was used in all lanes except lane 1, which received 0.4 μg.

loading of the two HANA preparations onto the gel, the lectins gave a stronger signal on BJx109 HA than on HKx31 HA whereas there was no apparent difference between signals on the NA molecules. Taken together, the binding data indicate that the additional glycosylation sites on BJx109 HA confer enhanced binding of the lectins and lead to greater sensitivity of the virus to neutralization in vitro.

Growth of influenza viruses in the respiratory tracts of mice. If collectins are an important component of host defense against influenza virus in vivo, virus strains that differ in their collectin sensitivity might be expected to differ also in their ability to infect the respiratory tract. The infectivity of HKx31 and BJx109 viruses for mice was therefore compared. Mice were inoculated intranasally with 10^4 PFU of virus under light anesthesia, and the titers of virus present in lung and tracheal extracts and in nasopharyngeal washings at 1 and 3 days postinfection were determined by the plaque assay. As shown in Fig. 4, HKx31 was recovered at high titer from all three sites whereas the recovery of BJx109 virus was very low, barely above the limit of detection for each sample.

The apparent link between lectin sensitivity in vitro and failure to grow in mouse lungs was examined further by testing

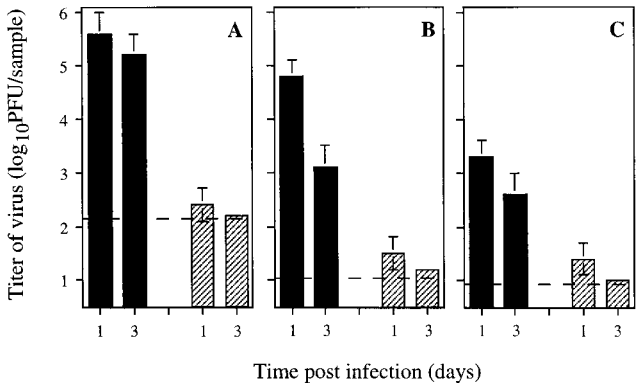

FIG. 4. Replication of HKx31 (■) and BJx109 (▨) viruses in the respiratory tracts of mice. Mice were inoculated intranasally under light anesthesia with 10^4 PFU of virus. Infectious virus present in lungs (A), tracheas (B), and nasal washings (C) was determined on days 1 and 3 postinfection. Virus titers shown are the means (±1 standard error) for five mice. The dashed line represents the lower limit of detection of virus in each sample.

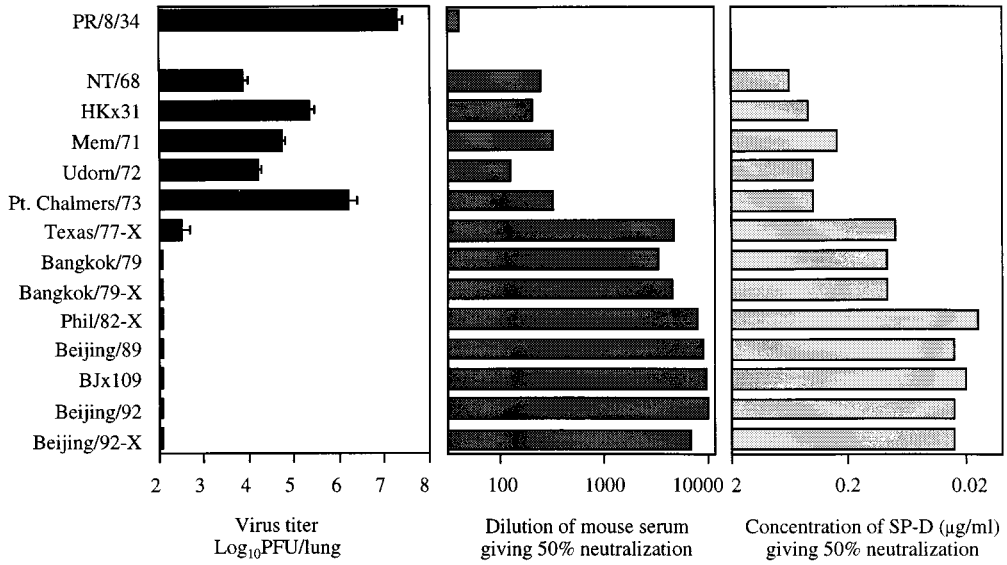

FIG. 5. Relationship between the sensitivity of H3 subtype influenza viruses to neutralization by mouse serum MBP and rat SP-D in vitro and their ability to replicate in the lungs of mice. Virus titers represent the mean lung titers (± 1 standard error) for five mice 3 days after intranasal inoculation of 10^4 PFU of virus. The neutralization titers were determined at least twice for each virus, with similar results, and the data from a single experiment with each lectin are shown.

a number of other virus strains of the H3 subtype. These included wild-type isolates as well as high-growth reassortants, like HKx31 and BJx109, in which some or all of the genes for internal components were derived from A/PR/8/34 (H1N1), a highly mouse-adapted virus (27). The number and location of potential glycosylation sites on the HA molecules of these viruses are shown in Table 1. A marked difference was observed between viruses isolated from 1968 to 1973 and those isolated from 1977 to 1992 (Fig. 5). The latter strains, whether wild type or reassortant, replicated very poorly, if at all, in the lungs at the inoculum given (10^4 PFU/mouse) and were highly sensitive to neutralization by the mouse serum lectin and rat SP-D in vitro. In contrast, the group of viruses isolated from 1968 to 1973 grew moderately to very well in the lungs and were 10- to 20-fold less sensitive to neutralization by the lectins in vitro than the viruses isolated later. There was thus a strong inverse correlation between the lectin sensitivity of H3 subtype influenza viruses and their replication in the lungs. Also shown in Fig. 5 are data for A/PR8 (H1N1) virus, which grows to a very high titer in mouse lungs and is markedly resistant to neutralization by both the serum and lung lectins.

Effect of saccharides on growth of influenza virus in the lungs. The data presented above strongly suggested that growth of the more highly glycosylated H3 virus strains in mouse lungs was limited by the sensitivity of the strains to serum- or lung-associated lectins. To test this hypothesis, we examined whether the inclusion of a ligand of the collectins in the virus inoculum would allow virus growth. As shown in Fig. 6B, growth of BJx109 in the lungs was greatly enhanced by the inclusion of mannan (10 mg/ml) or α-methyl-D-mannoside (100 mM) in the virus inoculum. The saccharide had to be present at the time of infection; no increase in virus recovery was observed if the lungs from mice infected in the absence of mannan were extracted into mannan-containing medium at the time of harvest (data not shown). Growth of HKx31 virus in the lungs was also enhanced by the presence of the saccharides but to a lesser extent (Fig. 6A), consistent with the observed lower sensitivity of this virus to the lectins in vitro. On the other hand, growth of A/PR8 virus was not enhanced by mannan, whether the virus was given at 10^4 PFU per mouse ($\log_{10}$ virus titers in the lung after 24 h: without mannan, 6.22 ± 0.19 PFU per lung; with mannan, 6.16 ± 0.26 PFU per lung) or at a low inoculum of 3×10^2 PFU per mouse (without mannan, 5.04 ± 0.09 PFU per lung; with mannan, 4.85 ± 0.11 PFU per lung). The extent of saccharide-mediated enhancement of viral replication thus correlates directly with the collectin sensitivity of the virus. These observations argue against a general nonspecific effect of the saccharides on innate immunity and provide strong support for the existence of a lectin-based system of host defense operating in the lungs.

Effect of collectins on viral NA activity. In addition to mediating neutralization of virus infectivity, it was of interest to determine whether binding of collectins to influenza virus particles affected the activity of the viral NA. As shown in Fig. 7, NA activity was inhibited by SP-D, with the inhibition being abolished in the presence of D-mannose. Interestingly, BJx109 NA was inhibited more strongly than was HKx31 NA, even though binding of SP-D to the two NA molecules appeared to be equivalent in lectin blots (Fig. 3). This suggests that inhibition of NA activity may occur at least in part through steric hindrance of the NA active site by SP-D bound to neighboring HA molecules on the virus particle.

Inhibition of NA activity by mouse serum was also observed but was only partially reversed by mannose (data not shown); the residual inhibition presumably reflects the activity of α-type sialylated inhibitors present in normal serum.

Collectin sensitivity of lung-grown virus. The carbohydrate structures present on viral glycoproteins are characteristic of the particular host cell in which the virus was grown, and in all the experiments described to date, the stocks of influenza virus were grown in eggs. In natural infection, however, the viral carbohydrate would have been derived from growth of the virus in the respiratory epithelium of the previous host. We therefore examined whether the murine collectins were also able to neutralize virus that had been propagated in mouse lungs. For BJx109, the lung-grown virus stocks were prepared by including mannan in the inoculum as described above and semipurifying the resultant virus away from mannan by cen-

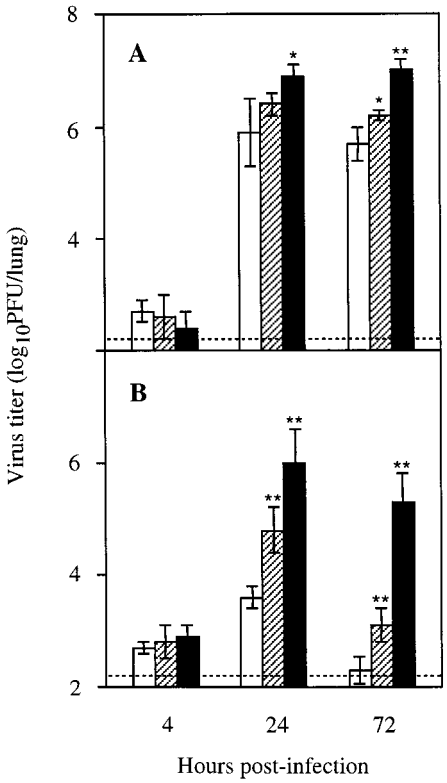

FIG. 6. Effect of saccharides on the growth of influenza virus in the lungs. Groups of four mice were infected intranasally with 10^4 PFU of HKx31 (A) or 10^5 PFU of BJx109 (B) diluted in 50 μl of PBS (□) or PBS supplemented with either 100 mM α-methyl-D-mannoside (▨) or 10 mg of mannan per ml (■). Lung virus titers were determined at 4, 24, and 72 h postinfection. The dotted line represents the lower limit of detection of virus in the sample. Columns marked with an asterisk indicate lung virus titers significantly different from those of mice receiving virus in PBS alone. *, $P < 0.05$; **, $P < 0.01$.

trifugation as described in Materials and Methods. As shown in Fig. 8, the lung-grown viruses were less sensitive than the egg-grown virus preparations (compare with Fig. 1); however, lung-grown HKx31 and BJx109 viruses were still neutralized by mouse serum MBL and rat SP-D, and the hierarchy in the sensitivity of the two viruses was maintained. Furthermore, rat SP-D displayed similar activity against a stock of HKx31 virus that had been propagated in the lungs of 3-week-old Wistar rats to that against the mouse lung-grown stock (data not shown). Both SP-D and serum MBL therefore recognize influenza virus grown in cells of the homologous host.

SP-D and MBL levels in BAL fluid and nasal washings. To gain insight into the relative importance of SP-D and serum MBL in the defense of the respiratory tract against influenza virus, we assayed nasopharyngeal washings and BAL fluids from uninfected mice by ELISA for the presence of these two collectins; we also examined BAL fluids taken from mice infected 1 or 3 days previously with 10^5 PFU of BJx109, which replicates only very poorly in the lungs, or 10^4 PFU of PR/8 virus, which is highly virulent for mice and replicates to high titer. As shown in Table 2, SP-D was readily detectable in the BAL fluids of uninfected mice, as expected, and the levels increased on infection with either virus. SP-D was also detected in the nasal washings of uninfected mice (Table 2). In contrast, MBL was not detected in nasal washings or in the BAL fluids of uninfected or infected mice, except for BAL fluid samples taken 3 days after infection with PR/8 virus. The

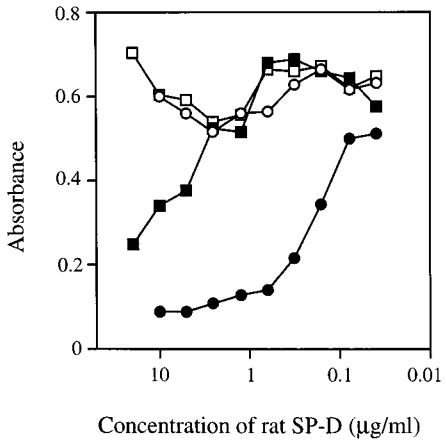

FIG. 7. Inhibition of influenza virus NA activity by SP-D. HKx31 (■, □) and BJx109 (●, ○) viruses were mixed with rat SP-D in the absence (solid symbols) or presence (open symbols) of 100 mM D-mannose and assayed for NA activity as described in Materials and Methods. The dose of virus used was chosen such that the two virus preparations displayed equivalent NA activity in the absence of SP-D (final dilution of allantoic fluid: HKx31, 1/45; BJx109, 1/16).

MBL present in these samples (103 ± 52 ng/ml) was not due to the presence of contaminating blood; all samples were monitored by erythrocyte count, and the maximum blood contamination found was 0.01% (vol/vol), which would contribute an MBL concentration of only 5 ng/ml. We conclude that MBL is not normally present in the alveolar spaces or nasal passages, but transudation of MBL into the lungs can occur by day 3 under the conditions of inflammation that occur on infection with PR/8 virus. In contrast, SP-D is present in both the upper and lower respiratory tracts of normal mice.

DISCUSSION

The results of this study point to an important role for collectins in vivo in host defense of the lungs against influenza virus. A marked inverse relationship was observed between the collectin sensitivity of H3N2 subtype influenza A viruses in vitro and their ability to replicate in the respiratory tracts of mice. Strains of virus isolated after 1977 were particularly sensitive to both SP-D and MBL and grew poorly in mouse lungs, but their growth was greatly enhanced in the presence of

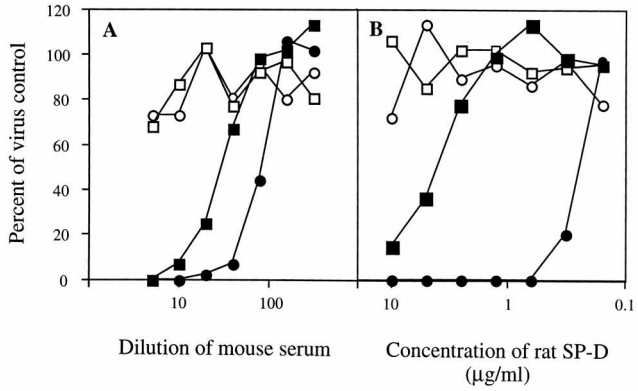

FIG. 8. Neutralization of lung-grown HKx31 (■, □) and BJx109 (●, ○) virus by mouse serum MBL (A) and rat SP-D (B). Lung-grown virus was added to mouse serum or rat SP-D in CFT (solid symbols) or CFT supplemented with α-methyl-D-mannoside to give a final concentration of 100 mM (open symbols).

TABLE 2. Concentration of SP-D and MBL in nasopharyngeal washings and BAL fluids of uninfected and influenza virus-infected mice[a]

Collectin	Collectin concn (ng/ml)[b] in:					
	Nasal washings (uninfected)[c]	BAL fluid				
		Uninfected[d]	BJx109 infected		PR/8 infected	
			Day 1[e]	Day 3[e]	Day 1[e]	Day 3[e]
SP-D	56	552 ± 174	1,261 ± 375[f]	2,937 ± 554[f]	1,430 ± 469[f]	1,506 ± 223[f]
MBL	<10	<10	<10	<10	<10	103 ± 52[f]

[a] Collectins were assayed by ELISA on mannan-coated wells with recombinant rat SP-D and normal mouse serum as standards. Mouse serum was assumed to contain 50 μg of MBL per ml (22). Samples contained <0.01% blood contamination as determined by erythrocyte count in a hemocytometer.
[b] Concentrations are expressed as mean ± 1 standard error.
[c] Pooled nasal washings from five mice.
[d] $n = 7$.
[e] $n = 4$.
[f] Significantly different from levels in uninfected mice ($P < 0.01$).

the saccharide mannan or α-methylmannoside. These and other observations discussed below strongly suggest that growth of virus in normal lungs is limited by collectins or related lectin-like molecules, with SP-D playing a major role.

The sensitivity of influenza virus strains to the collectins SP-D and MBL was related to the level of glycosylation of the HA molecule, particularly of its globular head. Early isolates (1968 to 1972), which show the lowest sensitivity and grow well to moderately well in the lungs, carry seven potential glycosylation sites, all of which are known to be glycosylated in HKx31 (53). Of these, only two (at residues 81 and 165) are located on the head of the HA molecule. The remaining sites, at residues 8, 22, 38, and 285 of HA1 and 154 of HA2, are located on the HA stalk (55), and carbohydrate at these sites may be less accessible to collectin binding than that on the head of the molecule. We have shown previously that the high-mannose glycan at residue 165 is critical for the sensitivity of H3 subtype viruses to bovine conglutinin, rat SP-D, and the MBP-like lectins in guinea pig and mouse serum (1, 15, 38). This site is conserved in all H3 subtype viruses, as are the glycosylation sites on the HA stalk. The later isolates, which show increased sensitivity to SP-D and MBL and grow poorly in the lungs, have all acquired additional glycosylation sites on the head of the HA molecule (Table 1). Increased sensitivity cosegregates with the presence of a glycosylation site at residue 126, and additional sites at 144 and/or 246 appear to further enhance sensitivity, particularly to SP-D. In contrast, the Mt. Sinai strain of A/PR/8 (H1N1) virus, which is highly virulent and largely resistant to SP-D and MBL (Fig. 5), does not carry any glycosylation sites on the head of HA (8).

Lectin blotting of the viral glycoproteins of HKx31 and BJx109 viruses confirmed a greater level of binding of SP-D and MBL to the more highly glycosylated BJx109 HA and showed binding of both collectins to the NA as well. This extends the findings of Malhotra et al. (30), who were the first to describe the binding of collectins (human MBP and SP-A) to NA, although binding to HA was not detected in that study, for reasons that are not clear. Binding of SP-D was shown to inhibit the NA enzymatic activity of influenza virus virions; interestingly, BJx109 NA was inhibited more strongly than HKx31 NA, even though binding of SP-D to NA of the two strains appeared comparable in lectin blots. This suggests that inhibition of NA activity may result from steric hindrance of the NA active site by SP-D bound to neighboring HA molecules on the virion. Alternatively, inhibition may result from direct binding of SP-D to NA, with the difference in sensitivity to inhibition reflecting differences in fine specificity and/or affinity of the NA of the two viruses for the fetuin substrate. Differences in substrate specificity of the NA of early and late H3N2 strains of virus have been documented (3). Further experiments are required to determine the mechanism of inhibition of influenza virus NA by SP-D.

In addition to differences in collectin sensitivity shown here, previous studies have shown that glycosylation of the HA molecule can modulate other properties of the HA that may affect the virulence of influenza viruses. These include changes in the receptor-binding specificity of HA (11) and possibly in the susceptibility of HA to cleavage activation (54). Neither of these potential effects accounts for the low infectivity of the late H3 influenza viruses for mice in the present system, however, given the marked enhancement of infectivity observed in the presence of mannose-containing saccharides. This latter result would not be expected if the lower infectivity were due to a poor fit of the HA molecule to sialylated receptors on mouse cells or to blocking of access of a cellular protease to the cleavage site on HA by oligosaccharide side chains. We conclude that increased sensitivity to collectins conferred by high levels of glycosylation of HA represents a major factor in the low infectivity of late H3 influenza viruses for mice.

Collectins may interfere with influenza virus infection in vivo in a number of ways. Neutralization of virus infectivity by SP-D may result from direct blocking of virus attachment or entry into host cells or from the formation of viral aggregates as described by Hartshorn et al. (16, 17), leading to a reduction in the number of infectious virus units and to enhanced clearance by mucociliary and phagocytic mechanisms. Since antibody specific for viral NA inhibits the release of newly formed virus particles from the surface of infected cells (26) and markedly inhibits influenza virus replication in mouse lungs (43), the inhibition of viral NA by SP-D represents another mechanism whereby SP-D may act in vivo against influenza virus infection. MBL together with complement mediates irreversible inactivation of viral infectivity (2) (Fig. 1) and lysis of virus-infected cells (38). Furthermore, MBL and SP-D have both been shown to function as opsonins for influenza virus, promoting the activation of neutrophils and at the same time protecting against virus-induced depression of neutrophil function (16, 17), which may be important in protection against bacterial superinfection.

In host defense against influenza virus, SP-D and MBL may play distinct but complementary roles. SP-D, produced by alveolar type II pneumocytes and nonciliated bronchiolar cells (Clara cells) (10) and cells of the tracheal epithelium (56), is present in the trachea, bronchioles, and alveolar spaces of normal mice; furthermore, the concentration of SP-D in BAL fluids increased rapidly on influenza virus infection. SP-D is

thus available to act immediately against the infecting virus and during the early rounds of viral replication. (We confirmed in experiments with lung-grown HKx31 virus that both SP-D and MBL do in fact recognize virus that has been propagated in the respiratory epithelium of the homologous host, and not egg-grown virus only.) SP-D was also detected in nasopharyngeal washings of normal mice and so may contribute to protection at this site as well. The finding that BJx109 virus replicated poorly in the nose as well as the lungs of mice is consistent with this notion. MBL was not present in lung or nasal washings from normal mice but could be detected in lung washings of mice infected with A/PR/8 virus 3 days previously. Its presence there was not due to hemorrhage into the lungs and presumably reflects transudation of MBL from the blood due to the increased permeability of lung capillaries that accompanies pulmonary inflammation. MBL may thus be important in preventing the spread of influenza virus into the bloodstream and could contribute directly to the defense of the lungs later in infection, depending on the sensitivity of the infecting virus.

Lung surfactant contains another collectin, SP-A, which may also contribute to host defense against influenza virus infection. However, although binding of SP-A to influenza virus through its lectin domain has been reported (30), the neutralizing and opsonizing activities of SP-A for influenza virus were shown to be mediated through binding of the viral HA to sialic acid on SP-A (4, 5). Given the saccharide-mediated enhancement of viral growth in the lungs that we observed in this study, it is unlikely that direct neutralization or opsonization of influenza virus by SP-A is playing any protective role in this system. Other activities of SP-A against influenza virus that involve the lectin domain and are inhibitable by saccharides cannot be excluded, however. The interaction of influenza virus with other lectin-like molecules, for example the macrophage mannose receptor (39), may also be important in host defense against this virus.

In the process of antigenic drift, the acquisition of new glycosylation sites on the HA molecule and consequent masking of antigenic epitopes represents one mechanism whereby influenza virus escapes from preexisting antibody in the population (45). If in humans, as in mice, the additional carbohydrate on HA increases the sensitivity of the virus to collectins, the virulence of the virus may ultimately be compromised. Genetic or physiological differences in the expression of SP-D or MBL may then be a factor in individual susceptibility to influenza virus, particularly to the more highly glycosylated strains currently circulating. Of interest is a recent report that SP-D and SP-A levels are reduced in smokers (23).

Recent data on excess mortality from pneumonia and influenza for the U.S. winter seasons from 1972 to 1973 to 1991 to 1992 do not provide any indication that the more recent H3N2 strains are less virulent than earlier strains (44); however, the effects might be more subtle than would be required to influence excess mortality figures. Furthermore, if lectin-mediated defenses decline in the elderly, an effect of glycosylation on virulence could be masked. Recently, Pyhälä et al. (37) reported the loss of potential glycosylation sites (not associated with egg adaptation) at residues 246 and 278 of the HA of certain H3N2 field strains isolated in Finland in 1994 to 1995. Loss of glycosylation sites from HA was also found among H1N1 strains isolated in Finland and elsewhere in Europe in the period 1988 to 1993 (36) and among H1N1 strains isolated in 1983 to 1984 from specimens collected during persistent infection of an immunodeficient child (40). We have found H1N1 subtype viruses isolated after 1977 to be very sensitive to collectins, like the later H3N2 isolates. While it is possible that the loss of carbohydrate reported in these studies confers other advantages to the virus, for example, an improvement in receptor-binding properties, the observations are also consistent with the existence of a fine balance between protection from antibody and increased sensitivity to collectins afforded by high levels of glycosylation of the HA molecule. If this is so, there may be a limit to the amount of glycosylation that influenza viruses can carry while remaining virulent for humans.

ACKNOWLEDGMENTS

This work was supported by grant 940742 from the National Health and Medical Research Council of Australia (to E.M.A.) and NIH grant HL-29594 (to E.C.C.).

We thank Sharon Feigl for technical assistance and Donald Chang for assistance with preparing the recombinant SP-D.

REFERENCES

1. **Anders, E. M., C. A. Hartley, and D. C. Jackson.** 1990. Bovine and mouse serum β inhibitors of influenza A viruses are mannose-binding lectins. Proc. Natl. Acad. Sci. USA **87:**4485–4489.
2. **Anders, E. M., C. A. Hartley, P. C. Reading, and R. A. B. Ezekowitz.** 1994. Complement-dependent neutralization of influenza virus by a serum mannose-binding lectin. J. Gen. Virol. **75:**615–622.
3. **Baum, L. G., and J. C. Paulson.** 1991. The N2 neuraminidase of human influenza virus has acquired a substrate specificity complementary to the hemagglutinin receptor specificity. Virology **180:**10–15.
4. **Benne, C. A., B. Benaissa-Trouw, J. A. G. van Strijp, C. Kraaijeveld, and J. F. van Iwaarden.** 1997. Surfactant protein A, but not surfactant protein D, is an opsonin for influenza A virus phagocytosis by rat alveolar macrophages. Eur. J. Immunol. **27:**886–890.
5. **Benne, C. A., C. A. Kraaijeveld, J. A. G. van Strijp, E. Brouwer, M. Harmsen, J. Verhoef, L. M. G. van Golde, and J. F. van Iwaarden.** 1995. Interactions of surfactant protein A with influenza viruses: binding and neutralization. J. Infect. Dis. **171:**335–341.
6. **Both, G. W., M. J. Sleigh, N. J. Cox, and A. P. Kendal.** 1983. Antigenic drift in influenza virus H3 hemagglutinin from 1968 to 1980: Multiple evolutionary pathways and sequential amino acid changes at key antigenic sites. J. Virol. **48:**52–60.
7. **Brown, L. E., C. W. Ward, and D. C. Jackson.** 1982. Antigenic determinants of influenza virus hemagglutinin. IX. The carbohydrate side chains from an Asian strain. Mol. Immunol. **19:**329–338.
8. **Caton, A. J., G. G. Brownlee, J. W. Yewdell, and W. Gerhard.** 1982. The antigenic structure of the influenza virus A/PR/8/34 hemagglutinin (H1 subtype). Cell **31:**417–427.
9. **Crouch, E., D. Chang, K. Rust, A. Persson, and J. Heuser.** 1994. Recombinant pulmonary surfactant protein D. J. Biol. Chem. **269:**15808–15813.
10. **Crouch, E., D. Parghi, S.-F. Kuan, and A. Persson.** 1992. Surfactant protein D: subcellular localization in nonciliated bronchiolar epithelial cells. Am. Physiol. Soc. **263:**L61–L66.
11. **Deom, C. M., A. J. Caton, and I. T. Schulze.** 1986. Host cell-mediated selection of a mutant influenza A virus that has lost a complex oligosaccharide from the tip of the hemagglutinin. Proc. Natl. Acad. Sci. USA **83:**3771–3775.
12. **Ezekowitz, R. A. B., M. Kuhlman, J. E. Groopman, and R. A. Byrn.** 1989. A human serum mannose-binding protein inhibits in vitro infection by the human immunodeficiency virus. J. Exp. Med. **169:**185–196.
13. **Fischer, P. B., S. Ellerman-Eriksen, S. Thiel, J. C. Jensenius, and S. C. Mogensen.** 1994. Mannan-binding protein and bovine conglutinin mediate enhancement of herpes simplex virus type 2 infection in mice. Scand. J. Immunol. **39:**439–445.
14. **Hartley, C. A., D. C. Jackson, and E. M. Anders.** 1992. Two distinct serum mannose-binding lectins function as β inhibitors of influenza virus: identification of bovine serum β inhibitor as conglutinin. J. Virol. **66:**4358–4363.
15. **Hartley, C. A., P. C. Reading, A. C. Ward, and E. M. Anders.** 1997. Changes in the hemagglutinin molecule of influenza type A (H3N2) virus associated with increased virulence for mice. Arch. Virol. **142:**75–88.
16. **Hartshorn, K. L., E. C. Crouch, M. R. White, P. Eggleton, A. I. Tauber, D. Chang, and K. Sastry.** 1994. Evidence for a protective role of pulmonary surfactant protein D (SP-D) against influenza A viruses. J. Clin. Invest. **94:**311–319.
17. **Hartshorn, K. L., K. B. M. Reid, M. R. White, J. C. Jensenius, S. M. Morris, A. I. Tauber, and E. C. Crouch.** 1996. Neutrophil deactivation by influenza A viruses: mechanisms of protection after opsonization with collectins and hemagglutination-inhibiting antibodies. Blood **87:**3450–3461.
18. **Hartshorn, K. L., K. Sastry, D. Brown, M. R. White, T. B. Okarma, Y.-M. Lee, and A. I. Tauber.** 1993. Conglutinin acts as an opsonin for influenza A viruses. J. Immunol. **151:**6265–6273.
19. **Hartshorn, K. L., K. Sastry, M. R. White, E. M. Anders, M. Super, R. A. B. Ezekowitz, and A. I. Tauber.** 1993. Human mannose-binding protein func-

tions as an opsonin for influenza A viruses. J. Clin. Invest. **91:**1414–1420.
20. **Haurum, J. S., S. Thiel, I. M. Jones, P. B. Fischer, S. B. Laursen, and J. C. Jensenius.** 1993. Complement activation upon binding of mannan-binding protein to HIV envelope glycoproteins. AIDS **7:**1307–1313.
21. **Holmskov, U., R. Malhotra, R. B. Sim, and J. Jensenius.** 1994. Collectins: collagenous C-type lectins of the innate immune defense system. Immunol. Today **15:**67–74.
22. **Holt, P., U. Holmskov, S. Thiel, B. Teisner, P. Højrup, and J. C. Jensenius.** 1994. Purification and characterization of mannan-binding protein from mouse serum. Scand. J. Immunol. **39:**202–208.
23. **Honda, Y., H. Takahashi, Y. Kuroki, T. Akino, and S. Abe.** 1996. Decreased contents of surfactant proteins A and D in BAL fluids of healthy smokers. Chest **109:**1006–1009.
24. **Hoppe, H.-J., and K. B. M. Reid.** 1994. Collectins—soluble proteins containing collagenous regions and lectin domains—and their role in innate immunity. Protein Sci. **3:**1143–1158.
25. **Kilbourne, E. D.** 1969. Future influenza vaccines and the use of genetic recombinants. Bull. W. H. O. **41:**643–645.
26. **Kilbourne, E. D., W. G. Laver, J. L. Schulman, and R. G. Webster.** 1968. Antiviral activity of antiserum specific for an influenza virus neuraminidase. J. Virol. **2:**281–288.
27. **Kilbourne, E. D., J. L. Schulman, G. C. Schild, G. Schloer, J. Swanson, and D. Bucher.** 1971. Correlated studies of a recombinant influenza-virus vaccine. I. Derivation and characterization of virus and vaccine. J. Infect. Dis. **124:**449–462.
28. **Kuan, S. F., K. F. Rust, and E. Crouch.** 1992. Interactions of surfactant protein D with bacterial lipopolysaccharides. Surfactant protein D is an Escherichia coli-binding protein in bronchoalveolar lavage. J. Clin. Invest. **90:**97–106.
29. **Lambré, C. R., H. Terzidis, A. Greffard, and R. G. Webster.** 1990. Measurement of anti-influenza neuraminidase antibody using a peroxidase-linked lectin and microtiter plates coated with natural substrates. J. Immunol. Methods **135:**49–57.
30. **Malhotra, R., J. S. Haurum, S. Thiel, and R. B. Sim.** 1994. Binding of human collectins (SP-A and MBP) to influenza virus. Biochem. J. **304:**455–461.
31. **Malhotra, R., S. Thiel, K. B. M. Reid, and R. B. Sim.** 1990. Human leukocyte C1q receptor binds other soluble proteins with collagen domains. J. Exp. Med. **172:**955–959.
32. **Muster, T., B. Ferko, A. Klima, M. Purtscher, A. Trkola, P. Schulz, A. Grassauer, O. G. Engelhardt, A. Garcia-Sastre, P. Palese, and H. Katinger.** 1995. Mucosal model of immunization against human immunodeficiency virus type 1 with a chimeric influenza virus. J. Virol. **69:**6678–6686.
33. **Naeve, C. W., V. S. Hinshaw, and R. G. Webster.** 1984. Mutations in the hemagglutinin receptor-binding site can change the biological properties of an influenza virus. J. Virol. **51:**567–569.
34. **Nakajima, S., Y. Takeuchi, and K. Nakajima.** 1988. Location on the evolutionary tree of influenza H3 haemagglutinin genes of Japanese strains isolated during 1985-6 season. Epidemiol. Infect. **100:**301–310.
35. **Newton, S. E., G. M. Air, R. G. Webster, and W. G. Laver.** 1983. Sequence of the hemagglutinin gene of influenza virus A/Memphis/1/71 and previously uncharacterized monoclonal antibody-derived variants. Virology **128:**495–501.
36. **Pyhälä, R., N. Ikonen, T. Forsten, S. Alanko, and L. Kinnunen.** 1995. Evolution of the HA1 domain of human influenza A (H1N1) virus: loss of glycosylation sites and occurrence of herald and conserved strains. J. Gen. Virol. **76:**205–210.
37. **Pyhälä, R., N. Ikonen, M. Haanpää, and L. Kinnunen.** 1996. HA1 domain of influenza A (H3N2) viruses in Finland in 1989-1995: evolution, egg-adaptation and relationship to vaccine strains. Arch. Virol. **141:**1033–1046.
38. **Reading, P. C., C. A. Hartley, R. A. B. Ezekowitz, and E. M. Anders.** 1995. A serum mannose-binding lectin mediates complement-dependent lysis of influenza virus-infected cells. Biochem. Biophys. Res. Commun. **217:**1128–1136.
39. **Reading, P. C., J. L. Miller, and E. M. Anders.** Unpublished data.
40. **Rocha, E., N. J. Cox, R. A. Black, M. W. Harmon, C. J. Harrison, and A. P. Kendal.** 1991. Antigenic and genetic variation in influenza A (H1N1) virus isolates recovered from a persistently infected immunodeficient child. J. Virol. **65:**2340–2350.
41. **Rocha, E. P., X. Xu, H. E. Hall, J. R. Allen, H. L. Regnery, and N. J. Cox.** 1993. Comparison of 10 influenza A (H1N1 and H3N2) haemagglutinin sequences obtained directly from clinical specimens to those of MDCK cell- and egg-grown viruses. J. Gen. Virol. **74:**2513–2518.
42. **Sastry, K., K. Zahedi, J.-M. Lelias, A. S. Whitehead, and R. A. B. Ezekowitz.** 1991. Molecular characterization of the mouse mannose-binding proteins. The mannose-binding protein A but not C is an acute phase reactant. J. Immunol. **147:**692–697.
43. **Schulman, J. L.** 1969. The role of antineuraminidase antibody in immunity to influenza virus infection. Bull. W.H.O. **41:**647–650.
44. **Simonsen, L., M. J. Clark, G. D. Williamson, D. F. Stroup, N. H. Arden, and L. B. Schonberger.** The impact of influenza epidemics on mortality: Introducing a severity index. Am. J. Public Health, in press.
45. **Skehel, J. J., D. J. Stevens, R. S. Daniels, A. R. Douglas, M. Knossow, I. A. Wilson, and D. C. Wiley.** 1984. A carbohydrate side chain on hemagglutinins of Hong Kong influenza viruses inhibits recognition by a monoclonal antibody. Proc. Natl. Acad. Sci. USA **81:**1779–1783.
46. **Sumiya, M., M. Super, T. Tabona, R. J. Levinsky, T. Arai, M. W. Turner, and J. A. Summerfield.** 1991. Molecular basis of opsonic defect in immunodeficient children. Lancet **337:**1569–1570.
47. **Summerfield, J. A.** 1995. Mannose binding protein gene mutations associated with unusual and severe infections in adults. Lancet **345:**886–889.
48. **Super, M., S. Thiel, J. Lu, R. J. Levinsky, and M. W. Turner.** 1989. Association of low levels of a mannan-binding protein with a common defect of opsonisation. Lancet **ii:**1236–1239.
49. **Thiel, S., and K. B. M. Reid.** 1989. Structures and functions associated with the group of mammalian lectins containing collagen-like sequences. FEBS Lett. **250:**78–84.
50. **Turner, M. W.** 1996. Mannose-binding lectin: the pluripotent molecule of the innate immune system. Immunol. Today **17:**532–540.
51. **Verhoeyen, M., R. Fang, W. Min Jou, R. Devos, D. Huylebroeck, E. Saman, and W. Fiers.** 1980. Antigenic drift between the haemagglutinin of the Hong Kong influenza strains A/Aichi/2/68 and A/Victoria/3/75. Nature (London) **286:**771–776.
52. **Ward, C. W., L. E. Brown, J. C. Downie, and D. C. Jackson.** 1981. Antigenic determinants of influenza virus hemagglutinin. VII. The carbohydrate side chains of A/Memphis/102/72 hemagglutinin heavy chain which cross-react with host antigen. Virology **108:**71–79.
53. **Ward, C. W., and T. A. Dopheide.** 1981. Amino acid sequence and oligosaccharide distribution of the hemagglutinin from an early Hong Kong influenza virus variant A/Aichi/2/68 (X-31). Biochem. J. **193:**953–962.
54. **Webster, R. G., Y. Kawaoka, and W. J. Bean.** 1986. Molecular changes in A/chicken/Pennsylvania/83 (H5N2) influenza virus associated with acquisition of virulence. Virology **149:**165–173.
55. **Wilson, I. A., J. J. Skehel, and D. C. Wiley.** 1981. The haemagglutinin membrane glycoprotein of influenza virus: structure at 3 Å resolution. Nature **289:**366–373.
56. **Wong, C. J., J. Akiyama, L. Allen, and S. Hawgood.** 1996. Localization and developmental expression of surfactant proteins D and A in the respiratory tract of the mouse. Pediatr. Res. **39:**930–937.