Enhancement of Antiviral Activity of Collectin Trimers through Cross-Linking and Mutagenesis of the Carbohydrate Recognition Domain

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Abstract
Surfactant protein D (SP-D) plays important roles in innate defense against respiratory viruses [including influenza A viruses (IAVs)]. Truncated trimers composed of its neck and carbohydrate recognition domains (NCRDs) bind various ligands; however, they have minimal inhibitory activity for IAV. We have sought to find ways to increase the antiviral activity of collectin NCRDs. Cross-linking of the SP-D NCRD with nonblocking monoclonal antibodies (mAbs) markedly potentiates antiviral activity. In the present report, we demonstrate that F(ab')2 [but not F(ab')1] fragments of a cross-linking mAb have similar effects. Hence, cross-linking activity, but not the Fc domain of the mAb, is needed for increased antiviral activity. In contrast, the Fc domain of the mAb was important for increasing viral uptake or respiratory burst responses of human neutrophils. Our NCRD constructs contain an S protein binding site. Herein, we show that a multivalent S protein complex caused cross-linking and also increased the antiviral activity of NCRDs. NCRDs of conglutinin and CL43 had greater intrinsic antiviral activity than those of SP-D or mannose-binding lectin. Based on motifs found in these serum collectins, we have constructed mutant versions of the human SP-D NCRD that have increased antiviral activity. These mutant NCRDs also had potentiating activity after cross-linking with F(ab')2 fragments or S protein complexes. Hence, the antiviral activity of NCRDs can be increased by 2 distinct, complementary strategies, namely cross-linking of NCRDs through various means and mutagenesis of CRD residues to increase viral binding. These findings may be relevant for antiviral therapy.

Introduction
Surfactant collectins, including surfactant protein A and surfactant protein D (SP-D), play important roles in host defense against influenza A virus (IAV) and other pathogens [1]. The collectins are composed of 4 domains including an N terminus, a collagen domain, a neck domain and a carbohydrate recognition domain (CRD). The basic structural unit of the collectin is a trimer [2]. These trimers in most cases assemble into larger multimeric assemblies through disulfide bond formation at the
N terminus. Through extensive studies with SP-D, we have shown that the level of oligomerization has an important impact on antiviral activity [3–5]. SP-D most commonly forms dodecamers (with 4 trimers together making a cross-like structure). Human SP-D can also be present in vivo and in vitro as full-length trimers or high-molecular weight multimers (≥32 trimers per molecule). Dodecamers and multimers have greater antiviral activity than trimers. The neck domain of collectins mediates trimerization, and recombinant preparations containing just the neck and CRD (NCRD) of collectins spontaneously trimerize and have lectin activity.

NCRD preparations of human SP-D (hNCRD) are being explored as therapeutics because they are relatively easy to produce in bacteria and have beneficial activity in various in vitro or murine model systems [6–10]. For instance, hNCRD can restore some functions of SP-D–/– mice and can ameliorate respiratory syncitial virus infection or pulmonary hypersensitivity reactions. It should be noted that many of these studies employed an NCRD construct that also contains a short N-terminal collagen domain fragment which has impact on its activity [11]. We have explored the activities of the human SP-D NCRD with regard to IAV, using an NCRD without any collagen sequence but with an S protein binding site and His tag in the N terminus [12]. Unfortunately, these human SP-D NCRD trimers have minimal ability to bind IAV and greatly reduced antiviral activity compared to full-length SP-D. We have shown, however, that cross-linking hNCRDs with certain monoclonal antibodies (mAbs) directed against SP-D results in strong antiviral activity [3]. In addition, mutations introduced around the lectin site of the CRD confer antiviral activity on hNCRD without the need for cross-linking [12, 13].

The goals of the current project were to understand the mechanisms through which mAbs enhance the antiviral activity of NCRDs. Firstly, we wanted to determine if the Fc domain on the mAbs are needed to enhance specific functional activities of NCRDs. To this end, we evaluated the effectiveness of Fab fragments of an enhancing mAb in combination with wild-type SP-D NCRD. We also wanted to develop alternative methods of cross-linking of NCRDs so that we could directly compare the activity of NCRDs of various collectins after cross-linking, to confirm increased intrinsic antiviral activity of the NCRDs of some of the serum collectins. We achieved this using a multivalent preparation of S protein and a panel of collectin NCRDs all containing the S protein binding site. In this way we established a hierarchy of antiviral activity among wild-type collectin NCRDs as follows: CL43 > conglutinin > mannose binding-lectin (MBL) or SP-D. Finally, we wanted to determine if mutant versions of the human SP-D NCRD with increased antiviral activity would show a further increase in activity after cross-linking. In particular, the serum collectins all have a hydrophobic amino acid (valine or isoleucine) at position 343 (using SP-D numbering), whereas SP-D and surfactant protein A have arginine or lysine (for rodent SP-D) at this site. Human SP-D NCRDs containing R343V or R343I substitutions have increased antiviral activity compared to the NCRD of wild-type SP-D [13]. We now show that cross-linking of these mutants with either mAbs or S protein complexes caused a further marked increase in antiviral activity. Of note, the antiviral activity of R343V cross-linked with S protein complexes exceeded that of any of the wild-type collectin NCRDs treated in a similar manner.

**Materials and Methods**

**Virus Preparations**

IAV was grown in the chorioallantoic fluid of 10-day-old chicken eggs and purified on a discontinuous sucrose gradient as previously described [14]. The virus was dialyzed against PBS to remove sucrose, aliquoted and stored at −80°C until needed. Philippines 82/H3N2 (Phil82) and its bovine serum inhibitor resistant variants (Phil82/BS) were kindly provided by Dr. E. Margot Anders (University of Melbourne, Melbourne, Australia). After thawing, the viral stocks contained approximately 5 × 10⁸ plaque-forming units/ml. The Phil82/BS strain is relatively resistant to collectins and other lectins with affinity for mannose like cyanovirin [15].

**Collectin Preparations**

Dodecamers of wild-type recombinant human SP-D were used as a control preparation and expressed in CHO cells and purified as described elsewhere [16]. NCRD preparations, including the wild-type human NCRD (hNCRD), rat NCRD (rNCRD), NCRDs of MBL, conglutinin and CL43 and of the R343V and R343I mutants, were produced in E. coli as described elsewhere [12, 17]. The use of bacterially expressed proteins is feasible because there are no known post-translational modifications within the human SP-D CRD or neck domains, which self-assemble in vitro as stable trimers with lectin activity. All of the NCRD preparations contain an S protein binding site in the N terminus as described previously [17]. Sequences were verified by automated sequencing of the entire coding sequence of the fusion protein. RosettaBlue competent cells were transformed with the wild-type or mutant construct in PET-30a(+) vector, and expressed proteins were isolated from inclusion bodies. After refolding and oligomerization, the fusion proteins were purified by nickel affinity chromatography. Trimers were isolated by gel filtration chromatography on an Äkta system (24). All proteins were isolated in similar yields with similarly low levels of endotoxin (0.1 to approximately 0.5 EU/ml; Limulus Lysate Assay, Cambrex, Walkersville, Md., USA). The fusion
proteins showed the expected mobility on SDS-PAGE in the absence and presence of sulfhydryl reduction, as previously demonstrated for the wild-type protein [12].

The R343V and R343I mutants used in the current study form trimers like wild-type SP-D NCRD and they only differ in the single noted amino acid substitution [13, 18]. To produce R343V and R343I, site-directed mutagenesis was performed using a QuikChange II XL Site-Directed Mutagenesis Kit (200521, Stratagene, La Jolla, Calif., USA) and the human SP-D neck + CRD DNA as template.

Monoclonal Antibodies

mAbs 246-04 and 246-08 were raised against SP-D by inoculating mice with 10 µg/ml human SP-D as previously described [19]. Both of these mAbs bind to the NCRD of human SP-D although neither blocks binding of SP-D to mannan or IAV [20]. The Fab fraction of Hyb 246-08 was obtained by proteolysis of the whole immunoglobulins by papain (Sigma-Aldrich, Brøndby, Denmark). Antibody was dialyzed into 100 mM NaOAc, pH 5.5, and 1 mM EDTA. Papain and antibody were mixed together at a ratio of 1:100 by weight. The antibodies (1.5 mg) were dissolved in 1.5 ml of 100 mM NaOAc, pH 5.5, containing 1 mM EDTA and 50 mM cysteine. The solution was incubated at 37 °C for 5 h. The degree of formation of Fab fragments was monitored by SDS-PAGE after size selection using a Superdex 200 column (GE Healthcare, Brøndby, Denmark). F(ab')2 fragments were prepared using pepsin, as follows. Firstly, 2 mg of purified Hyb 246-08 (batch 040889) was digested with pepsin. After initial buffer exchange into 100 mM acetic acid buffer (pH 4.5) using an Äkta HiTrap Fast Desalting column, 3% (w/w) pepsin (P6887, Sigma-Aldrich) was added to the immunoglobulin solution. IgG was then incubated overnight at 37 °C and the fragments were eluted in TBS, pH 7.4, and 0.05% NaN3 by size fractionation with an Äkta Superdex 200 column. Pepsin cleavage yielded F(ab')2 fragments with an apparent molecular weight of 110 kDa as judged by SDS-PAGE.

Hemagglutination Inhibition Assay

Hemagglutination (HA) inhibition was measured by serially diluting collectins or other host defense protein preparations in round-bottom 96-well plates (Seroclueter U-Vinyl plates, Costar, Cambridge, Mass., USA) using PBS containing calcium and magnesium as a diluent [21]. After adding 25 µl of IAV, giving a final concentration of 40 HA units/ml or 4 HA units/well, the IAV-protein mixture was incubated for 15 min at room temperature, followed by addition of 50 µl of a type O human erythrocyte suspension. The minimum concentration of protein required to fully inhibit the hemagglutinating activity of the viral suspension was determined by noting the highest dilution of protein that still inhibited HA. Inhibition of HA activity in a given well is demonstrated by the absence of formation of an erythrocyte pellet. If no inhibition of HA activity was observed at the highest protein concentration used, then the value is expressed as being greater than the maximal protein concentration. For some experiments, the NCRDs were first preincubated with S protein, S protein-biotin or S protein-horseradish peroxidase (HRP) conjugate. The S protein preparations were purchased from Novagen.

Measurement of Viral Aggregation by Collectins

Viral aggregation was measured by assessing light transmission through stirred suspensions of IAV as previously described [22]. In addition, viral aggregates were visualized using electron microscopy as described elsewhere [23].

ELISA Assay for Measurement of Binding of NCRDs to IAV

Binding of trimeric NCRD fusion proteins to IAV was measured as previously described using the S protein-HRP conjugate [12]. In brief, ELISA plates were coated with virus followed by washing and addition of NCRD alone or NCRD that had been preincubated with S protein-HRP. After further washing, S protein-HRP was added to the wells that had only received NCRD. Binding was detected using peroxidase substrate (Pierce, Rockford, Ill., USA). All values are given as the mean ± standard error of the mean (SEM) of at least 3 independent experiments.

Fluorescent Focus Assay of IAV Infectivity

MDCK cell monolayers were prepared in 96-well plates and grown to confluency. These layers were then infected with diluted IAV preparations for 45 min at 37 °C in PBS and tested for the presence of IAV-infected cells after 7 h using an mAb directed against the influenza A viral nucleoprotein (provided by Dr. Nancy Cox, CDC, Atlanta, Ga., USA) as previously described [24]. IAV was preincubated for 30 min at 37 °C with SP-D or control buffer, followed by addition of these viral samples to the MDCK cells. Where indicated, SP-Ds were first incubated with mAbs prior to adding them to IAV.

Human Neutrophil Preparation

Neutrophils from healthy volunteers were isolated to >95% purity 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 [14]. Cell viability was determined to be >98% by trypan blue staining. The isolated neutrophils were re-suspended at the appropriate concentrations in control buffer (PBS) and used within 2 h. Neutrophil collection was performed with informed consent as approved by the Institutional Review Board of the Boston University School of Medicine. RAW 264.7 cells were obtained from the American Type Culture Collection (Manassas, Va., USA).

Measurement of IAV Uptake by Neutrophils

Fluorescein isothiocyanate (FITC)-labeled IAV (Ph182 strain) was prepared, and uptake of virus by neutrophils was measured by flow cytometry as described elsewhere [22, 25]. In brief, IAV was incubated with neutrophils for 30 min at 37 °C in the presence of control buffer. Trypan blue (0.2 mg/ml) was added to these samples to quench extracellular fluorescence. Following washing, the neutrophils were fixed with paraformaldehyde and neutrophil-associated fluorescence was measured using flow cytometry. The mean neutrophil fluorescence (>1,000 cells counted per sample) was measured.

Measurement of Neutrophil H2O2 Production

H2O2 production was measured by assessing the reduction in scopoletin fluorescence as previously described [26]. Measurements were made using a Polarstar Optima fluorescent plate reader (BMG Labtech, Durham, N.C., USA). Using this assay, a decrease in fluorescence occurs with the production of H2O2.
Statistics
Statistical comparisons were made using Student’s paired, two-tailed t test or ANOVA with a post hoc test (Tukey’s). ANOVA was used for multiple comparisons to a single control.

Results
Role of Fc Domain and Bivalency in the Ability of mAbs to Increase Antiviral Activity of hNCRD
As shown in figure 1, the 246-08 mAb and its F(ab’)2 fragments had nearly equivalent ability to increase HA inhibition and viral neutralization by the human SP-D NCRD (NCRD). Aliquots of the PhilB2 strain of IAV were preincubated with NCRD alone or NCRD combined with the intact 246-08 antibody to SP-D or F(ab’)1 or F(ab’)2 fragments of that antibody. Then the effects of these treatments on viral HA activity (a) and viral infectivity for MDCK cells (b) were tested. Results are means ± SEM of 3 or more experiments. a NCRD alone at concentrations up to 8 μg/ml did not cause any inhibition of HA activity. Addition of F(ab’)1 fragments did not alter this results; however, addition of the intact mAb or F(ab’)2 fragments of the mAb did result in HA inhibition (p < 0.05). Similarly, NCRD alone (12 μg/ml) or combined with F(ab’)1 fragments had no viral neutralizing activity, whereas the intact mAb or F(ab’)2 fragments caused significant inhibition (each was significantly less than control or NCRD alone as assessed by ANOVA). The mAb alone had no effect on viral infectivity (black bar in Control).

b Note that the scopoletin assay for H2O2 generation involves quenching of scopoletin fluorescence by H2O2, so that increased H2O2 generation is reflected in decreased fluorescence.

Multivalent S Protein Increases Viral Binding and Antiviral Activity of NCRDs
We wanted to develop an additional means of cross-linking NCRDs to confirm the findings obtained with F(ab’)2 fragments and to be able to compare activities of various mammalian NCRDs that do not bind mAbs di-
rected against human SP-D. Our NCRDs contain an S protein binding site which we exploited for this purpose. S protein conjugated with HRP can be used to detect binding of our NCRDs to various ligands. Previous binding studies have shown that different NCRD preparations are equivalently detected by the S protein-HRP conjugates. As shown in figure 3a, S protein-HRP is present as high-molecular weight aggregates on a nonreduced SDS-PAGE gel, whereas free S protein or S protein conjugated to biotin exist as monomers. This property of S protein-HRP was exploited for our studies to generate aggregates of NCRDs containing the S protein binding site. As shown in figure 3b, preincubation of hNCRD with the S protein-HRP complex resulted in substantially increased binding to IAV compared to the situation where hNCRD is added to the virus followed by addition of S protein-HRP. This implies that S protein-HRP forms a complex with hNCRD that is able to bind more avidly to IAV by presenting multiple hNCRD trimeric heads simultaneously (resembling an SP-D multimer). Preincubation of either hNCRD or rNCRD with S protein-HRP also allowed the NCRDs to inhibit viral HA activity (fig. 4a). Preincubation with free S protein or S protein-biotin did not confer HA-inhibiting activity. These preparations provide useful controls because (as demonstrated in fig. 3a) they are not multimeric complexes of S protein.

The results indicate that multivalency is required for S protein to potentiate the antiviral activity of NCRDs. The S protein-HRP conjugate was also able to potentiate the viral neutralizing activity of hNCRD (fig. 4b), conferred viral aggregating activity on hNCRD (fig. 5a) and allowed the hNCRD to increase neutrophil and RAW cell uptake of IAV (fig. 5b). This again suggests that increased phagocyte uptake of IAV can occur in the absence of N-terminal domains of collectins or the Fc domain of antibodies as long as viral aggregation occurs. Note that viral aggregation was measured by assessing light transmission through stirred suspensions of IAV in this assay, and viral aggregates reduce light transmission compared to untreated virus [21]. Samples treated in the same manner as for the
Fig. 3. S protein-HRP is present as aggregates of S protein and increases binding of hNCRD to IAV. a A nonreduced SDS-PAGE gel of S protein-HRP, free S protein, or S protein-biotin. The latter two preparations are in monomeric form, whereas the S protein-HRP is present as large aggregates of approximately 50 or 100 kDa. Lane 1: S protein (1 μg); lane 2: S protein-HRP (1 μg); lane 3: S protein-biotin (1 μg). b Preincubation of hNCRD with S protein-HRP markedly increased binding of hNCRD to IAV as assessed by ELISA (n = 3; p < 0.01 at all concentrations of hNCRD). Free S protein did not increase viral aggregation in response to hNCRD (data not shown).

Fig. 4. Addition of S protein-HRP to hNCRD allows it to inhibit viral HA activity and cause viral neutralization. The experiments were performed as in figure 1 except that S protein-HRP, free S protein or S protein-biotin were incubated with hNCRD instead of antibody fragments. S protein-HRP enabled hNCRD to significantly reduce viral HA activity (a) and viral infectivity (b; p < 0.01; n = 4 or more experiments). Free S protein and S protein-biotin did not enhance the HA-inhibiting activity of hNCRD. Similar results were obtained using rat SP-D NCRD (a). The concentration of hNCRD used in b was 2 μg/ml.
phagocyte uptake assays were analyzed by electron microscopy and showed clear viral aggregation (fig. 5c). S protein-HRP alone (in the absence of NCRD) did not inhibit HA activity or viral infectivity (data not shown) and did not induce viral aggregation (fig. 5a). S protein-HRP alone did not alter viral uptake by phagocytes or result in viral aggregating activity (data not shown).

Use of S Protein-HRP to Compare Antiviral Activities of Mammalian Collectin NCRDs after Cross-Linking

In figure 6, we compare the HA-inhibitory activity of several wild-type collectin NCRDs with that of the R343V mutant form of the human SP-D NCRD. As noted, we have reported that the R343V mutant form of human NCRD has substantially increased antiviral activity com-
pared to the wild-type hNCRD [13]. The only wild-type NCRDs that had measurable activity in the absence of cross-linking were those of CL43 (as previously reported [12]) and bovine conglutinin. Note that R343V had considerably greater activity than any of the wild-type collectin NCRDs. All the NCRDs contain equivalent S protein binding sites. The HA-inhibitory activity of all of the proteins was significantly increased by S protein-HRP. After cross-linking with S protein-HRP, the following hierarchy of activity was determined: R343V > CL43 or conglutinin > MBL or human SP-D > rat SP-D. NCRDs with intrinsic activity prior to cross-linking had the greatest activity after cross-linking. As above, free S protein or S protein-biotin did not increase the HA-inhibiting activity of R343V (data not shown).

Cross-Linking of a Mutant hNCRD that Has Increased Intrinsic Antiviral Activity Results in Further Increase in Antiviral Activity

Given that S protein-HRP caused a marked increase in the HA-inhibiting activity of R343V, we tested the ability of mAbs to increase various antiviral activities of R343V and R343I. R343I alone had lower antiviral activity than R343V but significantly greater than wild-type hNCRD in our prior studies [13]. Incubation of R343V with either the 246-04 or 246-08 mAb increased its binding to IAV (fig. 7a). These antibodies also significantly increased the HA-inhibiting activity of R343V and R343I (fig. 7b). This effect was reproduced using F(ab')2 fragments of the 246-08 mAb (fig. 7c). In contrast, F(ab')1 fragments significantly reduced the intrinsic HA-inhibiting activity of R343V (fig. 7c). Similarly, the full 246-08 mAb significantly increased the neutralizing activity of R343V (fig. 7d), whereas the F(ab')1 fragment of this antibody essentially abrogated the neutralizing activity of R343V.

Avian and pandemic viral strains have less glycosylation on their HA than strains that have evolved for years in the human population [27]. Therefore, we wanted to determine whether cross-linking of the R343V construct would have increased activity against a less glycosylated viral strain. For this purpose, we used the Phil82/BS strain, which is derived from the Phil82 strain used in this study but lacks a key oligomannose glycan attachment on the tip of the viral HA [28]. We tested the effects of hNCRD or R343V alone or after cross-linking with S protein-HRP, the 246-04 mAb or an isotype control (IgG2a) on the HA activity of the Phil82/BS strain. As shown in table 1, both S protein-HRP and the 246-04 mAb caused a considerable increase in the activity of R343V against the Phil82/BS strain. This strain was less sensitive to inhibition by cross-linked hNCRD or R343V preparations caused HA inhibition on their own, and this activity was again significantly increased by S protein-HRP. CL43 and conglutinin NCRDs + S protein-HRP caused significantly greater inhibition than SP-D NCRDs + S protein-HRP (p < 0.02), and R343V + S protein-HRP caused significantly greater inhibition than any of the other NCRDs.
than the parental Phil82 strain; however, the combination of R343V and either the mAb or S protein-HRP was inhibitory at nanomolar concentrations of R343V. In contrast, even after cross-linking, 10 μg/ml or more of hNCRD was needed to inhibit this strain. The isotype control antibody did not cause any increase in the activity of either hNCRD or R343V.

**Discussion**

Relevance of Findings to Understanding Mechanisms of Antiviral Activity of Collectins

In this study, we established that the ability of mAbs to increase the antiviral activity of either human or rat SP-D NCRDs does not depend on their Fc domain but does require bivalency. The use of F(ab′)2 fragments pro-

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**Fig. 7.** Effects of mAbs alone or Fab fragments of mAb 246-08 on the ability of R343V to bind to IAV (a), inhibit the HA activity of IAV (b, c) or neutralize IAV (d). a Binding of R343V to IAV was significantly increased as assessed by ELISA (250 ng/ml of R343V used; n = 6) when either mAb 246-04 or 246-08 was added. b Similarly, these mAbs significantly increased the ability of R343V and R343I to inhibit the HA activity of IAV (n = 4; p < 0.05). Note that R343V and R343I have measurable HA-inhibitory activity on their own (unlike the wild-type NCRD). c Addition of F(ab′)2 fragments of mAb 246-08 to R343V significantly increased its HA-inhibiting activity, whereas F(ab′)1 fragments significantly reduced it (n = 4; p < 0.01 for both). d Similarly, the intact mAb 246-08 significantly increased the viral neutralizing activity of R343V, whereas the F(ab′)1 fragments strongly reduced it (n = 4; p < 0.02 for both).
vides a minimal model of cross-linking and suggests that the formation of hexamers (i.e. 2 trimers together) can be sufficient for enhancement of viral binding. We reached a similar conclusion in a recent study involving a modified form of rat SP-D lacking one half of the collagen domain [4]. This molecule (called ‘mini-SP-D’) has increased antiviral and opsonizing activity compared to wild-type rat SP-D and on electron microscopy forms decamers in which 2 of the trimeric NCRD heads are adjacent to each other, raising the possibility that a hexameric binding surface may be more easily generated by this form of SP-D.

We utilized S protein-HRP as an alternative means of cross-linking NCRDs. The S protein-HRP conjugate presents S protein in a functionally multivalent manner, whereas free S protein and S protein-biotin do not. These results again show that formation of a multimeric array of hNCRD (or rNCRD) is important for generating antiviral activity since the complexes formed by S protein-HRP resulted in increased viral binding and neutralizing and HA-inhibiting activity compared to NCRD (or S protein-HRP) alone. The monomeric S protein preparations did not have these effects.

Relevance of Findings to Understanding the Mechanisms of Viral Opsonizing Activity of SP-D or of NCRDs Complexed with mAbs

Engagement of Fc receptors on neutrophils or monocytes by various antibody-antigen complexes can increase both phagocytosis of antigens and cell activation as assessed by respiratory burst or other activities. We have previously shown that complexes of antiviral antibodies and IAV particles result in marked potentiation of IAV-induced neutrophil respiratory burst responses [29]. Similarly, Tacken et al. [30] reported similar results using mAbs directed against IAV and a bispecific protein containing hNCRD coupled to a F(ab')1 fragment directed against a neutrophil Fc receptor that are able to simultaneously bind to Fc receptors and viral particles (through the NCRD domain). We have also shown that preincubation of IAV with full-length SP-D dodecamers markedly potentiates viral uptake by neutrophils and virus-induced respiratory burst responses [21]. We have speculated that these effects result in large part from SP-D-mediated viral aggregation [29, 31]. Another possibility is that the N-terminal and collagen domains of SP-D or other collectins engage specific receptors on phagocytes to trigger phagocytosis and increased cellular activation. For instance, Gardai et al. [32] have shown that such receptors are important for mediating macrophage phagocytosis and activation in response to complexes of SP-D and lipopolysaccharide or apoptotic cell fragments.

We used the Fab fragments and S protein-HRP to assess the role of Fc receptors in the ability of mAb 246-08 to confer opsonizing activity on the human SP-D NCRD. The wild-type hNCRD had no opsonizing activity on its own. This is similar to our prior finding that full-length trimers of SP-D do not cause viral aggregation or increase neutrophil uptake or respiratory burst responses to IAV [33]. While the F(ab')2 was essentially equivalent to the intact mAb 246-08 in increasing the neutralizing and HA-inhibiting activity of hNCRD, it was less active than the mAb in increasing the opsonizing activity hNCRD. Specifically, cross-linking of the hNCRD with F(ab')2 modestly increased uptake of IAV by neutrophils [and

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Results shown are the amounts of NCRDs in μg/ml needed to inhibit 40 hemagglutinin units of the indicated viral strains and are means ± SEM of 4 experiments. The amount of mAb or isotype control used was 1.25 μg/ml. * Significant reduction in the concentration needed for inhibition by S protein-HRP or the 246-04 mAb compared to NCRD alone.

1 The value is approximately 20 μg/ml because there was variable inhibition at this concentration at the limit of detection of the assay.
Opsonizing Activity of Trimeric Collectins

We have previously shown that mAbs can enhance the activity of NCRDs [12]. This implies that mAbs can increase viral uptake by phagocytes as long as viral aggregation occurs.

**Use of S Protein-HRP Aggregates to Compare Intrinsic Antiviral Activity of Wild-Type Collectin NCRDs**

We have previously shown that the intrinsic antiviral activity of hNCRD can be greatly increased by specific mutations of residues surrounding the lectin-binding site of the NCRD (as in R343V) [13]. These mutations increase the ability of the NCRD to bind high-mannose glycans of the type found on the viral HA of recent human strains. We have now demonstrated that the neutralizing activity of R343V can be strongly potentiated by cross-linking with mAb 246-08. The combined effects of the R343V mutation and cross-linking result in inhibition of IAV at concentrations of R343V under 100 ng/ml. The activity of R343V cross-linked with S protein-HRP is also notable, since it was greater than the activity of any of the wild-type collectins treated in a similar manner. These results support the concept that combining two methods of increasing the activity of NCRDs (i.e. mutagenesis of the binding region and cross-linking of trimers) can have additive or synergistic effects. Of interest, cross-linking of R343V with either an mAb or S protein-HRP also caused a marked increase in activity against a hypoglycosylated strain of IAV, suggesting that the combined strategy of mutagenesis of the NCRD and cross-linking could increase activity against pandemic viral strains that have diminished envelope protein glycosylation.

Our findings have potential relevance to therapeutics because NCRDs are relatively easy to produce in large quantities. In addition, the use of collectins without including the collagen domain could be advantageous because the collagen domain is associated with some proinflammatory effects of SP-D [32]. The use of S protein-HRP complexes to cross-link NCRDs was more valuable as a tool to evaluate the mechanisms of activity of SP-D than as a potential therapy. In contrast, the use of mAbs or fragments to cross-link NCRDs also has conceivable clinical application because many mAbs are now being used effectively in the treatment of infectious or inflammatory diseases or cancer. For instance, palivizumab, which is an mAb directed against the respiratory syncytial virus fusion protein, is effective in prophylaxis against severe infection [35]. This implies that mAbs can achieve significant levels in the lung and inhibit respiratory viral replication. Furthermore, F(ab')1 or F(ab')2 fragments are also used in treatment. Certolizumab is an F(ab')1 fragment of an mAb directed against tumor necrosis factor alpha that has significant therapeutic benefit in Crohn's disease [36]. In this case, conjugation of the F(ab')1 fragment with polyethylene glycol is used to prolong the serum half-life. In animal models, F(ab')2 frag-
ments directed against the severe acute respiratory syndrome coronavirus or highly pathogenic H5N1 IAV show therapeutic benefit, indicating that such antibody fragments can achieve meaningful levels in the respiratory tract [37, 38].

We are not aware of therapies using mAbs or fragments of antibodies to potentiate the activity of endogenous host defense molecules or to combine with therapeutic administered inhibitors (like NCRDs), but testing this approach in mouse models seems plausible. The specific advantage of the use of F(ab’)2 fragments to cross-link NCRDs (or possibly endogenous SP-D) is that because they lack the Fc domain, these fragments do not provoke complement activation, antibody-dependent cellular cytotoxicity or respiratory burst activation of neutrophils. There is evidence that reactive oxygen or nitrogen species are harmful in the context of severe IAV infection [1, 39, 40], so it could be advantageous to avoid triggering such responses. It is also of specific interest that cross-linking of the NCRD of MBL results in increased antiviral activity without the use of the N-terminal and collagen domains. This could be of therapeutic interest since the N-terminal and collagen domains of MBL mediate complement activation, which could be deleterious in some settings [41, 42].

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**References**

Opsonizing Activity of Trimeric Collectins


