Distinctive anti-influenza properties of recombinant collectin 43

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Collectins play important roles in innate defence against viral, fungal and bacterial pathogens. CL-43, a bovine serum collectin, which appears to have evolutionarily evolved from surfactant protein D (SP-D), shows unique structural and functional properties. In the present study, we describe the initial characterization of a recombinant CL-43 expressed in mammalian cells. Like natural CL-43, the recombinant is secreted as trimeric forms that show a preference for mannose and *N*-acetyl mannosamine. The natural and recombinant proteins have significantly higher haemagglutination-inhibiting activity against influenza A virus (IAV) than recombinant trimeric forms of SP-D. In contrast with the more highly multimerized forms of SP-D, namely conglutinin or mannose-binding lectin, CL-43 did not induce viral or bacterial aggregation and did not enhance IAV-induced neutrophil H_2O_2 generation. Like SP-D, CL-43 also strongly enhanced neutrophil uptake of IAV. However, the mechanism of this enhanced internalization is different from that of SP-D in that it did not require viral aggregation. These studies establish that the trimeric structure of CL-43 is specified by its primary sequence and indicate that this naturally occurring trimeric collectin has unique antiviral activities. These findings could facilitate the development of recombinant collectins with novel antimicrobial properties.

Key words: conglutinin, neutrophil, surfactant protein D.

INTRODUCTION

The collectins are a group of collagenous lectins present in mammalian serum and pulmonary secretions and expressed at various epithelial surfaces that form part of the innate immune system [1]. Collectins can mediate defence against a wide variety of micro-organisms. Our principal focus has been on the interactions of collectins with influenza A viruses (IAVs), both as an experimental model and also due to the clinical importance of these respiratory viruses [2–7]. In humans, two collectins, surfactant proteins A and D (SP-A and SP-D), are present in pulmonary secretions and other mucosal locations. SP-A and SP-D play an important role in the host defence against IAV infection [4,8,9]. The human serum collectin, mannose-binding lectin (MBL), is also capable of neutralizing IAV *in vitro* [2]. Furthermore, individuals having low levels of serum MBL are at increased risk for infection of various kinds, including respiratory viral infections [10]. Additional collectins have been identified in the bovine serum, including conglutinin and collectin-43 (CL-43). Although the functions of these proteins are not clearly established, there is evidence that they are also involved in host defence. Among the various natural collectins, serum conglutinin is the most potent inhibitor of infectivity of IAV [7]. Preliminary studies also demonstrated strong antiviral activity of CL-43 [11].

The basic structural unit of all collectins is the trimer. Collectins share certain structural elements, including an N-terminal domain that contains cysteine residues involved in the disulphide bond formation within or between trimers, a collagen domain of varying length, a neck region that is involved in trimerization and a globular carbohydrate-recognition domain that is involved in calcium-dependent carbohydrate binding. In most cases, collectin trimers are further assembled into larger multimeric structures composed of four or more trimers attached together with N-terminal disulphide bonds. Collectins differ in their tissue distribution, the length and conformation of their collagen domains, their degree of multimerization, location of asparagine-linked carbohydrate attachments and the preference of their carbohydrate recognition domains (CRDs) for specific glycoconjugates. All of these features appear to have an impact on the interactions of collectins with IAV and other pathogens [12].

CL-43 is distinct from the other collectins in its structure and monosaccharide binding affinities [13]. Like bovine conglutinin, CL-43 appears to have evolved through duplication of the SP-D gene in ruminants. However, CL-43 is isolated from the serum as a trimer and does not form higher order multimers [14]. It is not known whether the tendency of CL-43 to assemble as trimers results from differences in primary structure or cell-type specific differences in protein assembly or processing. In addition, limited amino terminal degradation is often observed in preparations of serum CL-43 [13,14].

The present study demonstrates that recombinant CL-43 (RCL-43) retains the structural and monosaccharide binding properties of the serum-derived protein. Furthermore, RCL-43 has stronger viral neutralizing activity than other trimeric collectins and enhances neutrophil uptake of IAV through a non-aggregation-dependent mechanism that is distinct from enhanced uptake induced by SP-D or conglutinin.

MATERIALS AND METHODS

Reagents

RPMI 1640, sodium citrate, dextran, Trypan Blue stain, Wright's Giemsa stain, horseradish peroxidase type II, scopoletin and mannan and other saccharides were purchased from Sigma. PBS and Dulbecco's PBS++ (PBS with calcium and magnesium) were purchased from Gibco BRL (Grand Island, NY, U.S.A.).

Abbreviations used: CL-43, collectin-43; CRD, carbohydrate recognition domain; HAU, haemagglutination unit; IAV, influenza A virus; MBL, mannose-binding lectin; MDCK, Madin–Darby canine kidney; RCL-43, recombinant CL-43; RhMBL, recombinant human mannose-binding lectin; SP-A, surfactant protein A.

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Ficoll-Paque was obtained from Pharmacia Biotech (Piscataway, NJ, U.S.A.). Mannose–Sepharose was prepared using the same procedure described for maltosyl-agarose [15]. The antibody to CL-43 was prepared as described previously [13].

Neutrophil preparation

Neutrophils from healthy volunteers were isolated to higher than 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 red blood cells, as described previously [16]. Cell viability was determined to be higher than 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

IAV was grown in the chorioallantoic fluid of 10-day-old chicken eggs and purified on a discontinuous sucrose gradient as described previously [16] The virus was dialysed against PBS to remove sucrose, aliquoted and stored at −80 °C until needed. Phillipines 82/H3N2 (Phil82) and Brazil 78/H1N1 (Braz78) strains and their bovine serum β inhibitor-resistant variants (Phil82/BS and Braz78/BS) were provided by Dr E. Margot Anders (Department of Microbiology, University of Melbourne, Melbourne, Australia). The PR-8 strain (H1N1) was provided by Dr Jon Abramson (Department of Pediatrics, Bowman Gray School of Medicine, Wake Forest University, Winston Salem, NC, U.S.A.). The haemagglutination titre of each virus preparation was determined by titration of virus samples in PBS++ with thoroughly washed human type O, Rh(−) red blood cells as described previously [16]. A 1 HAU (haemagglutination unit) is that concentration of virus needed to induce haemagglutination in a 100 μl solution of red cells. Post-thawing, the viral stocks contained approx. 160000 HAU/ml or approx. 5×10^8 focus-forming units/ml.

Haemagglutination inhibition assay

Haemagglutination inhibition was tested by serially diluting collectin preparations in round-bottom 96-well plates (Serocluster U-Vinyl Plates; Costar, Cambridge, MA, U.S.A.) followed by addition of a solution containing IAV (final concentration of 40 HAU/ml or 4 HAU/well) and then by the addition of red blood cells. The minimal concentration of a collectin required to inhibit haemagglutination caused by the virus was then determined.

Collectin preparations

Bovine serum CL-43 was purified as described previously [13]. Full-length RCL-43 was produced in Chinese-hamster ovary-K1 cells using a full-length cDNA [17]. The cells were transfected with pEE14–CL-43 and stable transfectants expressing RCL-43 were isolated as described for human SP-D and other collectins [5]. The protein was secreted at levels comparable with SP-D and was extracted from the medium by affinity chromatography on Mannose–Sepharose. The RCL-43 trimers were further purified by gel-filtration chromatography on a column of 4% agarose [5]. SDS/PAGE and silver staining confirmed the purity. The recovery was comparable with that obtained for wild-type SP-D, yielding > 5 μg/ml of culture medium. Amino acid analysis of hydroxyproline and proline was performed by Dr Robert Mecham (Department of Cell Biology, Washington University, St. Louis, MO, U.S.A.).

The degree of oligomerization of RCL-43 was assessed using gel filtration under non-denaturing conditions. Proteins were chromatographed under non-denaturing conditions using A15M gel filtration columns (Bio-Rad) [18]. The columns were calibrated with blue dextran, thyroglobulin and BSA, as well as with purified RrSP-D dodecamers and rat SP-D trimeric subunits (RrSP-Dser15,20) [19]. The state of oligomerization of SP-D dodecamers and trimeric subunits was originally established by correlative ultrastructural and biochemical analysis of natural and recombinant SP-D proteins resolved under identical conditions. Eluted proteins were resolved by SDS/PAGE and visualized by silver staining or immunoblotting as required. All of these experiments used a flow rate of 8 ml/h and a fraction size of 3 ml.

RrSP-D and RbConglutinin were also produced in CHO-K1 cells and assembled as dodecamers as described previously [18,20]. RrSP-Dser15,20 was produced through site-directed mutagenesis according to the procedure described in [19]. In this construct, N-terminal Cys^{15} and Cys^{20} were replaced with serines, resulting in a molecule that forms only trimers (i.e. no dodecamers or higher order multimers). Recombinant human mannose-binding lectin (RhMBL) was produced in murine Sp2 cells as described in [21] and assembled as octadecamers. RhMBL was the gift from R. A. B. Ezekowitz (Department of Pediatrics, Massachusetts General Hospital, Boston, MA, U.S.A.).

The collectin preparations used in the present study were tested for the degree of contamination with endotoxin using a quantitative endotoxin assay (Limulus Amebocyte Lysate; BioWhittaker, Walkersville, MD, U.S.A.). The stock preparations of collectins were found to contain 1.5–8 ng/ml of endotoxin. After accounting for dilution of collectins used in antiviral or neutrophil functional assays, the final concentrations of endotoxin in samples containing the highest concentrations of collectins were approx. 20–100 pg/ml (or 6–12 endotoxin units/ml using the internal assay standard). Similar concentrations of purified endotoxin did not significantly alter the results obtained on the assays shown in the present study (results not shown; see [22]).

Saccharide binding assays

The affinity of CL-43 for specific saccharides was tested as described previously [13]. Microtitre plates (Polysorb; Nunc, Kamstrup, Denmark) were coated with mannan (1 μg/ml) in a coating buffer overnight. This incubation and all the following steps were performed in a volume of 100 μl/well and at room temperature. Washes and incubations were performed with Tris-buffered saline/Tween containing 5 mM $CaCl_2$ unless otherwise stated. After washing, the plates were incubated with CL-43 (5 μg/ml) or RCL-43 (5 μg/ml) with twice diluted monosaccharides or disaccharides starting at 50 μg/ml and then incubated at 4 °C overnight. After washing, the plates were incubated with 1 μg/ml of monoclonal anti-CL-43 antibody (Hyb 260–01) in Tris-buffered saline/Tween for 2 h. The plates were washed and incubated for 2 h with alkaline phosphatase-coupled rabbit anti-mouse IgG diluted 1:2000. After a final wash, the bound enzyme was estimated by adding 1 mg/ml of *p*-nitrophenyl phosphate in diethanolamine buffer. The absorbance of the 96-wells was read at 405 nm by means of a multichannel spectrophotometer.

Fluorescent focus assay of IAV infectivity

Madin–Darby canine kidney (MDCK) monolayers were prepared in 96-well plates and grown to confluence. These layers were then infected with diluted IAV preparations (Phil82 strain) for 30 min at 37 °C, followed by washing of the monolayer three times in serum-free Dulbecco's modified Eagle's medium containing 1 % penicillin–streptomycin. The monolayers were then incubated for 7 h at 37 °C with 5 % CO_2 in Dulbecco's modified Eagle's medium. The monolayers were subsequently washed three times with PBS++ and fixed with 80 % (v/v) acetone for 10 min at 4 °C. The monolayers were then labelled by incubating with monoclonal antibody directed against the IAV nucleoprotein (mAb A-3; gift from Nancy Cox, CDC, Influenza Branch, Atlanta, GA, U.S.A.) for 30 min at 4 °C. The monolayers were washed three times in PBS++ and incubated with FITC-labelled goat anti-mouse IgG. The fluorescent foci were counted directly under fluorescent microscopy. Viral inocula were used that resulted in approx. 50 fluorescent foci per ×20 field. These foci appeared to be single infected cells in general. Subsequently, this dose of virus was incubated with different doses of collectins to test their ability to neutralize viral infectivity.

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 8000 C (SLM Instrument, Urbana, IL, U.S.A.) spectrofluorometer as described previously [3]. 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

FITC-labelled IAV (Phil82 strain) was prepared as described previously [3]. Uptake of virus by neutrophils was measured as described in [6]. In most experiments, IAV was preincubated with control buffer or various concentrations of collectins for 30 min at 37 °C, followed by incubation of aliquots of these samples with neutrophils for 30 min at 37 °C. In the experiments shown in Figure 9, the sequence of incubations was altered such that CL-43 was incubated with neutrophils for 30 min at 4 °C (to avoid internalization), followed by washing off the unbound CL-43 (twice in PBS) and then incubation with FITC-labelled IAV. After incubation of the virus with neutrophils, Trypan Blue (0.2 mg/ml) was added to all samples to quench extracellular fluorescence. After further washing, the neutrophils were fixed with paraformaldehyde and the neutrophil-associated fluorescence was measured using flow cytometry. The mean neutrophil fluorescence (> 1000 cells counted per sample) and the percentage of neutrophils with positive fluorescence (i.e. fluorescence more than that of 99 % of untreated neutrophils) were measured.

Measurement of neutrophil H_2O_2 production

H_2O_2 production was measured by assessing reduction in scopoletin fluorescence as described previously [23].

Statistics

Statistical significance was calculated using Student's paired *t* test with the help of a computerized program ('StatMost', DataMost Corp., Salt Lake City, UT, U.S.A.).

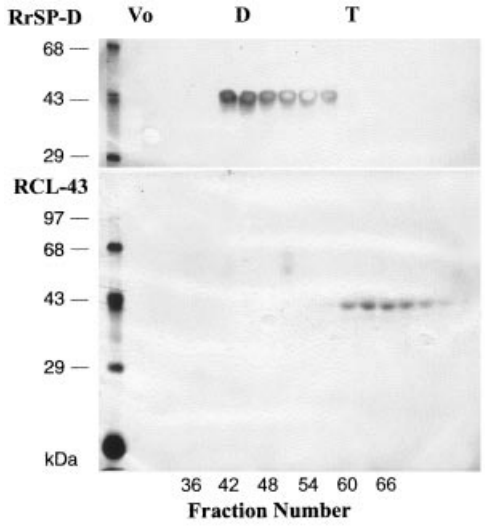

Figure 1 Gel-filtration chromatography of RCL-43 compared with RrSP-D

Affinity-purified RCL-43 was chromatographed on a calibrated agarose column under non-denaturing conditions as described in the Materials and methods section. Aliquots of every third, 3 ml fraction were reduced with dithiothreitol and examined by SDS/PAGE and silver staining. The profile of RCL-43 trimers (lower panel) is compared with a sequential run of RrSP-D dodecamers (D) using the same column (upper panel). RCL-43 elutes 1–2 fractions later than RrSP-D trimers (RrSP-Dser15,20, T). The position of the void volume (Vo) is shown.

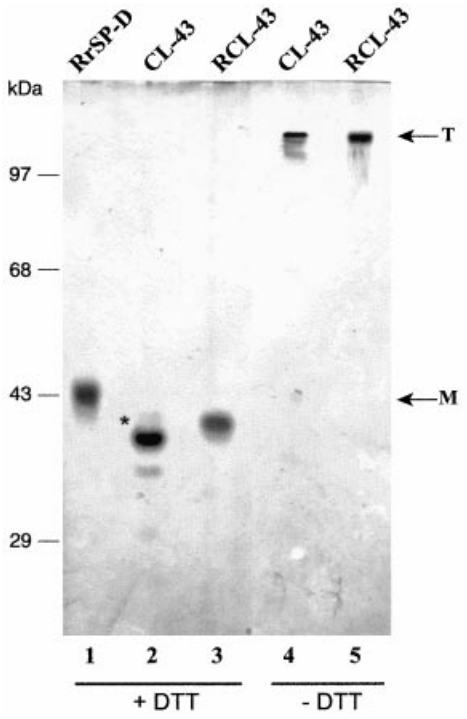

Figure 2 SDS/PAGE of natural CL-43 and RCL-43

Recombinant rat SP-D (lane 1); natural CL-43, lanes (2 and 4); and RCL-43 (lanes 3 and 5) were resolved by SDS/PAGE on 5/10% slab gels in the presence (lanes 1–3) or absence (lanes 4 and 5) of dithiothreitol (DTT) and visualized by silver staining. The preparation of the serum CL-43 used in this experiment contained predominantly a specific truncated form of CL-43 [13]. The position of the non-truncated form of serum CL-43 is indicated by an asterik. The expected positions of trimeric (T) and monomeric (M) forms of the collectins are shown, as are the positions of molecular-mass standards.

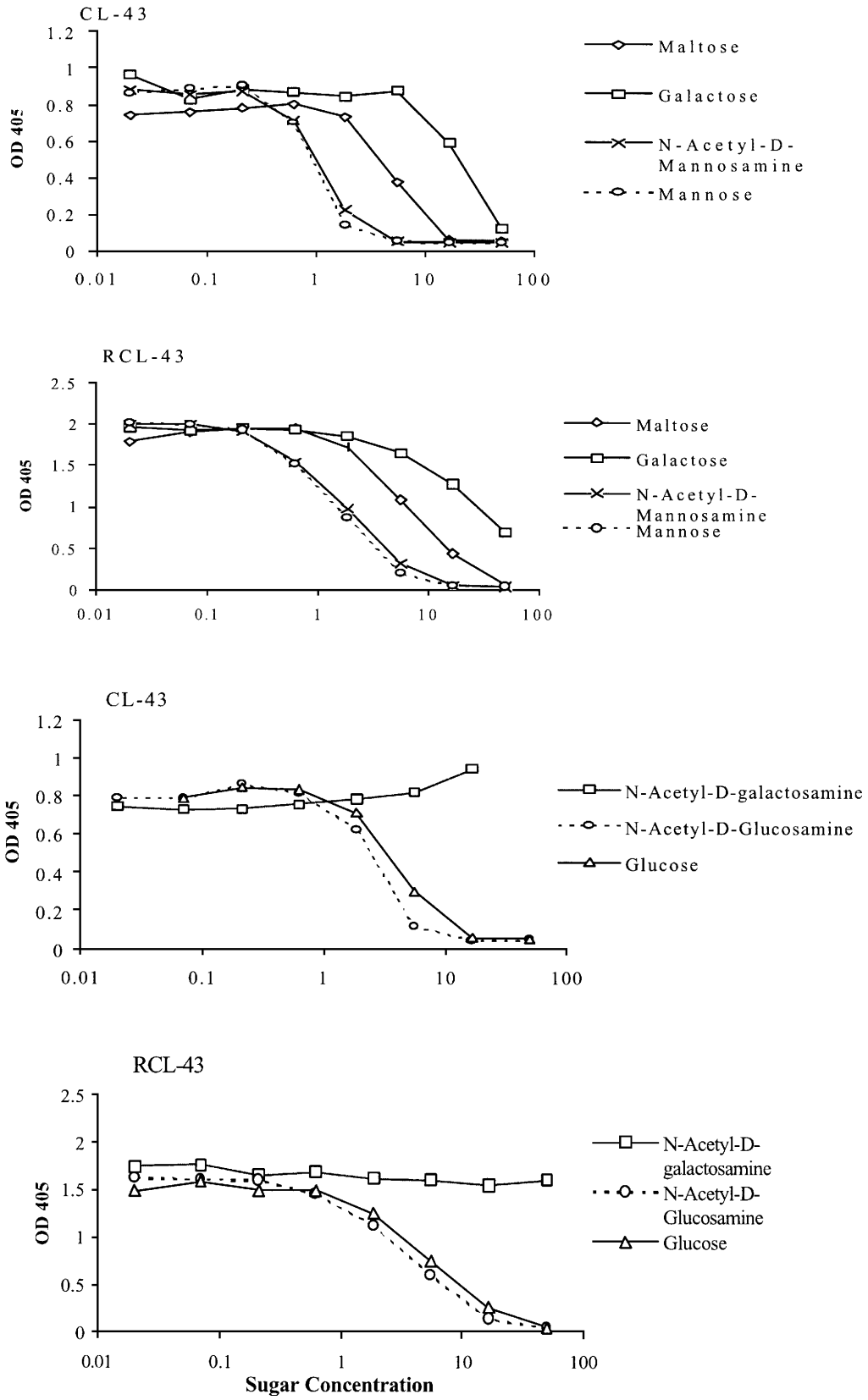

Figure 3 Saccharide selectivity of CL-43 and RCL-43

Equivalent amounts of the two proteins were incubated with mannan-coated wells, in the absence or presence of competing monosaccharides. Bound collectin was detected using monoclonal anti-CL-43 antibody and an indirect, biotinylated secondary antibody/avidin–alkaline-phosphatase detection system. Sugar concentrations (mM) are shown on the horizontal axes.

Table 1 Comparison of haemagglutination inhibiting activity of CL-43 with other collectins

Haemagglutination inhibition was measured as described previously [2]. The results shown are the concentrations of collectins (in ng/ml) found to inhibit fully haemagglutination activity of 40 HAU/ml of the indicated strains of IAV (means ± S.E.M.; $n \geqslant 3$). Experiments were performed in Tris-buffered saline with 2 mM calcium. Neither RCL-43 nor bovine serum CL-43 inhibited haemagglutination activity of Phil82 strain of IAV when the assay was performed in buffer containing 10 mM EDTA (results not shown). Results obtained with SP-D/$MBL_{neck+CRD}$ chimaera preparations were previously reported [22] and are shown here for purposes of comparison.

	Viral strain				
Collectin	Phil82	Phil82BS	Braz78	Braz78BS	PR-8
RCL-43	12 ± 1.7	115 ± 10	11 ± 2	26 ± 4.8	> 4400
Bovine CL-43	10 ± 1.2				> 2200
RrSP-D	19 ± 3.5	449 ± 61	28.5 ± 1.7	66 ± 10	> 4400
RrSP-Dser15/20	169 ± 51	406 ± 144	500 ± 90	1000 ± 164	
RbConglutinin	2 ± 0.6	917 ± 300			
RhMBL	58 ± 14	475 ± 43			
SP-D/$MBL_{neck+CRD}$ trimers	114 ± 26	> 1400	181 ± 30	> 1400	
SP-D/$MBL_{neck+CRD}$ dodecamers	7 ± 2.4	48 ± 17	7.5 ± 3	40 ± 3	

RESULTS

RCL-43 is secreted as trimers

Preliminary ELISA and immunoblotting assays using rabbit anti-bovine serum CL-43 [13] demonstrated that the protein is efficiently secreted into the culture medium (results not shown). In order to characterize further the protein and assess its degree of multimerization, the protein was isolated by saccharide affinity chromatography on Mannose–Sepharose and further purified by gel-filtration chromatography under non-denaturing conditions [5]. The protein bound to Mannose–Sepharose was specifically eluted with EDTA. On subsequent gel filtration, the protein eluted later than SP-D or conglutinin dodecamers and near the position of RrSP-Dser15,20 trimers (Figure 1) [19]. When examined by SDS/PAGE, the purified protein comigrated with intact serum CL-43 and slightly more slowly than the major degradation product found in some preparations of serum CL-43 (Figure 2, cf. lanes 2 and 3). As expected, the reduced protein migrated more rapidly than SP-D (Figure 2, lane 1), consistent with known differences in the size of their collagen domains. The natural and recombinant proteins also comigrated in the absence of thiol reduction (Figure 2, lanes 4 and 5), consistent with the known behaviour of trimeric subunits and the trimers derived from disulphide-cross-linked dodecamers [19]. Subcellular fractionation studies have shown that disulphide-cross-linked SP-D trimers (probably resembling CL-43) undergo a time-dependent rearrangement of disulphide bonds to form reducible trimeric species that contain the chain derived from two trimeric subunits [24].

Unlike SP-D, CL-43 has no consensus for N-linked glycosylation, so no glycosidase digestions were performed. However, the purified protein showed essentially normal hydroxylation of proline as assessed by amino acid analysis. The hypro or pro ratio of RCL-43 was 0.26, as compared with 0.23 for the published analysis. Interestingly, the immunoreactive, collagenase-resistant C-terminal domains of both natural and RCL-43 migrated more slowly than the corresponding fragment of SP-D, apparently reflecting a greater resistance of the contiguous collagen sequence to bacterial collagenase (results not shown).

RCL-43 and natural CL-43 have similar saccharide selectivity

The saccharide selectivities of the recombinant and natural proteins were directly compared with solid-phase binding assays using mannan as a ligand and selected monosaccharides as competitors. As shown in Figure 3, CL-43 and RCL-43 showed virtually indistinguishable competition profiles. Both showed preferential interaction with mannose and *N*-acetyl mannosamine as described previously [13]. There was less effective competition with *N*-acetyl glucosamine, glucose and maltose, whereas lactose and galactose were ineffective as competitors.

CL-43 is a potent inhibitor of influenza viral haemagglutination activity: comparison with other collectins and mechanism of action

The ability of natural CL-43 and RCL-43 to inhibit haemagglutination activity of various IAV strains was compared with that of various wild-type collectins (Table 1). The viral strains were selected based on known differences in N-linked glycosylation on the viral haemagglutinin that render some of the strains (e.g. Phil82BS, Braz78BS and PR-8) relatively resistant to inhibition by SP-D, conglutinin or MBL. The Phil82BS and Braz78BS strains differ from the parent Phil82 and Braz78 strains in that they lack a single high mannose oligosaccharide attachment on the viral haemagglutinin [25]. PR-8 is a mouse-adapted strain that lacks high mannose attachments on its haemagglutination [26].

RCL-43 was more potent at haemagglutination inhibition of the parent Phil82 and Braz78 strains than the other wild-type collectins tested, with the exception of conglutinin. Notably, RCL-43 and serum CL-43 had very similar haemagglutination inhibitory activity against the Phil82 IAV strain. This inhibitory activity was calcium-dependent, since addition of EDTA abrogated the activity. The Phil82BS and Braz78BS strains were relatively resistant to inhibition by RCL-43. However, RCL-43 was more potent than most of the other collectins tested at inhibiting haemagglutination activity of the Phil82BS or Braz78BS strains. Neither RCL-43 nor bovine serum CL-43 had discernible inhibitory activity against the PR-8 strain. In summary, these studies further confirm the functional similarities of natural and RCL-43 and indicate that CL-43 probably mediates inhibition of IAV, predominantly with calcium-dependent attachment to high mannose carbohydrates present on the viral haemagglutinin. However, the increased inhibitory activity of RCL-43 against the Phil82BS and Braz78BS strains suggests that CL-43 may have additional modes of attachment to some IAV strains.

Since CL-43 differs from the other wild-type collectins in that it exists only as a trimer, we directly compared the haemagglutination inhibitory activity of two recombinant trimeric

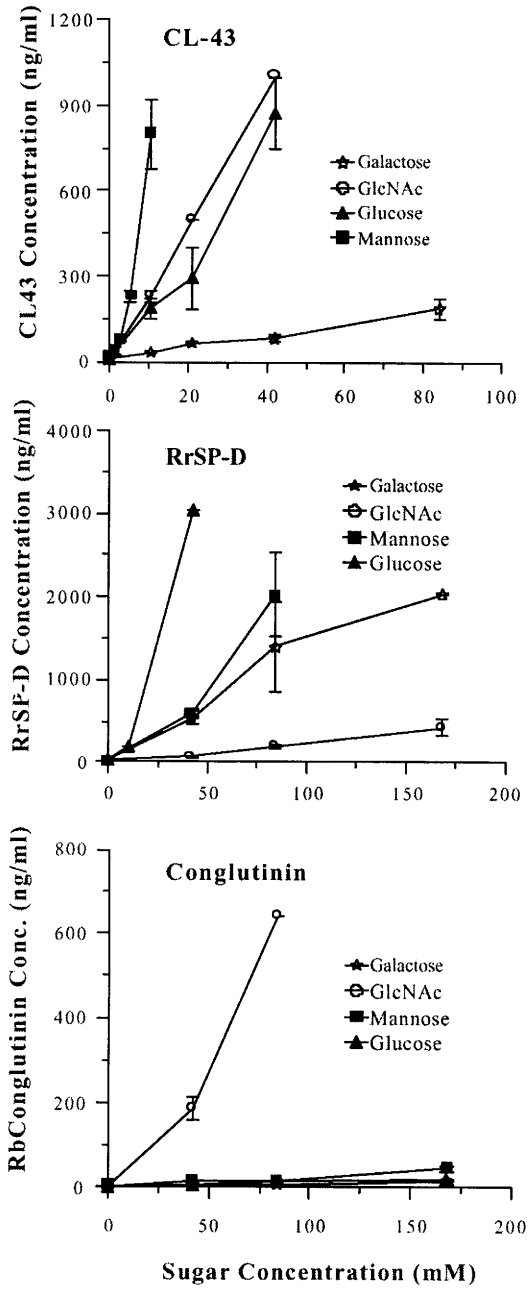

Figure 4 Effect of monosaccharides on the ability of recombinant bovine RCL-43, rat SP-D, or bovine conglutinin to inhibit IAV haemagglutination activity

Haemagglutination inhibitory activity was measured using type O human red blood cells as described in the Materials and methods section. The results shown are the collectin concentrations required to inhibit haemagglutination activity of a fixed concentration of IAV (Phil82 strain). Before haemagglutination inhibition assays, the collectins were preincubated in PBS alone, or PBS containing increasing concentrations of various monosaccharide preparations as indicated. Certain monosaccharides (e.g. GlcNAc in the case of conglutinin) competed against the haemagglutination-inhibitory activity of the collectins such that increasing concentrations of the collectins were required to achieve haemagglutination inhibition. Results shown are the means ± S.E.M. for 3 or more experiments. Note that CL-43 differed from both conglutinin and SP-D in terms of type and concentration of specific monosaccharides that competed against its haemagglutination inhibitory activity.

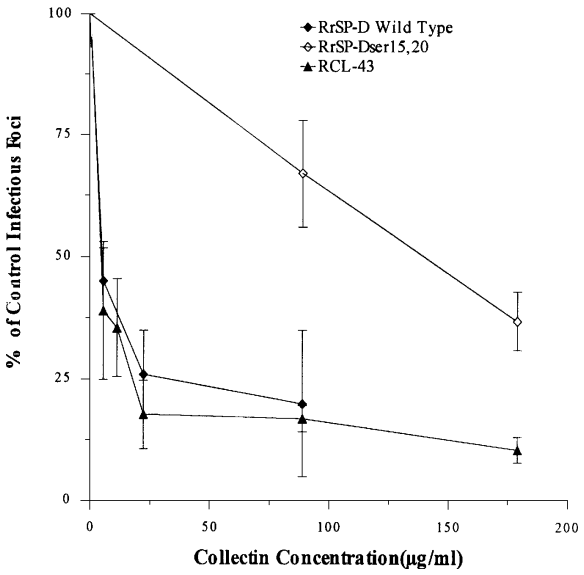

Figure 5 RCL-43 has greater potency in neutralizing the infectivity of IAV than RrSP-Dser15/20

Infectivity of IAV (Phil82 strain) for MDCK cells was assessed using a fluorescent focus forming assay as described in the Materials and methods section. Results are expressed as percentage of control foci present after infection with collectin-treated as compared with the same concentration of untreated virus. The neutralizing activity of RCL-43 is compared with that of RrSP-D and RrSP-Dser15/20. The neutralizing activity of RCL-43 was significantly higher than that of RrSP-Dser15/20 ($P \leqslant 0.05$ in all of the tested concentrations). Results are the means ± S.E.M. for 4 or more experiments.

collectin preparations with that of CL-43. Only RrSP-Dser15,20 forms trimers due to replacement of two N-terminal cysteines at positions 15 and 20 with serines, which precludes inter-subunit cross-links. CL-43 had significantly higher haemagglutination inhibitory activity than RrSP-Dser15,20 (Table 1). We have previously reported that dodecamers of a chimaeric collectin containing the N-terminal and collagen domains of human SP-D and the neck domain and CRD of MBL (SP-D/$MBL_{neck+CRD}$) has higher haemagglutination inhibitory or neutralizing activity than wild-type SP-D or MBL [22]. Using gel filtration, it was possible to isolate sufficient quantities of a trimeric fraction of the SP-D/$MBL_{neck+CRD}$ chimaera to compare its haemagglutination inhibitory activity with that of RCL-43. As shown in Table 1, trimeric SP-D/$MBL_{neck+CRD}$ had significantly lower haemagglutination inhibitory activity than RCL-43. Note that trimeric SP-D/$MBL_{neck+CRD}$ had significantly lower haemagglutination inhibitory activity than dodecamers of multimers of the chimaera. The increased activity of CL-43 trimers as compared with trimers of SP-D or SP-D/$MBL_{neck+CRD}$ suggests that the CRD of CL-43 has intrinsically higher anti-influenza activity than that of either SP-D or MBL.

To test further the hypothesis that distinctive properties of the CRD of CL-43 play a role in the enhanced ability of CL-43 to inhibit viral haemagglutination activity, we tested various monosaccharides as competitors of the collectins' haemagglutination inhibitory activity (Figure 4). We have previously demonstrated that SP-D and MBL have different profiles in this assay [22]. As shown in Figure 4, glucose and mannose effectively competed against the haemagglutination inhibitory activity of SP-D, whereas GlcNAc caused little competition. In contrast, GlcNAc was the most potent competitor of the haemagglutination inhibitory activity of conglutinin. RCL-43 appeared to combine features of SP-D and conglutinin in this assay. As predicted from the mannan binding assays, mannose was the most effective

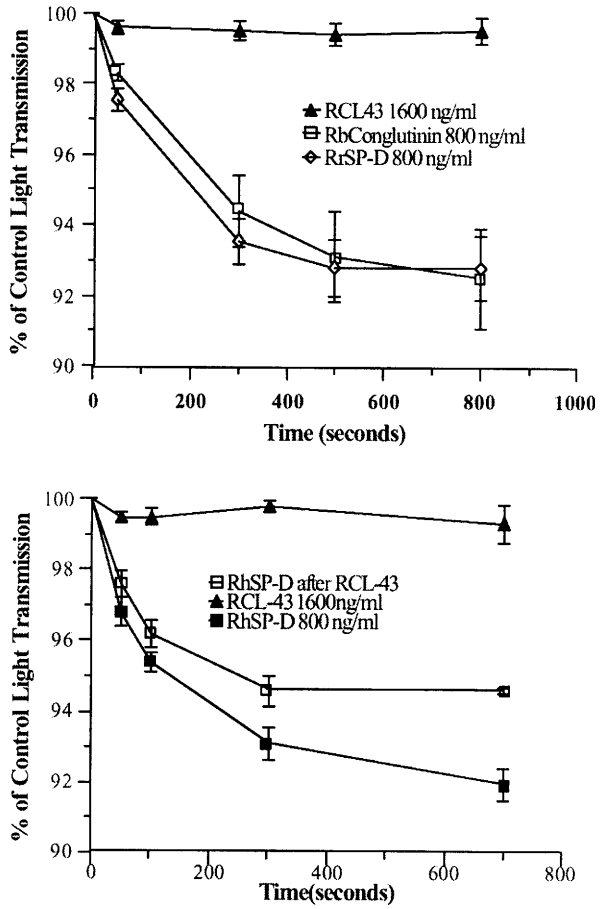

Figure 6 RCL-43 does not cause aggregation of IAV particles

Light transmission through stirred suspensions of IAV particles (Phil82 strain) was monitored after addition of the indicated collectin preparations at time 0. Results are means ± S.E.M. for 3 or more experiments and are expressed as percentage of control light transmission. No change in light transmission occurred in control samples not treated with collectins. RCL-43 caused no greater change in light transmission than control buffer alone. RrSP-D and RbConglutinin caused significant reduction in light transmission ($P < 0.005$ compared with control buffer or RCL-43). RhSP-D (0.8 μg/ml) also caused significant viral aggregation (lower panel). Preincubation of IAV with RCL-43 (1.6 μg/ml) significantly decreased aggregation caused by RhSP-D ($P < 0.02$; see RhSP-D after RCL-43; lower panel).

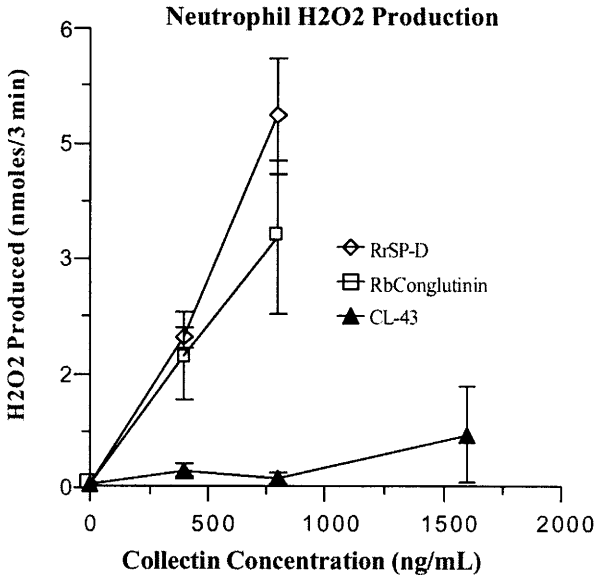

Figure 7 Preincubation of IAV with RCL-43 does not increase IAV-induced neutrophil H_2O_2 generation

IAV samples (Phil82 strain) were preincubated with collectins using the method shown in Figure 6. Freshly isolated neutrophils were added to these viral samples and H_2O_2 production was measured continuously using the fluorescent scopoletin method. Results shown are means ± S.E.M. for rate of H_2O_2 production in nmol · min^{-1} · $(4 \times 10^6$ neutrophils$)^{-1}$ · for three or more experiments. RrSP-D and RbConglutinin caused significant enhancement ($P < 0.05$) of neutrophil H_2O_2 production compared with that induced by IAV alone; however, preincubation with RCL-43 had no effect.

monosaccharide competitor of the haemagglutination inhibitory activity of CL-43. However, glucose and GlcNAc also strongly competed against RCL-43-mediated haemagglutination inhibition.

RCL-43 has higher viral neutralizing activity than other recombinant trimeric collectins

RCL-43 also strongly inhibited infectivity of the Phil82 strain of IAV as assessed using an assay for infectious foci formed in MDCK cell monolayers (Figure 5). RCL-43 had neutralizing activity comparable with wild-type RrSP-D. However, RCL-43 caused significantly greater reduction in infectivity than SP-D trimers, i.e. RrSP-Dser15/20 (Figure 5). RCL-43 also had substantially higher viral neutralizing activity than the trimeric fraction of SP-D/$MBL_{neck+CRD}$ [22]. Bovine serum CL-43 also significantly inhibited infectivity of IAV (90 ng/ml of bovine serum CL-43 decreased infectious foci to 49 ± 14 % of control; $n = 4$; $P < 0.03$).

RCL-43 does not induce viral aggregation

In marked contrast with results obtained for RbConglutinin or RrSP-D, RCL-43 did not induce viral aggregation (Figure 6). Preincubation of IAV with RCL-43 also significantly decreased the ability of SP-D to cause viral aggregation (Figure 6).

RCL-43 does not enhance IAV-induced neutrophil H_2O_2 generation

In our previous studies, the ability of various collectin preparations to enhance IAV-induced H_2O_2 production correlated with their ability to cause viral aggregation [19]. Results obtained with RCL-43 were consistent with previous findings (Figure 7). Whereas RrSP-D and RbConglutinin strongly enhanced IAV-induced respiratory burst responses, RCL-43 did not. Preincubation of IAV with RCL-43 (1.6 μg/ml) also significantly decreased the ability of SP-D (800 ng/ml) to enhance IAV-induced H_2O_2 production (i.e. H_2O_2 production was decreased by 28 ± 5 % as compared with IAV treated with SP-D alone; $n = 4$; $P < 0.01$).

RCL-43 increases neutrophil uptake of IAV over a broad range of concentrations

We have also previously reported that SP-D and conglutinin enhance neutrophil binding and uptake of IAV as assessed by mean fluorescence of neutrophils treated with FITC-labelled IAV [3,4]. Several lines of evidence indicated that this enhanced uptake resulted from binding of viral aggregates by neutrophils [27]. Therefore we expected that RCL-43 might not enhance viral uptake. However, as shown in Figure 8, RCL-43 caused similar or greater enhancement of viral uptake than RbConglutinin or RrSP-D. RbConglutinin and RrSP-D caused maximal enhance-

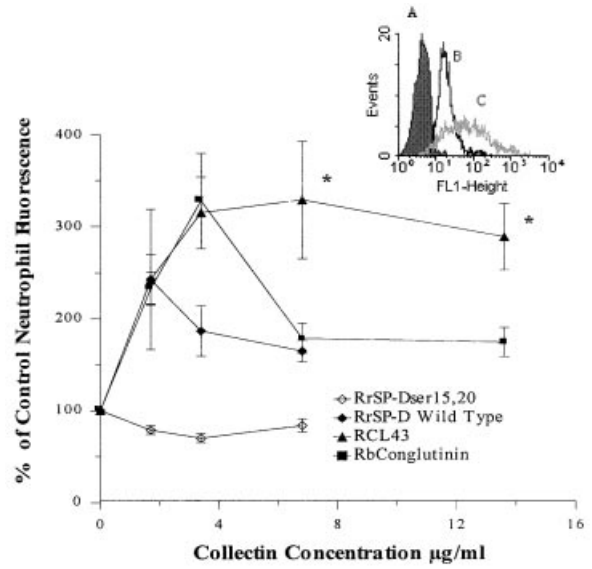

Figure 8 RCL-43 is more potent than RbConglutinin, wild-type RrSP-D or RrSP-Dser15,20 in increasing neutrophil uptake of IAV

FITC-labelled IAV (Phil82 strain) samples were treated with collectins as indicated and then incubated with human neutrophils. Viral uptake was then assessed by incubation of collectin-treated viral samples with neutrophils for 30 min at 37 °C, followed by addition of Trypan Blue to quench extracellular fluorescence, fixation of cells with paraformaldehyde and assessment of neutrophil associated fluorescence by flow cytometry. Results represent means ± S.E.M. for 3 or more experiments (using different blood donors) and are expressed as percentage of control neutrophil fluorescence in collectin-treated/control viral samples (> 1000 neutrophils counted/sample). All concentrations of RCL-43 shown caused significantly increased uptake of IAV as compared with uptake of untreated IAV ($P \leqslant 0.04$). The concentrations at which RCL-43 caused significantly greater increase in IAV uptake than RbConglutinin or RrSP-D are indicated by *. RrSP-Dser15,20 did not increase viral uptake at any concentration tested. The inset shows flow cytometry tracings from a representative experiment testing neutrophil uptake of unlabelled virus (A), FITC-labelled virus alone (B) or FITC-labelled virus pre-treated with 7 μg/ml of RCL-43 (C).

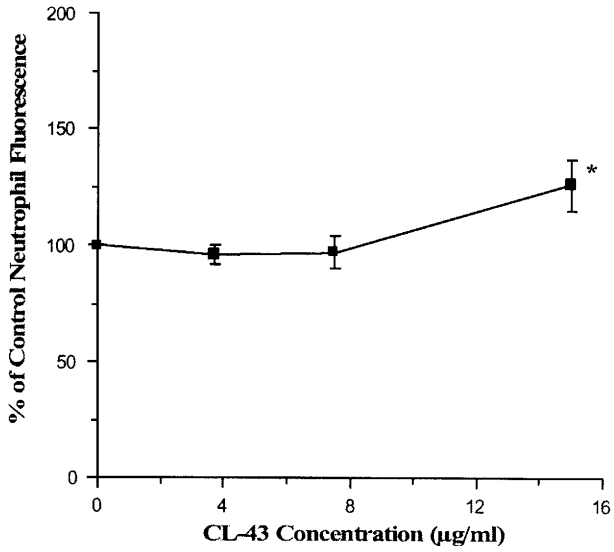

Figure 9 Effect of neutrophil preincubation with RCL-43 on subsequent uptake of IAV

These experiments were performed as described in Figure 8, except that RCL-43 was not preincubated with IAV before addition to neutrophils. Instead, RCL-43 was incubated with neutrophils for 30 min at 4 °C (to avoid internalization), followed by washing off of unbound CL-43 (twice in PBS) and then incubation of the neutrophils with FITC-labelled IAV. Using this method, uptake of IAV was only increased slightly at the highest concentration of RCL-43 used. Results are means ± S.E.M. for 4 experiments using different blood donors (*$P \leqslant 0.05$ compared with control).

ment of viral uptake at collectin concentrations of approx. 1.8–3 μg/ml. Higher concentrations of either RbConglutinin or RrSP-D caused less enhancement of uptake. A similar decline in viral uptake was not observed when concentrations of 7 or 14 μg/ml of RCL-43 were used (Figure 8). Results obtained with RrSP-Dser15,20 contrasted sharply with those obtained with RCL-43. RrSP-Dser15,20 significantly decreased viral uptake at all concentrations tested. Like RCL-43, bovine serum CL-43 increased uptake of IAV: 7 μg/ml of bovine serum CL-43 increased uptake to 256 ± 43 % of control (results not shown; $n = 4$; $P < 0.04$).

To clarify further the mechanism through which CL-43 increases neutrophil uptake of IAV, the cells were first pre-incubated with various concentrations of RCL-43 alone. This was done at 4 °C to avoid internalization of CL-43. The neutrophils were then washed to remove unbound RCL-43 and the FITC-labelled virus was added. Under these conditions RCL-43 caused only minimal increase in viral uptake at the highest concentration tested (Figure 9).

DISCUSSION

The absence of subunit multimerization and a unique profile of saccharide selectivity distinguish CL-43 from the other collectins. Present studies demonstrate that the distinctive biochemical properties of CL-43 isolated from bovine serum are retained in RCL-43. Both proteins are assembled as trimers (Figure 1), indicating that the primary sequence of the protein (and not secondary events like amino terminal degradation by proteases) is responsible for the failure to form higher order multimers. The distinctive monosaccharide binding affinities of serum CL-43 are also evident in RCL-43. The major species in our preparations of serum CL-43 showed slightly greater mobility than RCL-43 (Figure 2). As previously indicated, serum CL-43 often undergoes limited N-terminal degradation [14]. The development of RCL-43 allowed us to evaluate for the first time the functional properties of a fully intact preparation of the protein. Because the site of cleavage of serum CL-43 is N-terminal to the disulphide cross-links and remote from the CRD, this is not expected to result in differences in the oligomerization or lectin activity. At present, no specific functional activities have been localized to the first several amino acids. Thus it is not surprising that there were no major functional differences between the natural and recombinant proteins.

Distinctive biochemical properties of CL-43 are also reflected in distinctive interactions with IAV and neutrophils as compared with other collectins. The characteristic monosaccharide binding profile of CL-43 was evident in assays testing the ability of specific monosaccharides to compete against viral haemagglutination inhibition caused by CL-43 (Figure 4). SP-D, conglutinin and MBL bind to carbohydrates on viral envelope proteins (haemagglutination and neuraminidase) [7]. Binding of these collectins to the haemagglutination appears to be most important for viral neutralization. The mechanism of haemagglutination inhibition by CL-43 was similar to that of SP-D, MBL and conglutinin (e.g. calcium-dependent attachment to virus-associated carbohydrates). However, the potency of CL-43 at haemagglutination inhibition and viral neutralization was

remarkable in view of the fact that CL-43 does not form higher order multimers.

Previous studies have clearly demonstrated that the potency of various forms of SP-D in haemagglutination inhibition and neutralization assays is strongly associated with the degree of multimerization of the protein [5,6,19,22,28]. Trimeric forms of SP-D (or the SP-D/MBL chimaera) had markedly decreased activity in these assays as compared with dodecameric or more highly multimerized forms (see references cited above, Table 1 and Figure 5). RCL-43 had comparable haemagglutination inhibitory and neutralizing activity against wild-type IAV strains (e.g. Phil82 or Braz78) as fully multimerized RrSP-D and substantially higher activity than other trimeric collectins or MBL. Furthermore, the haemagglutination inhibitory activity of RCL-43 against bovine serum inhibitor resistant strains of IAV (e.g. Phil82BS or Braz78BS) was significantly higher than that of any of the other collectins tested. Enhanced haemagglutination inhibitory activity against Phil82 was also documented for bovine serum CL-43.

We have previously shown that another bovine serum collectin, conglutinin, also has strong anti-influenza activity mediated by its CRD [3,20]. Both CL-43 and conglutinin appear to have evolved from gene duplications of SP-D in cattle. It is unclear which evolutionary pressures lead to the development of additional collectins in cattle. However, our results support the concept that these molecules play a role in innate host defence. It is of interest in this regard that cows and related species have not been found to be a natural reservoir for influenza viruses despite living in herds in proximity to other species (i.e. humans, pigs, birds and horses) that are natural hosts for IAV [29]. This suggests that the additional collectins present in cows may increase their resistance to IAV.

The enhanced haemagglutination inhibitory activity of CL-43 most likely reflects specific binding attributes of its CRD. Hence, further study of binding of CL-43 to viral proteins as described in [7] is warranted. Furthermore, it appears likely that incorporation of the CRD of CL-43 into a chimaeric protein capable of forming dodecamers or higher order multimers (e.g. forming an SP-D/CL-43 chimaera) would result in a molecule with significantly higher antiviral activity than SP-D.

As predicted based on its trimeric structure, CL-43 did not induce viral aggregation. Previous studies have shown an association between the ability of SP-D preparations to induce viral aggregation and their ability to enhance neutrophil respiratory burst responses to IAV or to increase neutrophil uptake of IAV. Aggregates of SP-D- or conglutinin-treated IAV are taken up with a mechanism that appears to involve attachment of free viral haemagglutination molecules to sialylated receptors on the neutrophil surface [27]. IAV-induced H_2O_2 production is mediated by cross-linking of sialylated neutrophil surface receptors [30,31]. Larger viral particles, or aggregates of IAV formed by SP-D, appear to cause increased H_2O_2 production by causing more extensive cross-linking of sialylated receptors [27,32]. The failure of CL-43 to enhance IAV-induced neutrophil H_2O_2 production is consistent with these previous results, since CL-43 did not induce viral aggregation.

It was remarkable, however, that CL-43 and RCL-43 substantially increased neutrophil uptake of IAV in the absence of viral aggregation or enhancement of H_2O_2 production. We have also found that RCL-43 increases neutrophil uptake of *Escherichia coli* without causing aggregation of the bacteria (K. Hartshorn, M. R. White, E. Crouch and U. Holmekov, unpublished work). These findings suggest that CL-43 modifies neutrophil uptake of IAV or bacteria by mechanisms distinct from SP-D or conglutinin. This effect of CL-43 does not appear to involve prominently a direct effect of CL-43 on the neutrophils, since CL-43 caused minimal increase in viral uptake when neutrophils were first incubated with CL-43 followed by addition of the virus (Figure 9). Further studies will be needed to determine which domains of CL-43 contribute to enhanced uptake. For example, it is possible that the N-terminal domain of CL-43 differs from those of SP-D or conglutinin in terms of binding to certain phagocyte collectin receptors [14]. Definitive demonstration of the contribution of the CL-43 N-terminus to enhancement of viral or bacterial uptake by phagocytes will involve study of chimaeric collectins incorporating this domain, or mutagenesis of the domain.

In summary, we demonstrate that distinctive biochemical properties of the CL-43 N-terminus and CRD are retained in a recombinant form of the molecule. These biochemical features are associated with distinctive antiviral properties, suggesting that CL-43 may make specific contributions to innate host defence. Incorporation of domains of CL-43 in novel collectin constructs will be instructive for understanding the structural basis for specific functional attributes of collectins and could result in molecules with therapeutic potential in treatment of IAV or other infectious diseases.

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