Binding of human collectins (SP-A and MBP) to influenza virus

Rajneesh MALHOTRA,*§ John S. HAURUM,† Steffen THIEL‡ and Robert B. SIM*

*MRC Immunochemistry Unit, Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, U.K.,

†Institute of Molecular Medicine, John Radcliffe Hospital, Oxford OX3 9DU, U.K.,

and ‡Institute for Medical Microbiology, Bartholins Building, University of Aarhus, University Park, 8000 Aarhus, Denmark

Collectins are a group of soluble proteins each of which has collagenous domains and non-collagenous globular domains, the latter containing the consensus residues found in C-type lectins. Members of the collectin family are the serum proteins mannan-binding protein (MBP), conglutinin, CL-43, and the lung-associated proteins surfactant protein A (SP-A) and surfactant protein D (SP-D). MBP and conglutinin have been shown previously to bind to influenza viruses and to inhibit the infectivity and haemagglutinating activity of influenza viruses. We report here that the lung protein SP-A, like MBP, can bind to influenza

virus (strain A/X31) through its lectin domain and inhibit the virus-mediated agglutination of red cells. The binding of SP-A or MBP to influenza virus was saturable, concentration-dependent, and required the presence of Ca^{2+} ions. Ligand-blot analysis, using MBP as ligand, of the virus lysate indicated that MBP binds to a 68 kDa viral species. The 68 kDa species was isolated to homogeneity and was shown to be the viral neuraminidase. The purified 68 kDa species inhibited the binding of both MBP and SP-A to influenza virus.

INTRODUCTION

Collectins are a group of soluble proteins each of which has collagenous domains and non-collagenous globular domains, the latter containing the consensus residues found in C-type lectins. Members of the collectin family are the serum proteins mannan-binding protein (MBP), conglutinin, CL-43 and the lung-associated proteins, surfactant protein A (SP-A) and surfactant protein D (SP-D) [1–3]. SP-A is the most abundant surfactant-associated protein present in the lung and the respiratory tract. It is a complex molecule composed of 18 identical or very similar polypeptide chains. Each polypeptide consists of a short N-terminal segment, followed by a region of collagen-like sequence (characterized by the repeating triplet sequence Gly-Xaa-Yaa, where Yaa is often a hydroxylated amino acid). The C-terminal half of the polypeptide is non-collagenous, and contains a structure known as a C-type lectin domain. C-type lectins are characterized by Ca^{2+} -dependent binding to carbohydrates, and this is a property of SP-A [2–5]. Subunits of the protein are each built up by association of three polypeptide chains, the collagenous regions of which intertwine to form a collagen triple helix. The non-collagenous C-terminal halves form a globular ‘head’. Six such subunits associate to form the characteristic ‘bunch of tulips’ structure seen in electron microscopy of SP-A. In this structure, the globular heads form the ‘flowers’, and the collagen helices form the stalks [2,3,6]. This structure is also characteristic of the complement protein C1q, and of MBP, both of which activate the classical pathway of the complement system [2,7]. SP-A, however, does not activate complement [2,7,8]. Three other proteins, conglutinin, CL-43 [8,9] and lung SP-D, which also do not activate complement [2,7,8], have similar structures made up of four subunits, each with a globular head containing C-type lectin domains, connected to collagen triple helices which are longer than those found in C1q, SP-A and MBP [7–11]. C1q, SP-A, MBP, CL-43 and conglutinin all bind in a non- Ca^{2+} -dependent manner to the collectin receptor (C1q receptor) [8,12–14] which is present on lung epithelium [15] and

on many other cell types [16]. In C1q, a region of high charge density in the collagenous domain interacts with the receptor [14,17], and this is the likely site of receptor interaction in the other proteins [14]. SP-D may also be a ligand for this receptor, but this has not yet been demonstrated conclusively. C1q, MBP and conglutinin all have host defence roles, as opsonins [2,3,10]: they bind to targets via their globular head regions, and also to the collectin receptor through the collagen domain. SP-A appears to have a physiological role in regulating uptake and secretion of surfactant [18,19]. However, the function of the lectin domains of SP-A is not clearly established: i.e. it is not known what range of carbohydrate-bearing targets is recognized by the lectin domains. A developing hypothesis is that SP-A is likely to bind to a wide range of inhaled materials via its lectin domains, and to mediate their interaction with cells (phagocytes or specialized epithelium) as part of a natural defence system [1–3,7,10]. SP-A has been shown to play a role in host defence mechanisms [20,21] and is known to bind to *Pneumocystis carinii* [22], pollen grains and pollen grain extracts [23] through its lectin domain. Recently McNeely and Coonrod [24] reported that SP-A interacts with *Staphylococcus aureus* carbohydrate and mediates its attachment to alveolar macrophages.

Human MBP and bovine conglutinin have been shown to inhibit the infectivity and haemagglutinating activity of influenza virus of the H1 and H3 subtypes [25,26]. We considered it likely that SP-A, like MBP and conglutinin, could interact with carbohydrates on the surface of influenza virus and influence infectivity in the lung. In this paper we have investigated the characteristics of binding of human SP-A and human MBP to influenza virus strain A/X31.

MATERIALS AND METHODS

Purification of SP-A and MBP

MBP and SP-A were purified as described previously [5,12]. SP-A was biotinylated by incubating 2 ml of SP-A (100 $\mu\text{g}/\text{ml}$) with 10 μl of biotinyl-*N*-hydroxysuccinimide ester (‘BNHS’; Sigma

Abbreviations used: MBP, mannan-binding protein; SP-A, surfactant protein A; SP-D, surfactant protein D; HAU, haemagglutination units; MBPT-INF, MBP target from influenza virus.

§ To whom correspondence should be addressed.

Chemical Co., Poole, Dorset, U.K.) (5 mg/ml) in 0.1 M NaHCO₃ buffer (pH 8.0) for 3 h at room temperature. Biotinylated protein was dialysed three times against 10 mM sodium phosphate buffer (pH 7.4) to remove unbound labelling reagents.

SDS/PAGE

This was carried out as described by Laemmli [27]. Samples were prepared (reduced and/or alkylated) as described by Fairbanks et al. [28]. Proteins on gels were detected with Coomassie Brilliant Blue [28].

Interaction of SP-A and MBP with Influenza virus

Influenza virus (strain A/X31) (H3N2) was generously provided by Professor A. J. McMichael, Institute of Molecular Medicine, Oxford. The virus was grown in embryonated eggs [29]. Virus concentration in allantoic fluid was estimated by haemagglutination assay as described by Barrett and Inglis [29], and expressed as haemagglutination units (HAU). Monoclonal anti-haemagglutinin antibody was purified on Protein A-Sepharose from culture supernatant of the hybridoma MHM.3 (also provided by Professor A. J. McMichael). Microtitre plate wells (Nunc, Kamstrup, Roskilde, Denmark), were coated with purified monoclonal anti-haemagglutinin antibody MHM.3 (100 µl; 2.5 µg per well) in 100 mM Na₂CO₃ buffer (pH 9.6) for 12 h at 4 °C. Non-specific sites were blocked with BSA (200 µl per well; 5 mg/ml) in coating buffer. Influenza-virus suspension (250 HAU per well) in 10 mM Tris/HCl buffer (pH 7.4) containing 150 mM NaCl was placed in the anti-haemagglutinin-coated wells for 3 h at room temperature. After extensive washing with 10 mM Tris/HCl/150 mM NaCl, pH 7.4, the bound virus was incubated with serial dilutions of biotinylated SP-A (100 µl; maximum amount 1.25 µg) or MBP (100 µl; maximum amount 0.625 µg/well) in 10 mM Tris/HCl/150 mM NaCl, pH 7.4, containing either 5 mM CaCl₂ or 5 mM EDTA for 3 h at 37 °C. For inhibition studies, 50 µl of MBP (0.15 µg per well) or biotinylated SP-A (0.6 µg per well) was loaded on to virus-coated wells in the presence of mannose (50 µl; final concn. 250 mM) or other competitors in 10 mM Tris/HCl/150 mM NaCl, pH 7.4, containing 5 mM CaCl₂. After washing with the same buffer, bound MBP was detected by adding to each well 100 µl of horseradish-peroxidase-conjugated G2a monoclonal antibody (0.2 µg/ml) specific for MBP [30] for 2 h at room temperature. Bound biotinylated SP-A was detected with 100 µl of horseradish-peroxidase-conjugated avidin (2.5 µg/ml) (Sigma). After extensive washing, the horseradish-peroxidase activity was detected with *o*-phenylenediamine dihydrochloride (Fast enzyme substrate; Sigma, Catalogue no. P-9187). The reaction was stopped with 4 M H₂SO₄, and A₄₉₂ was measured.

Ligand-blot analysis of virus lysate, using MBP

Influenza-virus suspension (500 HAU per sample) was run on SDS/PAGE under reducing conditions, and transferred to nitrocellulose as described previously [31] (Hybond C; Amersham International, Amersham, Bucks., U.K.). The non-specific sites were blocked with BSA (3 mg/ml) in 10 mM Tris/HCl buffer (pH 7.4) containing 150 mM NaCl. Different lanes containing identical quantities of the virus proteins were treated separately for 14 h at 4 °C with MBP (5 ml; 0.2 µg/ml) in (a) 10 mM Tris/HCl/150 mM NaCl (pH 7.4) containing BSA (3 mg/ml) and 5 mM CaCl₂, or (b) the same buffer but with 5 mM EDTA instead of CaCl₂, or (c) the same buffer with 5 mM CaCl₂ plus 250 mM mannose. After extensive washing with buffer *a*, *b* or *c* as appropriate, the blots were incubated with horseradish-

peroxidase-conjugated G2a monoclonal antibody (0.2 µg/ml) for 2 h at room temperature in buffer *a*, *b* or *c*. After extensive washing with the same buffer, the horseradish-peroxidase activity was detected with 3,3'-diaminobenzidine tetrahydrochloride (Fast enzyme substrate tablets; Sigma, catalogue no. D-4293).

Purification from influenza virus of proteins which are recognized by MBP

Influenza virus suspension (25000 HAU; 1 ml) in allantoic fluid was diluted with 10 ml of 10 mM Tris/HCl buffer (pH 7.4) containing 0.15 mM CaCl₂, 0.5 mM MgCl₂, 1% (v/v) Nonidet P40, 100 µg/ml soya-bean trypsin inhibitor (Sigma, type I-S), 5 mM iodoacetamide, 2.5 mM Pefabloc SC [4-(2-aminoethyl)-benzenesulphonyl fluoride: Pentapharm A.G., Basel, Switzerland], 20 µM 1,10-phenanthroline and 5 µg/ml pepstatin A (Sigma) for 1 h at 4 °C. The lysate was frozen and thawed twice, and the insoluble material was spun down at 15000 rev./min for 25 min. The supernatant was loaded on to a Mono Q FPLC column (1 ml; Pharmacia, Milton Keynes, U.K.), pre-equilibrated with 10 mM Tris/HCl buffer (pH 7.4). The column was washed extensively and the bound proteins were eluted with a linear gradient (25 ml) (flow rate 1 ml/min) of 0–500 mM NaCl in 10 mM Tris/HCl (pH 7.4). The eluted fractions were tested for MBP-binding activity. The fractions positive for MBP binding were pooled and were designated as MBPT-INF (MBP target from influenza virus). The pooled samples were dialysed against 10 mM Tris/HCl (pH 7.4) containing 5 mM EDTA and were re-loaded on to the Mono Q FPLC column, equilibrated with 10 mM Tris/HCl (pH 7.4) containing 5 mM EDTA. The bound proteins were eluted with a linear NaCl gradient (40 ml; 0–500 mM) in 10 mM Tris/HCl (pH 7.4) containing 5 mM EDTA.

Haemagglutination-inhibition test [29]

Influenza virus suspension (25 µl; 5 HAU) in 10 mM Tris/HCl/150 mM NaCl, pH 7.4, containing 5 mM CaCl₂ or 5 mM EDTA was incubated with serial dilutions of MBP (25 µl; maximum amount 1.25 µg) or SP-A (25 µl; maximum amount 1.25 µg) in 10 mM Tris/HCl/150 mM NaCl, pH 7.4, for 1 h at room temperature. The mixture was then incubated with 50 µl of 1% (v/v) human type O red blood cells in 10 mM Tris/HCl/150 mM NaCl, pH 7.4, containing 5 mM CaCl₂ or 5 mM EDTA at room temperature for 45 min. The inhibition of virus-mediated agglutination of erythrocytes by collectins was observed.

Binding of MBP to influenza-virus proteins

Microtitre-plate wells were coated with individual fractions eluted from Mono Q FPLC chromatography of the virus lysate (diluted 4-fold in 100 mM Na₂CO₃ buffer, pH 9.6; final volume 100 µl) for 3 h at room temperature and non-specific binding sites were blocked with BSA as described above. Purified MBP (50 µl; amount 0.2 µg) in 10 mM Tris/HCl/150 mM NaCl, pH 7.4, containing 5 mM CaCl₂ or 5 mM EDTA was loaded on to protein-coated wells and left for 16 h at 4 °C. Bound MBP was detected, after extensive washing with the same buffer, with G2a monoclonal antibody as described above.

Binding characteristics of the purified MBPT-INF

Microtitre-plate wells were coated with 100 µl of the purified target glycoprotein (approx. 0.1 µg/well, as estimated by A₂₈₀) in 100 mM Na₂CO₃ buffer (pH 9.6), and non-specific sites were

blocked with BSA, as above. Serial dilutions of biotinylated SP-A (100 μ l; maximum amount 2.5 μ g) or MBP (100 μ l; maximum amount 1.25 μ g) were loaded on to MBPT-INF-coated wells in 10 mM Tris/HCl/150 mM NaCl, pH 7.4, containing 5 mM CaCl₂ or 5 mM EDTA or 5 mM CaCl₂/250 mM mannose. After extensive washing with the same buffers, the bound SP-A and MBP were detected as described above.

Neuraminidase assay [32]

Protein samples (65 μ l; amount 0.1 μ g) were incubated with 2'-(4-methylumbelliferyl)- α -D-N-acetylneuraminic acid (35 μ l; 1 mg/ml) (Sigma) in 0.4 M sodium acetate buffer, pH 4.2, for different time intervals at 37 °C. At the given time, the reaction was stopped by adding 1 ml of 0.085 M glycine/carbonate buffer (pH 10). The enzyme activity was measured by reading the fluorescence (365 nm excitation, 450 nm emission) on a fluorimeter. The amount of 4-methylumbelliferone (4-MU) released was estimated from a standard curve showing fluorescence intensity versus concentration of pure 4-MU; 1 unit of enzyme activity was defined as the activity required to release 1 mmol of 4-MU in 1 min at 37 °C.

Immunoassays (antigen-capture assays) and binding assay to identify the target glycoprotein

To identify the glycoprotein recognized by MBP, microtitre-plate wells were coated with serial dilutions of the glycoprotein or soya-bean trypsin inhibitor (as control), and the non-specific sites were blocked with BSA as described above. Monoclonal antibody (MHM.3) against haemagglutinin (100 μ l; 100 μ g/ml) was loaded on to the MBPT-INF-coated microtitre plate for 3 h at 37 °C in PBS/0.1% (v/v) Tween 20 (PBS/Tween 20). The plate was washed extensively and the bound antibody was detected with alkaline-phosphatase-conjugated anti-mouse IgG (dilution 1:8000) (Sigma).

Microtitre-plate wells were coated with 100 μ l of the anti-neuraminidase (N2) monoclonal antibodies N92, N81 or N154 at 50 μ g/ml, or with MBP (50 μ g/ml) or BSA (50 μ g/ml) in 100 mM Na₂CO₃ buffer (pH 9.6). The monoclonal antibodies were kindly provided by Dr. J. J. Skehel and Dr. A. R. Douglas, MRC National Institute of Medical Research, Mill Hill, London, and were raised against strain X31 (A/Aichi/2/68). Non-specific binding sites were blocked with BSA (3 mg/ml) in 10 mM Tris/HCl/150 mM NaCl, pH 7.4, containing 5 mM CaCl₂. Purified MBPT-INF was radioiodinated by the method of Fraker and Speck [33]. The radioiodinated MBPT-INF was analysed by SDS/PAGE on a 10%-acrylamide gel and the autoradiograph developed from the dried gel. ¹²⁵I-labelled MBPT-INF (5 $\times$ 10⁴ c.p.m. loaded per well; specific radioactivity 1 $\times$ 10⁷ c.p.m./ μ g) was added to the wells coated with anti-neuraminidase antibodies, MBP or BSA in 10 mM Tris/HCl/150 mM NaCl, pH 7.4, containing 5 mM CaCl₂. The plate was left for 16 h at 4 °C. The plate was washed extensively with 10 mM Tris/HCl/150 mM NaCl, pH 7.4, containing 5 mM CaCl₂. Bound radioactivity was solubilized and removed with 4 M NaOH. Radioactivity obtained from each well was measured on an LKB 1275 minigamma counter.

RESULTS

Binding of SP-A and MBP to immobilized influenza virus

Anti-haemagglutinin antibody (specific for H3 on influenza strains A/HK and A/X31) was used to immobilize the virus

Figure 1 Binding of MBP and SP-A to influenza virus

Serial dilutions of MBP or SP-A were incubated with solid-phase-immobilized influenza virus as described in the Materials and methods section. Ca²⁺-dependent binding of MBP (a) and SP-A (b) is shown. Binding of MBP and SP-A in the presence of EDTA was subtracted from the results shown. Results of a single experiment, with the average and the range of triplicate experimental points shown by error bars.

Table 1 Binding of MBP and SP-A to influenza virus

The binding of fixed quantities of MBP (0.15 μ g) or SP-A (0.5 μ g) to immobilized influenza virus was tested in the presence of 5 mM CaCl₂, 250 mM mannose and 5 mM CaCl₂, MBPT-INF (0.1 μ g/well) and 5 mM CaCl₂ or 5 mM EDTA, as described in the Materials and methods section. Binding is shown as colour development (A₄₉₂) in the e.i.s.a. Results are means $\pm$ S.D. of six determinations

Binding conditions	MBP binding	SP-A binding
CaCl ₂	0.272 $\pm$ 0.031	0.511 $\pm$ 0.09
Mannose/CaCl ₂	0.086 $\pm$ 0.010	0.328 $\pm$ 0.02
MBPT-INF (0.1 μ g)/CaCl ₂	0.164 $\pm$ 0.005	0.233 $\pm$ 0.04
EDTA	0.004 $\pm$ 0.001	0.209 $\pm$ 0.06

(H3N2) in microtitre-plate wells. Figure 1 shows that the binding of MBP (Figure 1a) and biotinylated SP-A (Figure 1b) to immobilized virus is saturable and concentration-dependent. Binding of MBP to virus was negligible in the presence of EDTA. The binding of MBP observed in the presence of Ca²⁺ ions was inhibited by approx. 70% in the presence of 250 mM mannose (Table 1). In the presence of EDTA the binding of SP-A to immobilized virus was decreased to 40% of that observed in the presence of Ca²⁺ ions. Binding of SP-A in the presence of Ca²⁺ ions was substantially inhibited in the presence of mannose

Figure 2 Inhibition of virus-induced haemagglutination by serial dilutions of SP-A and MBP in the presence of Ca^{2+} or EDTA

Haemagglutination assays were carried out as described in the Materials and methods section.

(Table 1). The results indicate that the binding of MBP to virus is mainly due to the interaction of the lectin domains of MBP with glycosylated species present on the surface of this virus. SP-A has the same mode of binding, but it is possible that SP-A has an additional, non- Ca^{2+} -dependent, mode of binding to the virus.

Inhibition of haemagglutination by MBP and SP-A

It has been previously reported that MBP in the presence of Ca^{2+} ions inhibits influenza-virus-induced agglutination of erythrocytes [26]. In Figure 2 it is shown that the virus-induced agglutination of human blood-group O erythrocytes is inhibited by both SP-A and MBP in the presence of Ca^{2+} ions, whereas no inhibition of agglutination was observed when SP-A and MBP were preincubated with virus in the presence of EDTA. The results shown in Figure 2 indicate that at a given concentration MBP is more effective in inhibiting the agglutination than SP-A, but the concentrations at which inhibition of agglutination of erythrocytes was observed with SP-A (between 0.312 and 0.624 $\mu\text{g}/\text{well}$, i.e. final concentration 3.12–6.24 $\mu\text{g}/\text{ml}$) were below the suggested physiological concentration range (100–140 $\mu\text{g}/\text{ml}$) [32] of SP-A in the lung. Inhibition of agglutination is observed with less than 78 ng of MBP/well, i.e. with a

Figure 4 Elution profile of chromatography of influenza-virus lysate on Mono Q FPLC

(a) Elution profile and Ca^{2+} -dependent MBP-binding activity of the eluted samples from Mono Q FPLC is shown. Binding to MBP in the presence of EDTA was subtracted from the results shown. Fractions containing MBPT-INF are shown by a solid bar. (b) Fractions containing MBPT-INF from (a) were reloaded on Mono Q FPLC in the presence of EDTA. The elution profile of the eluted material from the second Mono Q FPLC is shown. Fractions containing MBPT-INF are shown by a solid bar.

final concentration of 0.78 $\mu\text{g}/\text{ml}$, which is comparable with the average concentration of MBP in serum (about 1 $\mu\text{g}/\text{ml}$) [34].

Figure 3 Ligand-blot and SDS/PAGE analysis of the influenza-virus proteins

Influenza-virus lysate was analysed on SDS/PAGE with Coomassie Blue staining (lane 4). The proteins were transferred on to nitrocellulose and the blot was incubated with MBP in the presence of CaCl_2 (lane 1), EDTA (lane 2) or mannose and CaCl_2 (lane 3) as described in the Materials and methods section. Purified MBPT-INF (SDS/PAGE analysis, Coomassie Blue stain) and the radiolabelled MBPT-INF analysed by SDS/PAGE and autoradiography are shown in lanes 5 and 6 respectively. All samples were reduced.

Purification of the SP-A- and MBP-binding species from influenza virus

To investigate the molecules involved in the interaction of SP-A and MBP with influenza virus, ligand-blot analysis of the virus lysate was performed. In Figure 3 it is shown that MBP interacts specifically with a 68 kDa species in the presence of CaCl_2 (Figure 3, lane 1), but not in the presence of EDTA or mannose (Figure 3, lanes 2 and 3). To investigate further the properties of the 68 kDa species, Mono Q FPLC was used to separate the components of the virus lysate. The fractions eluted from the column were tested for MBP-binding activity. As shown in Figure 4(a), the fractions eluted between NaCl concentrations of 120 mM and 170 mM bound MBP. This activity was confirmed by showing that the same fractions competitively inhibited the binding of MBP to mannan-coated microtitre plates (results not shown). The fractions containing MBP-binding activity were pooled and further fractionated by Mono Q FPLC in the presence of EDTA. The elution profile of the pooled sample from Mono Q FPLC is shown in Figure 4(b). The purified MBP-binding protein from virus (MBPT-INF) behaves as a 68 kDa molecule on SDS/PAGE under reducing conditions (Figure 3, lane 5) and migrates identically with the band identified by the ligand-blot analysis (Figure 3, lane 1).

Figure 5 Characteristics of MBPT-INF

Serial dilutions of SP-A (▲) or MBP (○) were incubated with solid-phase-immobilized MBPT-INF in the presence of Ca^{2+} as described in the Materials and methods section. Binding of SP-A or MBP to MBPT-INF in the presence of EDTA was negligible, but was subtracted from the results shown. Results of a single experiment with the average and the range of triplicate experimental points are shown.

Table 2 Binding of MBP and SP-A to MBPT-INF

Binding of SP-A and MBP to immobilized MBPT-INF was tested in the presence of 5 mM CaCl_2 , 250 mM mannose and 5 mM CaCl_2 or 5 mM EDTA, as described in the Materials and methods section. Binding is expressed as colour developed (A_{492}) in the e.i.s.a. Results are means $\pm$ S.D. of six determinations.

Ligand	Binding		
	CaCl_2	Mannose/ CaCl_2	EDTA
SP-A	0.436 ± 0.005	0.257 ± 0.085	0.043 ± 0.012
MBP	0.402 ± 0.014	0.199 ± 0.021	0.075 ± 0.016

Binding characteristics of MBPT-INF

In Figure 5 it is shown that binding of SP-A and MBP to MBPT-INF is concentration-dependent and shows saturation, or approach to saturation, in the presence of Ca^{2+} ions. Table 2 shows that binding of SP-A and MBP to MBPT-INF was very low in the presence of EDTA. The binding of SP-A and MBP to MBPT-INF was inhibited by 41% and 50% respectively in the presence of 250 mM mannose (Table 2). The maximum obtainable inhibition with mannose was not investigated. In further sets of experiments, inhibition by MBPT-INF of binding of biotinylated SP-A and of MBP to immobilized virus was studied. Table 1 shows that the binding of biotinylated SP-A and MBP was inhibited by 55% and 40% respectively in the presence of a single fixed concentration of purified MBPT-INF.

The binding characteristics of MBPT-INF to MBP and SP-A, together with SDS/PAGE analysis, indicate that MBPT-INF is a glycoprotein. The major glycoproteins of the viral surface are haemagglutinin or neuraminidase [35,36]. The e.i.s.a. using anti-haemagglutinin monoclonal antibody indicated that MBPT-INF is not haemagglutinin (results not shown). To investigate the second possibility, a neuraminidase enzymic assay was performed. Figure 6 shows that the MBPT-INF preparation contains neuraminidase activity, with specific activity of approx. 0.1 unit/mg of protein, or 100 $\mu\text{mol}/\text{min}$ per mg. The specific activity associated with the MBPT-INF fraction is considerably

Figure 6 Neuraminidase enzymic activity

Time course for the neuraminidase activity of MBPT-INF (●) and the control protein BSA (○). Conditions for the assay are described in the Materials and methods section.

Table 3 Interaction of MBPT-INF with monoclonal anti-neuraminidase antibodies and MBP

Binding of ^{125}I -labelled MBPT-INF to anti-neuraminidase antibodies or MBP, bound to microtitre-plate wells, was tested as described in the Materials and methods section. Results are means $\pm$ S.D. of six determinations.

Ligand/antibody	Binding (c.p.m.)
M154	2688 ± 371
M81	5336 ± 528
M192	149 ± 100
MBP	5462 ± 624

lower than that reported for purified *Salmonella typhimurium* sialidase activity (3.9 units/mg), using the same substrate, by Hoyer et al. [32], but falls within the range (0.2–863 $\mu\text{mol}/\text{min}$ per mg) of specific activities, with a variety of substrates, reported by Hoyer et al. [32]. Neuraminidases have very wide variations in turnover of artificial substrates [32], and there are no published data for influenza-virus N2 neuraminidase with this substrate. However, an easily measurable level of neuraminidase activity is present in the purified protein. To establish further the identity of the MBPT-INF, the radioiodinated MBPT-INF was incubated with immobilized anti-neuraminidase monoclonal antibodies and MBP. Table 3 shows that two out of three monoclonal antibodies interacted with the purified radioiodinated MBPT-INF, establishing that MBPT-INF is the viral neuraminidase.

DISCUSSION

Hartley et al. [25] reported that two non-human collectins, mouse MBP and bovine conglutinin, inhibit infectivity and haemagglutinating activity of influenza A virus. Recently Harts-horn et al. [26] reported that human MBP functions as an opsonin and inhibits virus-induced haemagglutination of erythrocytes. The inhibition of haemagglutination activity by MBP and conglutinin was Ca^{2+} -dependent and therefore presumably involved the binding of the lectin domain of the collectins

Figure 7 Schematic illustration of the interaction of collectins with influenza-virus surface molecules

Values for the size of the neuraminidase (□) and haemagglutinin (■) and their respective numbers and relative orientation on the virus surface were obtained from [36]. Relative numbers of neuraminidase and haemagglutinin molecules vary from strain to strain, and no information was available on the strain tested in this work. The work cited ([36]) refers to strains BEL (AO) and X-7F1.

to the surface-expressed carbohydrate on the virus. In this paper we have shown that two human proteins of the collectin family, SP-A and MBP, bind to influenza virus in the presence of Ca^{2+} ions and inhibit the haemagglutinating activity of the virus. A molecule of 68 kDa was identified by the ligand-blot analysis as showing Ca^{2+} -dependent binding to MBP. The isolated 68 kDa species was shown to have SP-A- and MBP-binding activity. A monoclonal antibody against haemagglutinin did not interact with this material, but anti-N2 neuraminidase antibodies did, and the purified molecule showed neuraminidase activity. Thus the Ca^{2+} -dependent binding of SP-A and MBP to influenza virus strain A/X31 takes place through neuraminidase. The molecular mass of neuraminidase from various influenza virus strains has been reported to vary from 48 to 70 kDa [36]. The binding of MBP to virus was completely inhibited in the presence of EDTA, whereas the binding of SP-A to virus was inhibited by only 60% in the presence of EDTA, suggesting more than one type of interaction of SP-A with the surface of the virus and more than one site on SP-A for binding of the virus. However, the inhibition by SP-A of virus-induced haemagglutination (Figure 2) was seen only in the presence of Ca^{2+} ions. It has been reported that SP-A interacts with herpes simplex virus via the charged carbohydrate present on the SP-A molecule [21], but it was not indicated whether the binding required Ca^{2+} ions. Binding of haemagglutinin to sialic acid does not require Ca^{2+} , so it is possible that the binding of SP-A to influenza virus in the presence of EDTA might be through sialic acid on SP-A. If such an interaction does occur, it does not inhibit haemagglutination. The results presented here suggest that SP-A and MBP do not interfere directly with the sialic acid-binding site on haemagglutinin, but the inhibition of haemagglutination of erythrocytes observed is more likely due to steric effects. In Figure 7,

a diagrammatic view of the arrangement of haemagglutinin and neuraminidase on the influenza virus surface is shown. Structurally, SP-A and MBP resemble C1q, and it has been reported that the angle between any two collagenous 'arms' of C1q can vary between 30° and 105° [37]. Assuming that the arms of SP-A and MBP have similar flexibility, it is probable that one collectin molecule can interact with a maximum of six molecules of neuraminidase. The arrangement of bound SP-A or MBP shown in Figure 7 strongly suggests that binding of the collectin to neuraminidase will cause considerable steric interference with the binding of other macromolecules to the haemagglutinin. Steric-hindrance effects of these very large flexible collectins may be an important general feature of their effects on infectivity or adherence of virus to host cells.

Hartshorn et al. [26] reported that human MBP functions not only as an inhibitor of haemagglutination activity, but also as an opsonin, and enhances neutrophil reactivity against influenza virus. They suggested that the interaction of MBP with neutrophils takes place through the collagen domain of MBP. We previously reported a receptor for the collectin family of proteins, the collectin receptor [12,13]. Collectin receptor is widely distributed and has been shown to be present on many cell types, including leucocytes, platelets, endothelium and specialized epithelia [1-3,7]. The binding of collectins to collectin receptor takes place through the collagen domain of the collectins [14]. As MBP interacts with virus through the lectin domain, it is likely that the enhancement of neutrophil activity observed by Hartshorn et al. [26] is mediated by interaction of virus-collectin complex with the collectin receptor.

SP-A is the most abundant surfactant-associated protein present in the lung; therefore interaction of SP-A with virus through the lectin domain may play a major role in neutralizing

the infectivity of the virus and removing the virus by phagocytosis. As binding of SP-A to virus inhibits the haemagglutinating activity of the virus, SP-A is likely to inhibit the haemagglutinin-mediated interaction of virus with host cells, and thus the infectivity. Additionally SP-A, like MBP, may act as an opsonin and thus help in removal of virus from the host lung. McNeely and Coonrod [24] have shown that *Staphylococcus aureus* interacts with SP-A through the lectin domain and SP-A mediates the binding of bacteria to alveolar macrophages. This effect was only observed when SP-A was preincubated with bacteria, followed by incubation with macrophages, whereas preincubation of SP-A with macrophages did not result in increase in binding of bacteria to macrophages. McNeely and Coonrod [24] suggested that the binding of SP-A to the macrophages takes place through the collagen domain and requires multiple binding of SP-A molecules to the cell-surface receptors present on macrophages. It is likely that physiologically relevant interactions of collectins with collectin-receptor-bearing cells require 'aggregation' or 'polymerization' of the ligand, by binding of the ligand to a surface, and facilitating interaction of multiple ligand molecules with multiple receptor molecules [1]. For both C1q and SP-A, interaction of monomeric ligand with monomeric soluble collectin receptor is ionic-strength-dependent, and is weak at physiological salt strength [13]. It is established that, in physiological conditions, binding of C1q to collectin-receptor-bearing cells is readily observed when C1q is aggregated [38] or when C1q is bound to immune complexes [17]. Several workers have reported that interaction of C1q with collectin-receptor-bearing cells induces the internalization of immune complexes bound to C1q (for review see [16]). Similarly, SP-A bound to pollen grains mediates adhesion of the grains of A549 cells in physiological conditions [23]. Interaction of SP-A with influenza virus, via neuraminidase (Figure 7), will fix SP-A in a regular array for interaction with multiple receptor molecules on cell surfaces. Binding of SP-A is likely to inhibit the normal haemagglutinin-mediated adhesion of virus to cells, but will instead promote adhesion via the collectin receptor. This altered mode of interaction of virus with cells is likely to promote phagocytosis of the virus, but could in some circumstances represent another mode of cell invasion by the virus.

We thank Miss B. Moffatt and Mrs. J. U. Newell for technical assistance. We thank Professor A. J. McMichael, Institute of Molecular Medicine, Oxford, for useful suggestions and for providing influenza virus and anti-haemagglutinin antibody. We also thank Professor K. B. M. Reid for useful discussion and advice. We are grateful to Dr. M. Super and Professor R. A. B. Ezekowitz, Division of Hematology/Oncology, Harvard University, Department of Paediatrics, Boston, MA, U.S.A., for providing horseradish-peroxidase-labelled anti-MBP monoclonal antibody, to Dr. C. Milner, MRC Immunochemistry Unit, for providing the neuraminidase assay, and to Dr. J. J. Skehel and Dr. A. R. Douglas, National Institute for Medical Research, London, for the provision of anti-neuraminidase antibodies. This project was funded by the British Lung Foundation and the Colt Foundation. R. M. is a British Lung Foundation Fellow.

REFERENCES

- Malhotra, R., Lu, J., Holmskov, U. and Sim, R. B. (1994) Clin. Exp. Immunol. **97**, suppl. 2, 4–9
- Holmskov, U., Malhotra, R., Sim, R. B. and Jensenius, J.-C. (1994) Immunol. Today **15**, 67–74
- Sastry, K. and Ezekowitz, R. A. B. (1993) Curr. Opin. Immunol. **5**, 59–66
- Haagsman, H. S., Hawgood, S., Sargeant, T., Buckley, D., White, R., Drickamer, K. and Benson, B. J. (1987) J. Biol. Chem. **262**, 13877–13880
- Haurum, J. S., Thiel, S., Haagsman, H. P., Laursen, S. B., Larsen, B. and Jensenius, J.-C. (1993) Biochem. J. **293**, 873–878
- Hawgood, S. (1989) Am. J. Physiol. **257**, L13–L22
- Lu, J. and Sim, R. B. (1994) in New Aspects of Complement Structure and Function (Erdei, A., ed.), R. G. Landes Co., Austin, TX, in the press
- Holmskov, U., Laursen, S. B., Malhotra, R., Wiedemann, H., Timpl, R., Stuart, G. R., Tornøe, I., Madsen, P. S., Reid, K. B. M. and Jensenius, J. C. (1994) Biochem. J., in the press
- Holmskov, U., Teisner, B., Willis, A. C., Reid, K. B. M. and Jensenius, J.-C. (1993) J. Biol. Chem. **268**, 10120–10125
- Thiel, S. and Reid, K. B. M. (1989) FEBS Lett. **250**, 78–84
- Lu, J. H., Thiel, S., Wiedmann, H., Timpl, R. and Reid, K. B. M. (1990) J. Immunol. **144**, 2287–2294
- Malhotra, R., Thiel, S., Reid, K. B. M. and Sim, R. B. (1990) J. Exp. Med. **172**, 955–959
- Malhotra, R., Haurum, J., Thiel, S. and Sim, R. B. (1992) Eur. J. Immunol. **22**, 1437–1445
- Malhotra, R., Laursen, A. B., Willis, A. C. and Sim, R. B. (1993) Biochem. J. **293**, 15–19
- Malhotra, R., Willis, A. C. and Sim, R. B. (1993) Mol. Immunol. **30**, suppl. 1, 30
- Ghebrehwet, B. (1989) Behring Inst. Mitt. **84**, 204–215
- Arvieux, J., Reboul, A., Bensa, J. C. and Colomb, M. G. (1984) Biochem. J. **218**, 547–555
- Wright, J. R., Wager, R. E. and Hawgood, S. (1987) J. Biol. Chem. **262**, 2888–2894
- Dobbs, L. G., Wright, J. R., Hawgood, S., Gonzalez, R., Venstrom, K. and Nellenbogen, J. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 1010–1014
- Tenner, A., Robinson, S. L., Borchet, J. and Wright, J. R. (1989) J. Biol. Chem. **264**, 13923–13928
- Van Iwaarden, J. F., Van Strijp, J. A., Visser, H., Haagsman, H. P., Verhoef, J. and Van Golde, L. M. (1992) J. Biol. Chem. **267**, 25039–25043
- Zimmerman, P. E., Voelker, D. R., McCormack, F. X., Paulsrud, J. R. and Martin, W. J. (1992) J. Clin. Invest. **89**, 143–149
- Malhotra, R., Haurum, J., Thiel, S., Jensenius, J.-C. and Sim, R. B. (1993) Biosci. Rep. **13**, 79–90
- McNeely, T. B. and Coonrod, J. D. (1993) J. Infect. Dis. **167**, 91–97
- Hartley, C. A., Jackson, D. C. and Anders, M. (1992) J. Virol. **66**, 4358–4363
- Hartshorn, K. L., Sastry, K., White, M. R., Anders, E. M., Super, M., Ezekowitz, R. A. and Tauber, A. I. (1993) J. Clin. Invest. **91**, 1414–1420
- Laemmli, U. K. (1970) Nature (London) **227**, 680–685
- Fairbanks, G., Steck, T. L. and Wallach, D. F. H. (1971) Biochemistry **10**, 2602–2610
- Barrett, T. and Inglis, S. C. (1991) in Virology: A Practical Approach (Mahy, B. W. J., ed.), pp. 119–151, IRL Press, Oxford
- Super, M., Gillies, S. D., Foley, S., Sastry, K., Schweinle, J. E., Silverman, V. A. and Ezekowitz, R. A. B. (1992) Nature Genet. **2**, 50–55
- Malhotra, R., Willis, A. C., Jensenius, J.-C., Jackson, J. and Sim, R. B. (1993) Immunology **78**, 341–348
- Hoyer, L. L., Roggentin, P., Schauer, R. and Vimr, E. R. (1991) J. Biochem. (Tokyo) **110**, 462–467
- Fraker, P. J. and Speck, J. C., Jr. (1978) Biochem. Biophys. Res. Commun. **80**, 849–857
- Lipscombe, R. J., Sumiya, R. J., Hill, A. V. S., Lau, Y. L., Levinsky, R. J., Summerfield, J. A. and Turner, M. W. (1992) Hum. Mol. Genet. **1**, 709–715
- Gregory, T. J., Longmore, W. J., Moxley, M. A., Whitsett, J. A., Reed, C. R., Fowler, A. A., Hudson, L. D., Maunders, R. J., Crim, C. and Hyers, T. M. (1991) J. Clin. Invest. **88**, 1976–1981
- Fenner, F., McAuslan, B. R., Mims, C. A., Sambrook, J. and White, D. O. (1974) in The Biology of Animal Viruses, 2nd edn. (Fenner, F., ed.), pp. 72–149, Academic Press, New York
- Perkins, S. J. (1989) Behring Inst. Mitt. **84**, 129–142
- Daha, M. R., Miltenburg, A. M. M., Hiemstra, P. S., Klar-Mohamad, N., Van Es, L. A. and Van Hinsbergh, V. W. M. (1989) Eur. J. Immunol. **18**, 783–787