Biochemical and Biophysical Research Communications **286,** 206–213 (2001)
doi:10.1006/bbrc.2001.5373, available online at http://www.idealibrary.com on IDEAL®

Increased Antiviral and Opsonic Activity of a Highly Multimerized Collectin Chimera

Mitchell R. White,* Erika Crouch,† Donald Chang,† and Kevan L. Hartshorn*,[1]
**Department of Medicine, Boston University School of Medicine, Boston, Massachusetts 02118; and*
†Department of Pathology, Washington University School of Medicine, St. Louis, Missouri 63110

Received July 11, 2001

Altering the carbohydrate binding properties of surfactant protein D (SP-D) [e.g., by replacing its carbohydrate recognition domain (CRD) with that of either mannose binding lectin (MBL) or conglutinin] can increase its activity against influenza A virus (IAV). The current study demonstrates that the degree of multimerization of SP-D is another independent determinant of antiviral activity. A chimeric collectin containing the N-terminus and collagen domain of human SP-D and the CRD of MBL formed high-molecular-weight multimers similar to those previously described for human SP-D. Using several complementary assays, and diverse viral strains, the chimeric multimers showed greater anti-IAV activity than similarly multimerized preparations of SP-D or incompletely oligomerized preparations of the chimera. More highly multimerized preparations of the chimera also caused greater increases in uptake of IAV by neutrophils. These studies may have implications for development of collectins as therapeutic agents and understanding of natural variations in susceptibility to IAV infection.

Key Words: **influenza virus; collectins; neutrophils; surfactant protein D; mannose binding lectin.**

The collectins are a group of collagenous lectins present in mammalian serum, and pulmonary and gastrointestinal secretions that participate in first line host defense against a variety of pathogens including bacteria, viruses, and yeast (1). The surfactant collectins, surfactant proteins A and D (SP-A and SP-D) appear to play an important role in initial defense against respiratory infections, including infection with IAVs (2–5).

[1] To whom correspondence and reprint requests should be addressed at Boston University School of Medicine, Evans Biomedical Research Center, 80 East Concord Street, Boston, MA 02118. Fax: 617-638-7530.

Our major goals have been to delineate the mechanisms through which SP-D neutralizes the infectivity of IAV and causes viral aggregation or promotes uptake of the virus by phagocytes, and to generate novel collectins with enhanced antiviral activities compared to the natural proteins. Furthermore, we have sought to develop molecules that have a broadened range of reactivity with various strains of influenza, particularly to strains which are resistant to the natural collectins.

SP-D has the greatest intrinsic antiviral activities against IAV of the wild-type human collectins. Modification of the carbohydrate recognition properties of SP-D can result in significant increase or decrease in anti-influenza activity. For instance, replacement of the CRD of SP-D with that of either MBL or bovine conglutinin results in increased IAV neutralizing and aggregating activity (6, 7). In contrast, reducing the affinity of SP-D for mannose through site-directed mutagenesis of the CRD reduces anti-influenza activity (8). Properties of the N-terminal and collagen domain of SP-D also appear to contribute to its anti-influenza activity. As with all collectins, the basic structural subunit of SP-D is a trimer. The N-terminal domain of SP-D contains cysteine residues required to stabilize higher order multimeric structures including dodecamers and higher molecular weight structures. Previous studies have also shown that dodecamers of SP-D (or of the SP-D/$MBL_{neck+CRD}$ chimera) have greater anti-influenza activity than trimeric preparations. However, a significant proportion of native or recombinant human SP-D is made up of higher molecular weight multimers. Whereas dodecamers contain 4 trimers (and hence four globular CRD domains) bound together at the N terminus, the higher order multimers can contain ~32 CRD domains.

In this paper we demonstrate that SP-D/$MBL_{neck+CRD}$ also forms higher order multimers. This indicates that the propensity to form higher order multimers is an intrinsic property of the N-terminal domains of human

0006-291X/01 $35.00

SP-D. Higher order multimers of human SP-D or of SP-D/MBL$_{neck+CRD}$ are shown to have greater anti-IAV activity than less multimerized SP-D or SP-D/MBL$_{neck+CRD}$ preparations. Furthermore, SP-D/MBL$_{neck+CRD}$ multimers had significantly greater antiviral activity based on a variety of assays than similarly a multimerized fraction of RhSP-D. Importantly, SP-D/MBL$_{neck+CRD}$ multimers had greater activity against collectin resistant strains than any collectin preparation described to date.

MATERIALS AND METHODS

Reagents. RPMI 1640, sodium citrate, dextran, trypan blue stain, Wright's Giemsa stain, horseradish peroxidase-Type II, and scopoletin were purchased from Sigma Chemical Co. (St. Louis, MO). Dulbecco's phosphate-buffered saline with (PBS++) and without (PBS) calcium and magnesium were purchased from GIBCO BRL (Grand Island, NY). Ficoll–Paque was obtained from Pharmacia Biotech (Piscataway, NJ).

Neutrophil preparation. Neutrophils from healthy volunteers were isolated to >95% purity by using dextran precipitation, followed by Ficoll–Paque gradient separation for the removal of mononuclear cells, and then hypotonic lysis to eliminate any contaminating erythrocytes, as previously described (9). Cell viability was determined to be >98% by trypan blue staining. The isolated neutrophils were resuspended at the appropriate concentrations in control buffer (PBS++) and used within 2 h.

Virus preparation. Influenza A virus was grown in the chorioallantoic fluid of ten day old chicken eggs and purified on a discontinuous sucrose gradient as previously described (9). Philippines 82/H3N2 (Phil82) and Brazil 78/H1N1 (Braz78) strains and their bovine serum β inhibitor resistant variants (Phil82/BS and Braz78/BS) were kindly provided by Dr. E. Margot Anders (University of Melbourne, Melbourne, Australia). Viral stocks contained $\sim 5 \times 10^8$ plaque-forming units/ml.

Collectin preparations. Recombinant human mannose binding lectin (RhMBL) was produced in murine Sp2 cells as described (10, 11). The RhMBL used was of the more common allelic variant (termed MBP$_G$). As previously demonstrated this RhMBL preparations is composed predominantly of multimers containing 5 or 6 trimers (i.e., octadecamers) in association (10). Recombinant human SP-D (RhSP-D) was produced in CHO-K1 cells as previously described (12). For these studies the high molecular weight multimeric fraction of RhSP-D was used. A chimeric collectin containing the human SP-D N-terminal and collagen domains and human MBL neck and CRD domains (called SP-D/MBL$_{neck+CRD}$) was constructed using a PCR based method and human SP-D and MBL cDNAs as previously described (7).

SP-D/MBL$_{neck+CRD}$ was purified from supernatants of CHO-K-1 cells using sequential mannose-affinity chromatography and gel filtration chromatography as previously described for RhSP-D (6, 12). The recovery of chimeric protein was approximately 2 μg/ml of conditioned medium. The majority of the recombinant protein eluted from A15M columns in the position of human SP-D dodecamers, but significant fractions eluted in the position of human SP-D trimers and high-molecular-weight multimers, as previously observed for RhSP-D (12). Figure 1 depicts the gel-filtration pattern of dodecamers and multimers of RhSP-D and the SP-D/MBL$_{neck+CRD}$ chimera. Previous electron microscopic studies have demonstrated that the high molecular weight fraction of RhSP-D is mainly composed of highly multimerized SP-D molecules containing up to 32 trimeric SP-D arms (12).

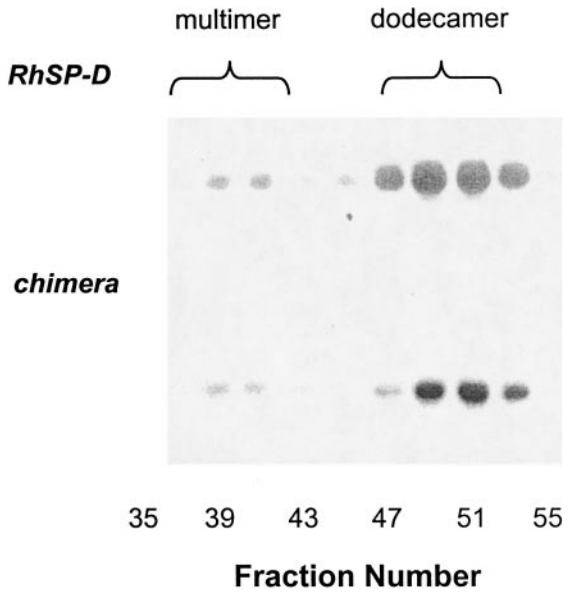

FIG. 1. Gel-filtration analysis of RhSP-D and SP-D/MBL$_{neck+CRD}$. As indicated the most abundant fractions of RhSP-D and the SP-D/MBL$_{neck+CRD}$ chimera eluting from the A15M column corresponded to dodecamers. However, both collectins formed a significant amount of higher molecular weight multimers as well. The high-molecular-weight multimer fractions of each collectin were pooled for use in experiments described in this paper.

The collectin preparations and buffers were assayed for endotoxin using a quantitative assay (Limulus Amebocyte Lysate; Bio-Whittaker, Walkersville, MD). Buffers and virus stocks contained $\leq$1 pg/ml (or $\leq$1 EU/ml) of endotoxin. The stock preparations of collectins contained between 1.5 and 8 ng/ml of endotoxin. After accounting for dilution of collectins for use in antiviral or neutrophil function assays, the final concentrations of endotoxin in samples containing the highest concentrations of collectins were $\sim$20–100 pg/ml (or 6–12 endotoxin units/ml using internal assay standard). These concentrations of endotoxin did not significantly alter results obtained in the assays described in this study (7).

Fluorescent focus assay of IAV infectivity. Infectivity of influenza virus was assessed by incubation of MDCK cell monolayers with the virus for 7 h, followed by detection of infectious foci using a monoclonal antibody directed against the influenza A viral nucleoprotein (mAb A-3; gracious gift of Dr. Nancy Cox, Centers for Disease Control, Atlanta, GA) as previously described (7). Viral samples were either untreated or pre-treated for 30 min. with various concentrations of collectins. The fluorescent foci were counted directly under fluorescent microscopy. Results were expressed as % of control infectious foci (i.e., number of foci in wells infected with collectin-treated IAV/number of foci in control wells $\times$ 100).

Measurement of aggregation of IAV particles. Aggregation of IAV particles was assessed following addition of various concentrations of collectins by monitoring changes in light transmission on a highly sensitive SLM/Aminco 8000C (SLM Instrument, Urbana, IL) spectrofluorometer as described (13). The aggregation of viral particles or liposomes is demonstrated by a decline in light transmission (i.e., increased turbidity).

Measurement of IAV uptake by neutrophils. IAV was FITC-labeled and aliquots were incubated with neutrophils for 30 minutes at 4°C. IAV was then preincubated with various concentrations of collectins for 30 min at 37°C. Viral uptake by neutrophils was as-

TABLE 1

Inhibition of HA Activity of Wild-Type and Serum β-Inhibitor-Resistant Strains of IAV by Collectins

Collectin	Collectin concentrations (ng/ml) causing HA inhibition of the following IAV strains				
	Brazil78	Brazil78/BS	Phil82	Phil82/BS	PR-8
SP-D/MBL$_{neck+CRD}$ multimers	1.6 ± 0.1	7.5 ± 1.5	3.7 ± 0.8	13 ± 3.5	>250
SP-D/MBL$_{neck+CRD}$ dodecamers	7.5 ± 3	40 ± 3	8.7 ± 2.4	32 ± 8	
RhSP-D multimers	7 ± 1.8	13.5 ± 3.8	5.5 ± 1.1	37 ± 7	>250
RhSP-D dodecamers	7.5 ± 3	63 ± 29	13 ± 6.5	133 ± 25	
RhMBL	254 ± 58	122 ± 30	58 ± 1.1	458 ± 43	

Note. HA inhibition was measured using Type O erythrocytes as described (11). Results are means ± SEM of three or more experiments. SP-D/MBL$_{neck+CRD}$ multimers inhibited HA activity of all viral strains tested at significantly lower concentrations than any of the other collectin preparations. No detectable HA inhibition of any of the viral strains was induced by any of the collectins when the assays were carried out in TBS with 10 mM EDTA (data not shown).

sessed by flow cytometry as previously described (14). Briefly, aliquots of collectin-treated virus were incubated with neutrophils for 30 min at 37°C, followed by addition of 0.2 mg/ml of trypan blue to quench extracellular fluorescence, prior to measurement of neutrophil fluorescence using flow cytometry.

Measurement of neutrophil H_2O_2 production. H_2O_2 production was measured by assessing reduction in scopoletin fluorescence as previously described (15).

RESULTS

High-Molecular-Weight Multimers of SP-D/MBL$_{neck+CRD}$ Have Greater Ability to Inhibit Hemagglutination Activity of IAV Than RhMBL, SP-D/MBL$_{neck+CRD}$ Dodecamers or High-Molecular-Weight Multimers of RhSP-D

We compared the ability of SP-D/MBL$_{neck+CRD}$ multimers, SP-D/MBL$_{neck+CRD}$ dodecamers, RhSP-D multimers, and RhMBL (predominantly octadecamers) to inhibit HA activity of the representative wild-type strains of IAV, Philippines 1982/H3N2 (Phil82) and Brazil 1978/H1N1 (Braz78). The SP-D/MBL$_{neck+CRD}$ multimers were markedly more potent than RhMBL against either strain (Table 1). Furthermore, SP-D/MBL$_{neck+CRD}$ multimers were also more potent than either SP-D/MBL$_{neck+CRD}$ dodecamers or RhSP-D multimers against these strains.

We also tested the ability of the collectins to inhibit HA activity of bovine serum β inhibitor resistant strains of IAV. The bovine serum β inhibitor resistant strains have been shown to be highly resistant to HA inhibitory activity of conglutinin and MBL [see Refs. (11, 13, 16)] as a result of loss of a single high mannose oligosaccharide attachment on the viral HA. Although all of the collectins had reduced HA inhibitory activity against these strains compared to their activity against wild type strains, SP-D/MBL$_{neck+CRD}$ multimers were again more potent than all the other collectins preparations tested. RhSP-D multimers were more potent against the β-inhibitor resistant strains than RhSP-D dodecamers. However, SP-D/MBL$_{neck+CRD}$ multimers had greater activity than RhSP-D multimers. None of the collectins had inhibitory activity against the PR-8 strain of IAV. PR-8 is a mouse adapted strain which does not have any high mannose oligosaccharide attachments on its hemagglutinin (17).

HA Inhibitory Activity of SP-D/MBL$_{neck+CRD}$ Multimers Is More Resistant to Competition by Monosaccharides Than That of RhSP-D Multimers or SP-D/MBL$_{neck+CRD}$ Trimers

Figure 2 compares the ability of monosaccharides to block HA inhibitory activity of RhSP-D or SP-D/MBL$_{neck+CRD}$. SP-D/MBL$_{neck+CRD}$ multimers had a distinctive pattern of interference by monosaccharides compared to RhSP-D multimers. SP-D/MBL$_{neck+CRD}$ multimers were resistant to competition by glucose and galactose, but showed some degree of competition by GlcNAc. All of the monosaccharides caused some degree of competition of the HA inhibitory activity of RhSP-D multimers. However, RhSP-D multimers were most susceptible to competition by mannose and glucose. Overall, SP-D/MBL$_{neck+CRD}$ multimers were more resistant to competition by monosaccharides than RhSP-D multimers. For comparison, we tested the effect of monosaccharides on HA inhibition by the trimeric fraction of SP-D/MBL$_{neck+CRD}$. Trimers of the chimera were significantly more susceptible to competition by the monosaccharides than SP-D/MBL$_{neck+CRD}$ multimers. These results suggest that SP-D/MBL$_{neck+CRD}$ multimers bind IAV with high affinity, and that this is accounted for in part by their degree of multimerization.

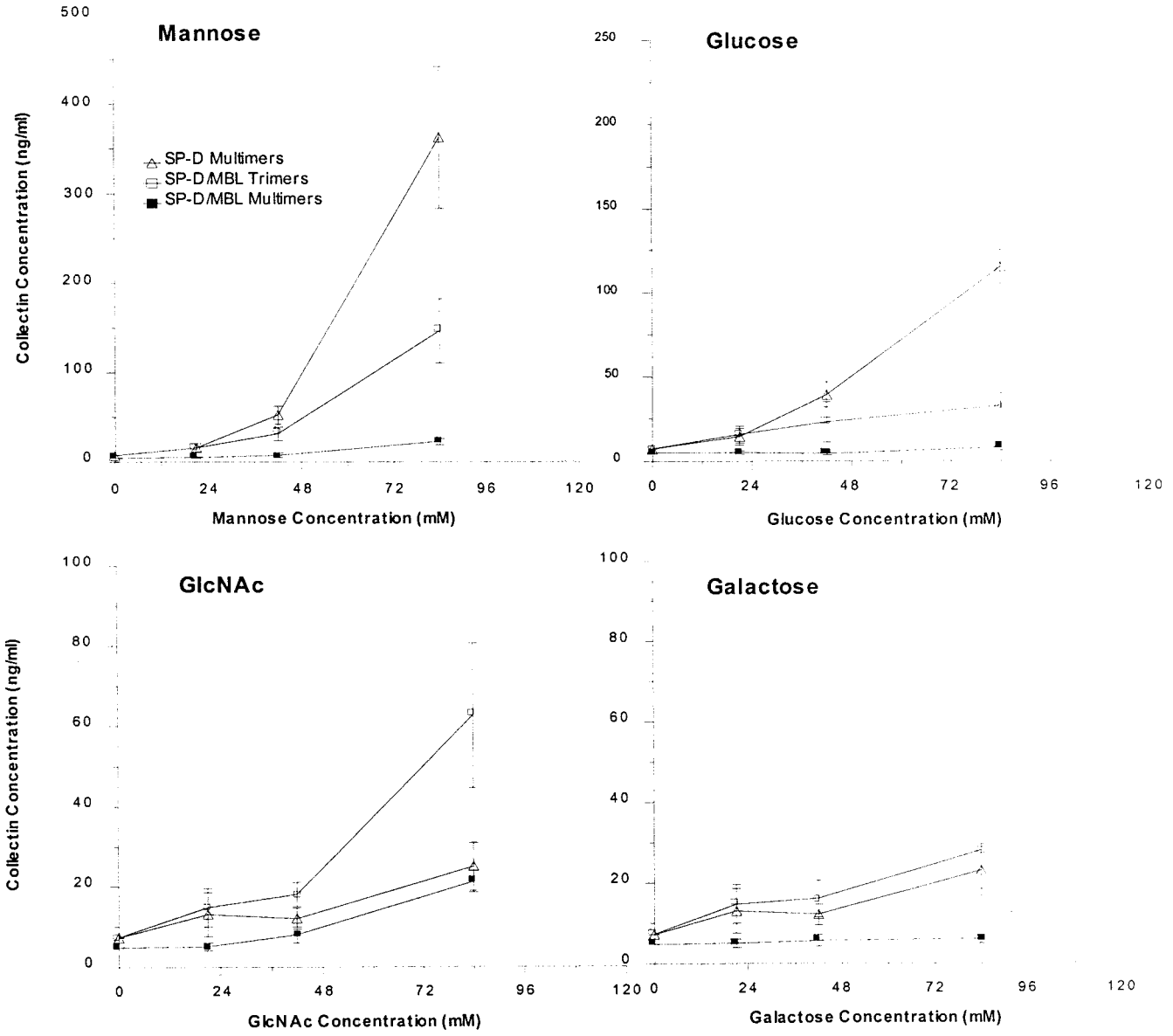

FIG. 2. Effect of monosaccharides on the ability of collectins to inhibit IAV hemagglutination (HA) activity. HA inhibitory activity was measured using Type O human erythrocytes as described under Materials and Methods. Prior to HA inhibition assays, the collectins were preincubated in PBS alone, or PBS containing increasing concentrations of various monosaccharide preparations as shown. Note that substantially greater concentrations of RhMBL or SP-D/MBL$_{neck+CRD}$ were required to inhibit viral HA activity as concentrations of mannose or GlcNAc were increased. Mannose and glucose (but not GlcNAc) had similar effects on HA inhibition by RhSP-D. Galactose did not alter HA inhibition by RhMBL or SP-D/MBL$_{neck+CRD}$ at the concentrations tested. The highest concentration of galactose tested (166 mM) did cause statistically significant ($P < 0.05$), although subtle, interference with HA inhibition by RhSP-D. Results shown are means ± SEM of three or more experiments.

SP-D/MBL$_{neck+CRD}$ Multimers Cause Significantly Greater Inhibition of Infectivity Than RhSP-D Multimers

RhSP-D multimers caused greater inhibition of infectivity of IAV than RhSP-D dodecamers (Fig. 3A). However, SP-D/MBL$_{neck+CRD}$ multimers (Fig. 3B) caused significantly greater inhibition of infectivity than RhSP-D multimers. The inhibition caused by SP-D/MBL$_{neck+CRD}$ multimers was also significantly greater than that of SP-D/MBL$_{neck+CRD}$ dodecamers or rhMBL.

SP-D/MBL$_{neck+CRD}$ Causes Significantly Greater Aggregation of IAV Than RhMBL or RhSP-D

As shown in Fig. 4, a concentration of 200 ng/ml of SP-D/MBL$_{neck+CRD}$ multimers caused markedly greater aggregation of IAV than the same concentration of

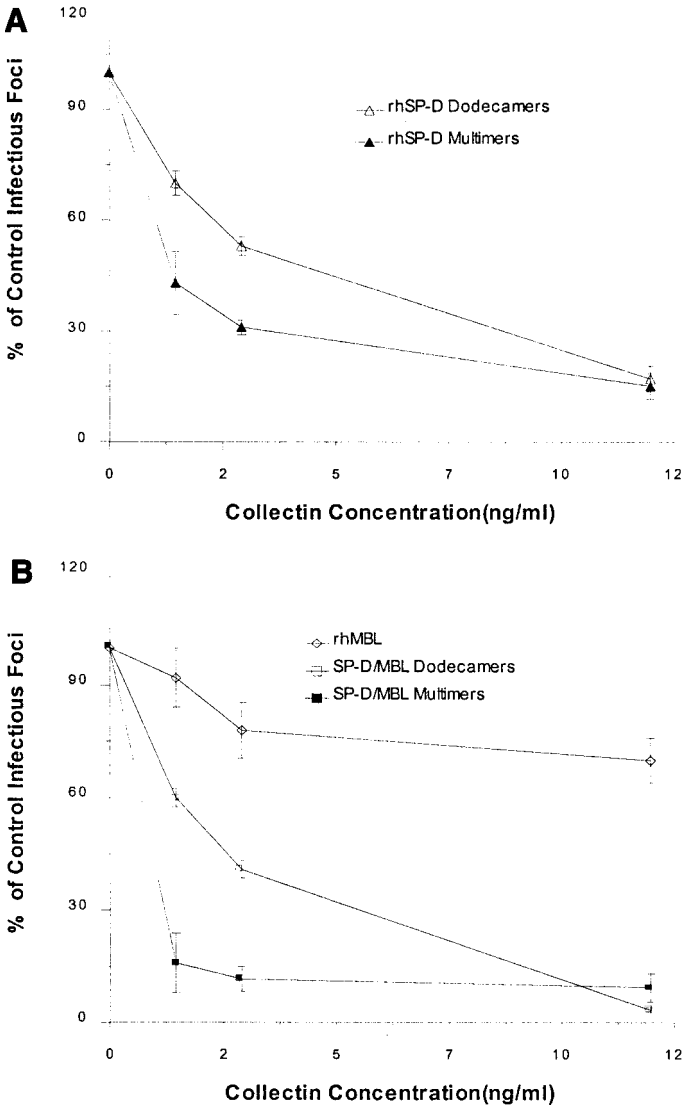

FIG. 3. Neutralization of infectivity of IAV by collectins. Infectivity of IAV for MDCK cells was assessed using a fluorescent focus forming assay as described under Materials and Methods. Results are means ± SEM of at least three experiments and are expressed as percent of control foci present after infection with collectin-treated/control virus. A compares the effect of rhSP-D dodecamers to that of rhSP-D multimers. B compares the effect of SP-D/MBL multimers to those of SP-D/MBL dodecamers and to wild type rhMBL. Results shown for rhSP-D dodecamers, SP-D/MBL dodecamers and rhMBL were previously reported (7) and given here for comparison. RhSP-D multimers caused significantly greater inhibition of viral infectivity than rhSP-D dodecamers. Concentrations of 1.4 or 2.8 ng/ml of SP-D/MBL$_{neck+CRD}$ multimers caused significantly greater viral neutralization than the same concentrations of either rhSP-D multimers or rhSP-D dodecamers. SP-D/MBL$_{neck+CRD}$ multimers also caused greater inhibition of infectivity than SP-D/MBL$_{neck+CRD}$ dodecamers or rhMBL.

RhMBL or RhSP-D multimers. SP-D/MBL$_{neck+CRD}$ multimers also caused significantly greater viral aggregation than RhSP-D multimers when concentrations of 100 or 400 ng/ml of the collectins were compared (see Table 2). The HA activity of the aggregation samples were also tested after completion of the aggregation assay. SP-D/MBL$_{neck+CRD}$ multimers also caused greater inhibition of viral HA titers in these samples than either RhSP-D multimers or RhMBL (Table 2).

SP-D/MBL$_{neck+CRD}$ Multimers Show Greater Enhancement of Viral Internalization Than SP-D/MBL$_{neck+CRD}$ Trimers or Dodecamers or Than RhSP-D Multimers or RhMBL

SP-D/MBL$_{neck+CRD}$ multimers caused much greater enhancement of neutrophil uptake of FITC-labeled IAV than RhSP-D multimers or RhMBL (Fig. 5A). The degree of multimerization of SP-D/MBL$_{neck+CRD}$ directly correlated with its ability to enhance neutrophil uptake of IAV (Fig. 5B). Preincubation of neutrophils with SP-D/MBL$_{neck+CRD}$ multimers caused greater enhancement of IAV-induced neutrophil H_2O_2 responses than either RhMBL or RhSP-D multimers (Table 2).

DISCUSSION

These studies demonstrate that a chimera constructed using the N-terminal and collagen domains of human SP-D and the neck and CRD regions of MBL can form high order multimers similar to those previously reported for human SP-D. EM analysis of SP-D eluting at this high-molecular-weight fraction has shown the multimers to be structurally stable stellate

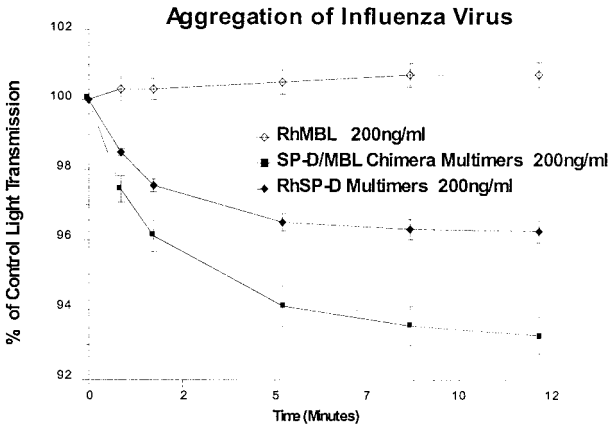

FIG. 4. Collectin-induced aggregation of IAV particles. Light transmission through stirred suspensions of IAV particles was monitored after addition of the indicated collectin preparations. Results are means ± SEM of three or more experiments and are expressed as percent of control light transmission. No change in light transmission occurred in control samples not treated with collectins. This figure compares results obtained using 0.2 μg/ml of RhSP-D or SP-D/MBL$_{neck+CRD}$ multimers or rhMBL (predominantly octadecamers). SP-D/MBL$_{neck+CRD}$ caused significantly greater aggregation than the wild-type collectins ($P < 0.05$). Higher or lower concentrations of the SP-D/MBL$_{neck+CRD}$ chimera also induced greater aggregation than rhSP-D or rhMBL (see Table 2).

TABLE 2

Comparison of Ability of Collectins to Induce Aggregation of, Inhibit HA Activity of, and Enhance Neutrophil H_2O_2 Responses to Influenza Virus

		Collectin concentration (ng/ml)			
Collectin	Assay	0	100	200	400
SP-D/MBL multimers	Aggregation	100%	97 ± 0.4*	93 ± 0.5*	91 ± 1.3*
	H_2O_2	0.14 ± 0.1	1.1 ± 0.7	2.8 ± 1*	4.7 ± 0.3
	HA titer	2160 ± 348	950 ± 275	240 ± 65*	0*
RhSP-D multimers	Aggregation	100%	99 ± 0.5	96 ± 0.5	93.4 ± 0.6
	H_2O_2	0.14 ± 0.1	0.51 ± 0.4	1.23 ± 0.5	4.2 ± 0.3
	HA titer	2160 ± 348	1900 ± 443	500 ± 122	112 ± 38
RhMBL	Aggregation	100%		100 ± 0.3	98.4 ± 0.9
	H_2O_2	0.14 ± 0.1	0.04 ± 0.01	0.13 ± 0.09	0.28 ± 0.23
	HA titer	2160 ± 348	2400 ± 800	2133 ± 533	900 ± 100

Note. SP-D/$MBL_{neck+CRD}$ multimers caused significantly more viral aggregation, enhancement of neutrophil responses, and depression of viral HA activity than RhMBL at all concentrations shown ($P \leq 0.05$). * indicates where results obtained with SP-D/$MBL_{neck+CRD}$ multimers differed significantly from those obtained with RhSP-D multimers.

appearing molecules with up to 32 trimeric domains attached at the N-terminus (12). Rat SP-D also forms such multimers but does so to a much more limited extent than human SP-D (18). Bovine conglutinin does not appear to form such multimers (6). Hence, the propensity to form high order multimers appears to be an intrinsic property of the N-terminal domains of human SP-D.

We previously demonstrated that dodecameric SP-D/$MBL_{neck+CRD}$ or SP-D/$Conglutinin_{neck+CRD}$ chimeras have greater IAV-neutralizing activity than wild type SP-D or conglutinin dodecamers or wild type RhMBL (6, 7). An important and novel finding of this paper is that increasing the extent of multimerization of SP-D or SP-D/$MBL_{neck+CRD}$ increases antiviral activity independently of changes in carbohydrate binding properties. The highly multimerized preparation of SP-D caused greater inhibition of HA activity and neutralization of infectivity of IAV than dodecameric SP-D. Furthermore, the highly multimerized forms of SP-D/$MBL_{neck+CRD}$ caused greater inhibition of HA activity and infectivity, and induced more viral aggregation, than either RhSP-D multimers or less multimerized forms of SP-D/$MBL_{neck+CRD.}$ Of interest, the HA inhibitory activity of SP-D/$MBL_{neck+CRD}$ multimers was also more resistant to competition by monosaccharides than RhSP-D multimers or less multimerized forms of SP-D/$MBL_{neck+CRD}$. These results are consistent with the hypothesis that more highly multimerized forms of SP-D or SP-D/$MBL_{neck+CRD}$ bind more strongly to influenza virus particles because of increased cooperativity of binding.

The more highly multimerized collectin preparations (either RhSP-D or SP-D/$MBL_{neck+CRD}$) were particularly effective in inhibiting the HA activity of bovine serum β-inhibitor resistant strains. In fact, SP-D/$MBL_{neck+CRD}$ multimers had greater ability to inhibit HA activity of serum collectin-resistant viral strains than any collectin preparation we have studied to date. The mechanism through which the chimera exerts antiviral effects against the resistant strains is not fully elucidated. We have demonstrated, however, that a similar SP-D/Conglutinin chimera is able to bind to the hemagglutinin of Phil82/BS, while wild-type conglutinin is not (8).

Our findings also demonstrate a clear association between the degree of multimerization and the ability of SP-D/$MBL_{neck+CRD}$ to enhance neutrophil uptake of IAV. SP-D/$MBL_{neck+CRD}$ multimers more effective at enhancing neutrophil uptake of IAV (or IAV stimulated H_2O_2 responses) than chimeric dodecamers (or RhSP-D multimers), which were, in turn, more effective than SP-D/$MBL_{neck+CRD}$ trimers. Hence, the degree of multimerization impacts on direct antiviral and opsonic effects of SP-D/$MBL_{neck+CRD}$. These findings are relevant to strategies for production of recombinant collectins with broader or more potent antiviral activity.

Our findings also suggest that patients with less highly multimerized SP-D might show greater susceptibility to IAV infection. This might especially be true in the case of infection with a relatively collectin-resistant IAV strain. Differences in multimerization could be age- or disease-related or could result from polymorphisms of SP-D genes that might influence assembly (19). A polymorphism characterized by the presence of threonine, rather than methionine, at position 11 of the mature protein immediately amino terminal to the site of interchain disulfide crosslinks has been reported to be associated with a higher risk of tuberculosis (20). Some humans with threonine in position 11 have been shown to accumulate a trimeric species in their lavage (21). Whether this polymor-

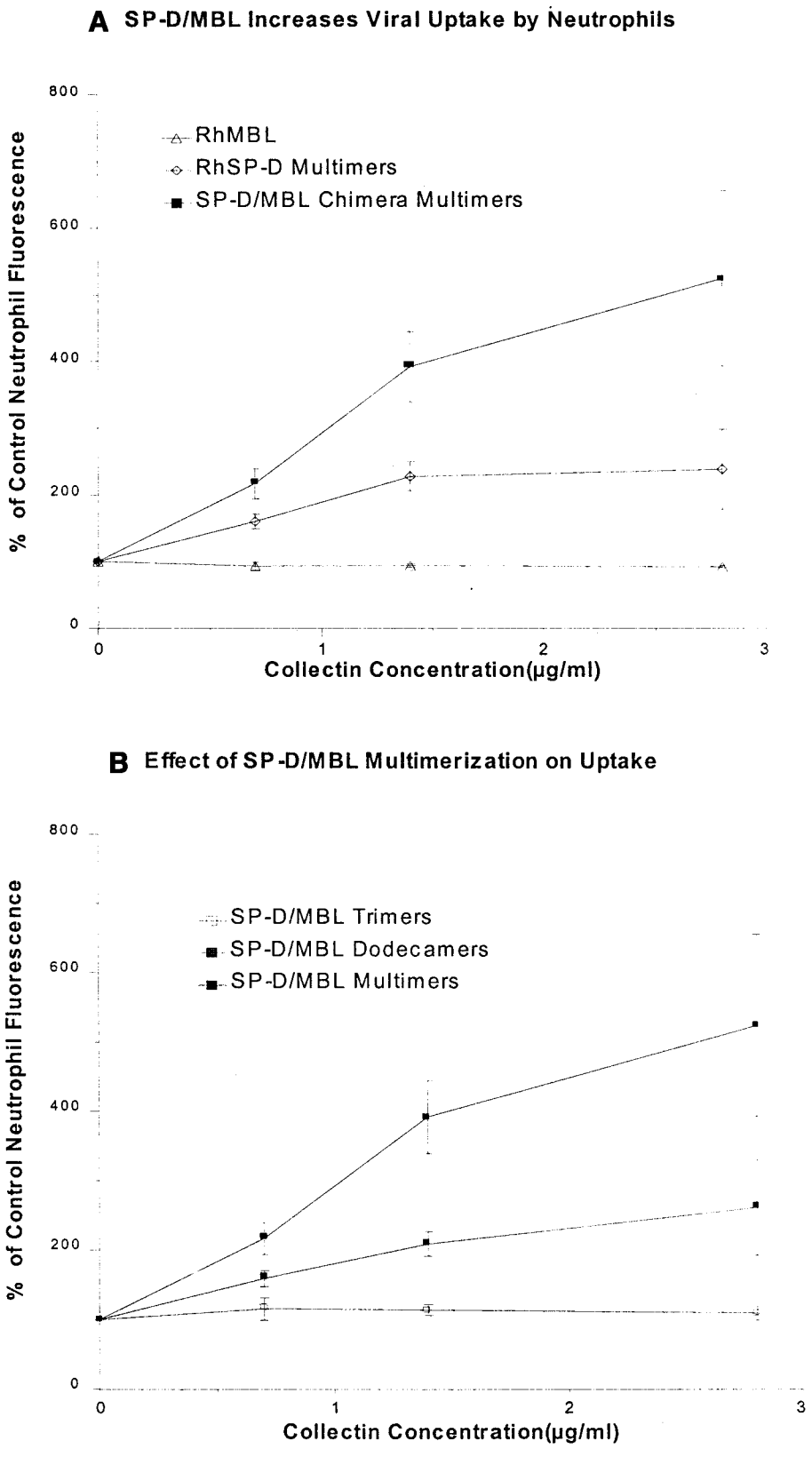

FIG. 5. Effect of collectins on neutrophil uptake of IAV. FITC-labeled IAV samples were treated with collectins as indicated and then incubated with human neutrophils followed by measurement of viral binding. Viral uptake was assessed by incubation of collectin-treated viral samples with neutrophils for 30 min at 37°C, followed by addition of trypan blue and fixing of cells with paraformaldehyde. Assessment of neutrophil associated fluorescence with flow cytometry. Results represent means ± SEM of three or more experiments (using different blood donors) and are expressed as mean neutrophil fluorescence. Multimers of SP-D/MBL$_{neck+CRD}$ caused significantly greater enhancement of viral binding than either the SP-D multimers or rhMBL (A) or the trimeric or dodecameric fraction of SP-D/MBL$_{neck+CRD}$ (B).

phism affects susceptibility to IAV infection is worthy of study.

ACKNOWLEDGMENTS

This work was supported by NIH Grants HL58910 (K.L.H.) and HL29594 (E.C.). We greatly appreciate Dr. R. Alan B. Ezekowitz and Kazue Takahashi (Harvard Medical School, Boston, MA) for providing recombinant human MBL.

REFERENCES

1. Sastry, K., and Ezekowitz, R. (1996) *in* Collectins and Innate Immunity (Ezekowitz, R., Sastry, K., and Reid, K., Eds.), pp. 1–7, Landes, Austin, TX.
2. Crouch, E., Hartshorn, K., and Ofek, I. (2000) Collectins and pulmonary innate immunity. *Immunol. Rev.* **173,** 52–65.
3. LeVine, A., Bruno, M., Huelsman, K., Ross, G., Whitsett, J., and Korfhagen, T. (1997) Surfactant protein A deficient mice are susceptible to group B streptococcal infection. *J. Immunol.* **158,** 4336–4340.
4. LeVine, A., Gwozdz, J., Stark, J., Bruno, M., Whitsett, J., and Korfhagen, T. (1999) Surfactant protein-A enhances respiratory synctitial virus clearance *in vivo. J. Clin. Invest.* **103,** 1015–1021.
5. Reading, P., Morey, L., Crouch, E., and Anders, E. (1997) Collectin-mediated antiviral host defense of the lung: Evidence from influenza virus infection of mice. *J. Virol.* **71,** 8204–8212.
6. Hartshorn, K., Sastry, K., Chang, D., White, M., and Crouch, E. (2000) Enhanced anti-influenza activity of a recombinant pulmonary surfactant protein D and serum conglutinin fusion protein. *Am. J. Physiol.* **278,** L90–L98.
7. White, M., Crouch, E., Chang, D., Sastry, K., Guo, N., Engelich, G., Takahashi, K., Ezekowitz, R., and Hartshorn, K. (2000) Enhanced antiviral and opsonic activity of a human mannose binding lectin and surfactant protein D fusion protein. *J. Immunol.* **165,** 2108–2155.
8. Hartshorn, K., White, M., Voelker, D., Coburn, J., Zaner, K., and Crouch, E. (2000) Mechanism of binding of surfactant protein D to influenza A viruses: Importance of binding to hemagglutinin to antiviral activity. *Biochem. J.* **351,** 449–458.
9. Hartshorn, K. L., Collamer, M., Auerbach, M., Myers, J. B., Pavlotsky, N., and Tauber, A. I. (1988) Effects of influenza A virus on human neutrophil calcium metabolism. *J. Immunol.* **141,** 1295–1301.
10. Super, M., Gillis, S., Foley, S., Sastry, K., Schweinle, J. E., Silverman, V. J., and Ezekowitz, R. A. B. (1992) Distinct and overlapping functions of allelic forms of human mannose binding protein. *Nat. Genet.* **2,** 50–55.
11. Hartshorn, K. L., Sastry, K., White, M. R., Anders, E. M., Super, M., Ezekowitz, R. A., and Tauber, A. I. (1993) Human mannose-binding protein functions as an opsonin for influenza A viruses. *J. Clin. Invest.* **91,** 1414–1420.
12. Hartshorn, K., Chang, D., Rust, K., White, M., Heuser, J., and Crouch, E. (1996) Interactions of recombinant human pulmonary surfactant protein D and SPD multimers with influenza A. *Am. J. Physiol.* **271,** L753–L762.
13. Hartshorn, K. L., Sastry, K., Brown, D., White, M. R., Okarma, T. B., Lee, Y., and Tauber, A. I. (1993) Conglutinin acts as an opsonin for influenza A viruses. *J. Immunol.* **151,** 1–9.
14. Hartshorn, K., White, M., Shepherd, V., Reid, K., Jensenius, J., and Crouch, E. (1997) Mechanisms of anti-influenza activity of pulmonary surfactant proteins A and D: Comparison with other collectins. *Am. J. Physiol.* **273,** L1156–L1166.
15. Hartshorn, K. L., Collamer, M., White, M. R., Schwartz, J. H., and Tauber, A. I. (1990) Characterization of influenza A virus activation of the human neutrophil. *Blood* **75,** 218–226.
16. Hartley, C. A., Jackson, D. C., and Anders, M. E. (1992) Two distinct serum mannose-binding lectins functions as B inhibitors of influenza virus: Identification of bovine serum B inhibitor as conglutinin. *J. Virol.* **66,** 4358–4363.
17. Schwarz, R., and Klenk, H. (1981) Carbohydrates of influenza virus. *Virology* **113,** 584–593.
18. Crouch, E., Chang, D., Rust, K., Persson, A., and Heuser, J. (1994) Recombinant pulmonary surfactant protein D. *J. Biol. Chem.* **269,** 15808–15813.
19. DiAngelo, S., Lin, Z., Wang, G., Phillips, S., Ramet, M., Luo, J., and Floros, J. (1999) Novel, nonradioactive, simple and multiplex PCR–cRFLP methods for genotyping human SP-A and SP-D marker alleles. *Dis. Markers* **15,** 269–281.
20. Floros, J., Lin, H., Garcia, A., Salazar, M., Guo, X., DiAngelo, S., Montano, M., Luo, J., Pardo, A., and Selman, M. (2000) Surfactant protein marker alleles identify a subgroup of tuberculosis patients in a Mexican population. *J. Infect. Dis.* **182,** 1473–1478.
21. Mason, R., Nielsen, L., Kuroki, Y., Matsuura, E., Freed, J., and Shannon, J. (1998) A 50-kDa variant form of human surfactant protein D. *Eur. Respir. J.* **12,** 1147–1155.