Surfactant protein D binds to human immunodeficiency virus (HIV) envelope protein gp120 and inhibits HIV replication

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The envelope protein (gp120) of human immunodeficiency virus (HIV) contains highly conserved mannosylated oligosaccharides. These glycoconjugates contribute to resistance to antibody neutralization, and binding to cell surface lectins on macrophages and dendritic cells. Mannose-binding lectin (MBL) binds to gp120 and plays a role in defence against the virus. In this study it is demonstrated that surfactant protein D (SP-D) binds to gp120 and inhibits HIV infectivity at significantly lower concentrations than MBL. The binding of SP-D was mediated by its calcium-dependent carbohydrate-binding activity and was dependent on glycosylation of gp120. Native dodecameric SP-D bound to HIV gp120 more strongly than native trimeric SP-D. Since one common polymorphic form of SP-D is predominantly expressed as trimers and associated with lower blood levels, these individuals may have less effective innate defence against HIV. A chimeric protein containing the N-terminal and collagen domains of SP-D linked to the neck and carbohydrate-recognition domains of MBL (called SP-D/MBL neck + CRD) had greater ability to bind to gp120 and inhibit virus replication than either SP-D or MBL. The enhanced binding of SP-D/MBL neck + CRD was dependent on assembly into higher molecular mass multimers (i.e. a trimeric form of the chimera did not bind to a greater extent than MBL). Hence, the enhanced binding of SP-D compared with MBL results from distinctive properties of its N-terminal and/or collagen domains. SP-D is present in lung and airway fluids, as well as in blood and various mucosal locations, and could, like MBL, play a role in restricting HIV transmission or replication in vivo.

INTRODUCTION

Glycosylation of the human immunodeficiency virus (HIV) envelope protein, gp120, plays an important role in the pathogenesis of the acquired immunodeficiency syndrome (AIDS). gp120 is very highly glycosylated with 24 utilized N-linked glycosylation sites on the well characterized HIV-1_LAI strain (Leonard et al., 1990). gp120 has a particularly high proportion of high mannose oligosaccharide attachments, and the high mannose and hybrid oligosaccharides are conserved among different viral isolates (Sanders et al., 2002). In addition to possible roles of these glycans in the synthesis and folding of gp120 during assembly, important interactions with the immune system have been established. Recent studies indicate that gp120-associated glycans are modified among sequential isolates of HIV taken at different time points after infection in individual subjects (Wei et al., 2003). These modifications are associated with evolving resistance to antibody neutralization. These findings have given rise to the concept that gp120 remains resistant to antibody neutralization by use of a 'glycan shield', a protective coat of oligosaccharides that inhibits antibody binding to gp120 without blocking sites critical for binding to cellular receptors. A survey of sera from HIV-infected subjects showed a lack of effective neutralizing antibodies directed against carbohydrate epitopes on gp120 (Wei et al., 2003). One illustrative exception is a broadly neutralizing
monoclonal antibody, 2G12, that recognizes a specific con-
estellation of high mannose oligosaccharides on the protein
(Sanders et al., 2002). Of interest, the high mannose oligosaccharides recognized by 2G12 are clustered on the end of gp120 most distal from the viral envelope and adjacent to the cell-binding domain of the molecule. These glycans are, therefore, highly accessible to binding by soluble or cell surface lectins or other ligands.

Mannosylated oligosaccharides on gp120 also are involved in the binding of HIV to the DC-SIGN lectin or homologous C-type lectin, langerin, expressed on the surface of dendritic cells and Langerhan’s cells, respectively (Hong et al., 2002; Lue et al., 2002; Turville et al., 2003). Dendritic cell lectins appear to play an important role during initial infection with HIV. This receptor allows immature dendritic cells to bind HIV in mucosal locations (e.g. the vaginal mucosa) such that these cells then transmit the virus in viable form to lymphoid rich areas where T cells be infected (Turville et al., 2002). Mannosylated glycans also mediate uptake of the virus by macrophages via the macrophage mannose receptor that also allows transmission of the infection to T cells (Nguyen & Hildreth, 2003).

High mannose oligosaccharides on gp120 also represent a potential target for inhibition by antiviral agents, like the high mannose oligosaccharide-binding agent cyanovirin (Hong et al., 2002). Furthermore, mannose-binding lectin (MBL), a member of the collectin family of innate immune proteins, appears to contribute to host defence against HIV through a mechanism that involves binding to high mannose oligosaccharides on gp120 (Ezekowitz et al., 1989; van de Wetering et al., 2004). A recent study also demonstrated that MBL can inhibit DC-SIGN-mediated transfer of infectious HIV from dendritic cells to T cells (Spear et al., 2003).

Subjects having low blood levels of MBL due to polymorphic variations in the coding or promoter sequences of the protein have been found to have increased susceptibility to HIV infection in case–control studies (Boniotto et al., 2000; Garred et al., 1997; Pastinen et al., 1998). Variant forms of MBL have also been associated with vertical transmission of HIV (Boniotto et al., 2000). Whether low levels of MBL are associated with higher rates of disease progression or mortality after infection is controversial; some studies find an association (Garred et al., 1997) and others do not (McBride et al., 1998).

MBL binds to primary and highly passaged isolates of HIV via mannosylated carbohydrates on gp120 (Ezekowitz et al., 1989; Hart et al., 2003; Saifuddin et al., 2000). MBL neutralizes HIV in vitro, although the concentrations of MBL needed for neutralization are high (e.g. 20–50 µg ml⁻¹) (Ezekowitz et al., 1989; Hart et al., 2003). Removal of sialic acid from the viral surface not only increased infectivity of simian immunodeficiency virus (SIV) (Means & Desrosiers, 2000), but also increased susceptibility of HIV to neutralization by MBL (Hart et al., 2003). Removal of terminal sialic acids from complex oligosaccharides may facilitate viral binding by reducing negative charge, and/or increasing accessibility of high mannose or hybrid oligosaccharides.

Glycosylation of gp120 appears, therefore, to play a pivotal role in mediating interactions of the virus with innate and adaptive components of the immune system. Although mannosylated glycans on HIV are highly conserved, and therefore presumably advantageous to the virus overall, they render the virus susceptible to inhibition by collectins and (apparently rare) glycan-specific antibodies. Recent studies have shown that another member of the collectin family, surfactant protein D (SP-D), is present not only in the lung, but also in various mucosal locations (Madsen et al., 2000) and in blood (Husby et al., 2002), where it could interact with HIV. Importantly, SP-D, unlike MBL, is expressed in locations like the genito-urinary tract, oral cavity and gastrointestinal tract (Madsen et al., 2000), where it could impede transmission of HIV. Furthermore, alveolar macrophages are an important site of HIV replication in advanced HIV disease, and replication in the lung becomes particularly pronounced in the presence of opportunistic infection (e.g. tuberculosis) (Hoshino et al., 2002; Nakata et al., 1997). Hence, SP-D could have important interactions with HIV in the lung itself. SP-D has been shown to play an important role in innate defence against influenza virus and respiratory syncitial virus infection (Crouch et al., 2000; Hickling et al., 1999; LeVine et al., 2001). Polymorphisms of SP-D exist and affect susceptibility to some infections (Floros et al., 2000; Lahti et al., 2002). Recent studies have shown that serum levels and the extent of multimerization of SP-D vary depending on SP-D genotype as well (Husby et al., 2002; Leth-Larsen et al., 2005). In this study, we demonstrate that SP-D binds to gp120 and inhibits virus replication in culture with greater potency than MBL. The mechanism of this enhanced inhibition is also elucidated.

**METHODS**

**Buffers.** Dulbecco’s PBS with calcium and magnesium was purchased from Gibco-BRL.

**HIV envelope proteins.** The HIV envelope protein preparations were provided by the NIH AIDS research and reference reagent programme (www.aidsreagent.org). Three purified gp120 preparations were used: HIV_BAL gp120 recombinant (called ‘HIV_BAL’ hereafter), HIV_SSF162 gp120 (called ‘HIV_SSF2’ hereafter), and a nonglycosylated form of HIV-1SF2 gp120 (called ‘HIVSF2degly’ hereafter). The HIVSF2degly Preparation was also obtained from Chiron Corporation (Emeryville, CA, USA) as a gift after it was no longer provided by the NIH AIDS research and reference reagent programme. HIV_BAL is a recombinant protein produced in HEK293 cells; it was purified by immunoaffinity chromatography and was derived from a macrophage-tropic viral strain. HIVSF2 was prepared in CHO cells and was also derived from a macrophage-tropic strain of HIV. The endotoxin level of this protein is 26–7 U endotoxin ml⁻¹ and the protein was diluted 100-fold for use in ELISA (hence giving a 0.267 U endotoxin ml⁻¹ working dilution). HIV_BAL and HIVSF2 were both produced in mammalian cells, and hence have mammalian N-linked glycosylation. HIVSF2degly is a nonglycosylated form of HIVSF2 produced in the yeast strain 2150; it elicits neutralizing antibodies in animals effective against HIVSF2 (Haigwood et al., 1992).
Glycan blotting of the gp120 preparations was carried out using the DIG-glycan differentiation kit (Roche Diagnostics), according to the manufacturer’s specifications. In brief, the gp120 preparations were subjected to SDS-PAGE under reducing conditions and transferred to PVDF membranes. A positive control (carboxypeptidase Y, 63 kDa) was included. Presence of high mannose sugars was detected with the Galanthus nivalis agglutinin (GNA) coupled to digoxigenin.

**Virus preparations.** HIV Lai strain was used in the infectivity experiments. The virus preparation was propagated in stimulated peripheral blood mononuclear cells from an HIV-negative donor, and stored at a concentration of 10^6 particles ml^−1 at −80 °C until use. Influenza A virus (IAV) (Philippines 82/H3N2 strain) was grown in chicken eggs and purified by sucrose density gradients as described previously (Hartshorn et al., 1988).

**Collectin preparations.** Recombinant human SP-D (RhSP-D) and SP-D/MBLneck + CRD chimera were produced in CHO-K1 cells as previously described (Hartshorn et al., 1996a). The SP-D/MBLneck + CRD chimera was prepared by recombinant splicing of the neck domain and carbohydrate recognition domain (CRD) of MBL onto the N-terminus and collagen domain of human SP-D (White et al., 2000). The chimera forms trimers, dodecamers and high molecular mass multimers in a manner similar to RhSP-D (White et al., 2001). The chimera has a higher affinity for mannose than wild-type RhSP-D, and has increased ability to bind to, aggregate and neutralize IAV compared to either wild-type SP-D or MBL. For most experiments, the dodecameric RhSP-D and SP-D/MBLneck + CRD were used. However, to assess the role of collectin multimerization in the binding to HIV gp120 trimers, dodecamers and higher molecular mass multimers of SP-D/MBLneck + CRD were compared. The mode of purification of these fractions of RhSP-D or SP-D/MBLneck + CRD has been previously described in detail (Hartshorn et al., 1996a; White et al., 2000, 2001). Recombinant human MBL (RhMBL) was produced in murine Sp2 cells (Hartshorn et al., 1993; Super et al., 1992). RhMBL was kindly provided by Dr Kazue Takahashi and Dr R. A. B. Ezekowitz (Department of Pediatrics, Harvard Medical School, Boston, MA, USA). This RhMBL was the more common allelic variant (termed MBPc), and the preparation is composed predominantly of multimers containing five or six trimers (i.e. octadecamers) in association (Super et al., 1992).

Native human SP-D and SP-A were also tested. Native human SP-D was isolated from amniotic fluid (Leth-Larsen et al., 2005; Strong & Kishore, 1998) using maltose affinity chromatography. Gel filtration chromatography was used to separate trimeric and more highly multimerized (mainly dodecameric) forms (Hartshorn et al., 1996a).

The collectin preparations were tested for endotoxin contamination using a quantitative endotoxin assay (Limulus amebocyte lysate; Cambrex). The final concentrations of endotoxin in samples containing the highest concentrations of collectins were ~20–100 pg ml^−1 (or 6–12 U endotoxin ml^−1 using an internal assay standard).

**Assessment of the binding of collectins to HIV envelope proteins.** The binding of collectins to envelope proteins was measured by ELISA. The envelope proteins were diluted to 20 μg ml^−1 and allowed to adhere to ELISA plates overnight at 4 °C. The plates were then centrifuged at 2500 r.p.m. and fixed by brief incubation with 95% ethanol, followed by 10 min incubation with methanol. The plates were then dried for 30 min. at 37 °C and incubated with PBS containing 2.5% fatty-acid-free BSA (Sigma) for 2 h to block nonspecific binding. After washing, the plates were incubated with various concentrations of biotinylated collectins. Biotinylation of recombinant collectins has been previously described (Hartshorn et al., 2000). Background binding was determined using wells coated with BSA only (see Fig. 3). Wells coated with IAV were used as a positive control. The binding of the HIVSF2deglyc preparation to ELISA plates was confirmed by a separate ELISA using an goat polyclonal antibody raised against the protein [provided by the NIH AIDS research and reference reagent programme (www.aidsreagent.org)]. Maximal binding was seen at 2 μg ml^−1, and a concentration of 20 μg ml^−1 was used in the assay to assure maximal coating.

The binding of the recombinant collectins was also assessed using an alternative method in which unlabelled collectins were detected using mAbs. RhMBL or SP-D/MBLneck + CRD were detected using the 131I mAb (a gift from R. A. B. Ezekowitz) that recognizes the CRD of SP-D/MBL was detected using the 246-01 mAb. These experiments gave overall similar results to those obtained with biotinylated collectins. The binding of native SP-D to HIV envelope proteins was determined in the same manner except that the proteins were not biotinylated and detection of bound SP-D was made using a rabbit polyclonal antibody to SP-D as described (Leth-Larsen et al., 2005).

**Assessment of the effect of collectins on replication of HIV.** The ability of collectins to inhibit HIV replication was tested using U937 cells (ATCC) (Callahan et al., 2003; Hammer et al., 1986; Hartshorn et al., 1987; Zhang et al., 1995). The cells were cultured in 96-well plates to minimize the amounts of collectins needed. The plates were seeded with 3 × 10^5 cells in 200 μl RPMI containing 20% fetal bovine serum. The cells were then inoculated with HIV at an m.o.i. of 0.07. Prior to inoculation the virus was pre-incubated with various concentrations of collectins (or control buffer only) for 30 min at 37 °C. The cells were then maintained in culture in the presence of the same concentration of collectins. Every 3–4 days, 100 μl cell suspension was removed and cell-free supernatants were frozen for use in an HIV-1 p24 antigen assay by ELISA. Fresh media was added with collectin concentrations being maintained by the addition of sufficient amounts of the proteins along with the media.

**Fig. 1.** Comparison of high mannose oligosaccharides on gp120 preparations derived from HIVBAL, HIVSF2 and HIVSF2deglyc. The gp120 preparations were Western blotted and high mannose oligosaccharides were detected using the GNA (specific for terminal mannose residues) as described in Methods. Equal amounts of gp120 protein were added to each well. The control mannosylated protein carboxypeptidase has a molecular mass of 63 kDa (lane A). Note the absence of mannosylated sugars in HIVSF2deglyc (lane C) and greater intensity of staining of HIVBAL (lane D) as compared to HIVSF2 (lane B).
The level of virus replication was assayed by measurement of p24 antigen levels in the supernatant using a p24 antigen ELISA kit obtained from Perkin Elmer Life Sciences, following the manufacturer’s instructions. Briefly, 100 μl cell-free supernatants from HIV infected U937 cells were added to assay wells that were coated with mAb specific for the p24 protein of HIV-1. For each assay negative and positive controls were included, and standard curves were obtained through the addition of known amounts of purified p24 antigen. These controls showed consistent results through all the assays, and were within the expected range (according to the manufacturer’s instructions). The supernatants and controls were incubated for 2 h at 37 °C. After washing, the plates were incubated for 1 h at 37 °C with biotinylated polyclonal antibodies directed against HIV-1 p24 protein. Bound antibody was detected by the addition of streptavidin-HRP and OPD substrate solution.

Statistics. Statistical comparisons were made using Student’s paired t-test and ANOVA using the Statmost program.

RESULTS

Comparison of the high mannose oligosaccharide attachments of preparations of HIV gp120

As shown in Fig. 1, HIVBAL and HIVSF2 had extensive high mannose oligosaccharide attachments as assessed by lectin blotting, whereas the deglycosylated form of HIVSF2 had no such attachments. Note also that HIVBAL stained more strongly for high mannose oligosaccharides than HIVSF2. This difference in glycosylation could reflect either differences in the cell lines used to produce the two preparations or differences in N-linked glycosylation sites on the viral strains.

Recombinant SP-D binds to HIV envelope protein gp120 through its lectin activity

As shown in Fig. 2(a), RhSP-D dodecamers bound to gp120 of the HIVBAL strain. The binding of RhSP-D to IAV was tested in parallel as a positive control. The binding to gp120 of HIVBAL was approximately equal if the assay was performed without the ethanol and methanol fixation step (data not shown). The binding of SP-D was significantly greater than the binding of BSA, and was abrogated by the addition of mannose or EDTA during the ELISA (Fig. 2b). The binding of SP-D to gp120 was completely dependent on oligosaccharide attachments on gp120, since it bound...
SP-D binds to HIV and inhibits infectivity

**Fig. 3.** Binding of native SP-D to gp120 is dependent on the extent of high mannose oligosaccharides on gp120 and on the degree of multimerization of SP-D. The binding of high and low molecular mass forms of native SP-D (purified from amniotic fluid as described in Methods) to gp120 preparations was compared. The binding was measured by ELISA using polyclonal rabbit antibodies directed against SP-D, and the results represent the mean±SEM of three separate experiments using 160 ng ml⁻¹ of the SP-D preparations. The binding of high molecular mass SP-D (mainly composed of dodecamers) to gp120 of HIVBAL and HIVSF2 strains was significantly greater than the binding of low molecular mass SP-D (predominantly composed of trimers) (P<0.001; indicated by **). The binding of high molecular mass form of SP-D to gp120 of HIVBAL was significantly greater than the binding to that of HIVSF2 strains, which in turn was significantly greater than the binding to deglycosylated gp120 of HIVSF2 strain (P values shown). Note that gp120 of HIVBAL had more intense staining for high mannose oligosaccharides than HIVSF2 (Fig. 1). MM, molecular mass.

Strongly to HIVSF2 but not to the deglycosylated preparation HIVSF2deglyc (Fig. 2c).

**Binding of native human SP-D to gp120 depends on glycosylation of gp120 and multimerization of SP-D**

Native human SP-D varies in the degree of multimerization and serum concentration depending on a polymorphism in amino acid 11 in the N-terminal domain of the molecule (Leth-Larsen et al., 2005). Subjects homozygous for threonine at this position (17% of the Danish population) have lower serum levels of SP-D and the molecule exists predominantly in a trimeric form, whereas those homozygous for methionine (35% of the Danish population) have higher serum levels and the protein is predominantly present in the form of dodecamers. The trimeric form of SP-D has a reduced ability to bind to IAV and bacteria (Leth-Larsen et al., 2005). We isolated high molecular mass (predominantly dodecameric) and low molecular mass (predominantly trimeric) SP-D from amniotic fluid as described previously (Leth-Larsen et al., 2005), and tested their ability to bind to gp120 of HIV (Fig. 3). High molecular mass native SP-D bound to a significantly greater extent than low molecular mass SP-D to the glycosylated forms of gp120 (HIVBAL and HIVSF2). Both forms of native SP-D showed minimal binding to HIVSF2deglyc. Of note, high molecular mass SP-D bound to HIVBAL to a significantly greater degree than to HIVSF2, perhaps due to the increased presence of high mannose oligosaccharides on HIVBAL. Note that the binding of SP-D to IAV glycoproteins depends on the presence of high mannose oligosaccharides on these proteins as well (Hartshorn et al., 2000).

**SP-A does not bind to gp120 of HIV**

The mechanism of binding of SP-A to IAV differs from that of SP-D, and does not involve recognition of IAV-associated carbohydrates by the lectin activity of SP-A. Instead, SP-A binds to IAV via attachment of the viral haemagglutinin to sialic acids on SP-A (Benne et al., 1995; van Eijk et al., 2003, 2004). Despite strong binding to IAV, we found no measurable binding of SP-A to gp120 of HIVBAL (mean OD450 for the binding of 2·5 µg SP-A ml⁻¹ to gp120 was 0.0 7 ± 0.0 1 as compared to 0.86 ± 0.26 for the binding to IAV, n = 4, P<0.04, background binding to BSA subtracted prior to analysis).

**Binding of SP-D to gp120 exceeds the binding of MBL: the role of the CRD**

As shown in Fig. 4(a), RhMBL bound to HIVBAL to an extent comparable to its binding to IAV. The binding to HIVBAL was significantly greater than the binding to HIVSF2, which in turn exceeded the binding to HIVSF2deglyc, consistent with the previously demonstrated binding of MBL to HIV (or IAV) (Hartshorn et al., 1993) via its CRD. Note again that the relatively greater binding of MBL to HIVBAL compared to the binding to HIVSF2 could reflect a lower amount of high mannose oligosaccharides on the latter preparation. The binding of MBL was compared to the binding of SP-D using biotinylated preparations of the collectins (Fig. 4b, c, d). A comparable extent of biotinylation was confirmed by the similar binding of streptavidin-HRP to equal amounts of the proteins coated directly on ELISA plates (data not shown). SP-D bound significantly more than MBL to HIVBAL or HIVSF2 in these assays. Similar results have been obtained with IAV in prior studies (White et al., 2000).

To establish the role of the CRD of the collectins in this binding difference, we also tested the binding of dodecamers of the SP-D/MBLneck+CRD chimera. This chimera contains the N-terminal and collagen domains of SP-D, and the neck and CRD of MBL. The molecule structurally resembles SP-D and, like human SP-D, forms trimers, dodecamers and high molecular mass multimers than can be separated by gel filtration. The SP-D/MBLneck+CRD chimera is a useful tool because it allows comparison of the contributions of the N-terminal and carbohydrate-binding domains of SP-D to various functional activities. For the initial experiments
the dodecamer fractions of SP-D and SP-D/MBLneck + CRD were used. The SP-D/MBLneck + CRD chimera bound more strongly than either SP-D or MBL. Similar results were obtained when testing binding to IAV (White et al., 2000, 2001; data not shown).

The binding of the recombinant collectins was also tested by an alternative method in which the bound collectins were detected by specific mAbs (Fig. 5). The mAb directed against the CRD of MBL and the mAb directed against SP-D resulted in similar OD450 values when reacted with equal concentrations of various fractions of SP-D/MBLneck + CRD (for the MBL antibody) and RhSP-D dodecamers (for the SP-D antibody). When testing the binding of the collectins using these antibodies, dodecamers of SP-D/MBLneck + CRD again bound more strongly to gp120 of HIVBAL as compared to dodecamers of SP-D (Fig. 5). Higher molecular mass multimers (that contained as many as 32 trimeric CRD heads) and trimers of SP-D/MBLneck + CRD were also tested. The binding of the higher molecular mass multimers to HIV gp120 was not greater than the binding of dodecamers. However, the trimeric fraction of SP-D/MBLneck + CRD bound markedly less than multimers or dodecamers. Thus, the degree of multimerization was a critical determinant of the increased binding activity of SP-D/MBLneck + CRD.

As shown in Fig. 5, the binding of SP-D/MBLneck + CRD was also dependent on glycosylation of gp120 since it showed minimal binding to HIVSF2deglyc. Although comparisons of binding between SP-D, SP-D/MBLneck + CRD and MBL cannot be considered entirely conclusive because of possible differences in biotinylation or antibody recognition (despite using controls), the results strongly suggest that the binding of SP-D is greater than the binding of MBL, and that this is not the result of an intrinsically greater binding affinity of the CRD of SP-D compared to that of MBL. To further

![Graph](image)

**Fig. 4.** The binding of SP-D to HIV gp120 is greater than the binding of either SP-D or MBL. (a) Binding of RhMBL to HIV gp120 preparations and IAV. Note that RhMBL bound significantly more to HIVBAL than to HIVSF2. In addition, RhMBL bound significantly more to HIVSF2 than to HIVSF2deglyc. (b) Binding of RhMBL, RHSP-D dodecamers and SP-D/MBLneck + CRD dodecamers to the gp120 of HIVBAL. SP-D bound more strongly than either SP-D or MBL (P < 0.05; indicated by *), while dodecamers of SP-D/MBLneck + CRD bound more strongly than either SP-D or MBL (P < 0.05; indicated by **). (c) Similar results were obtained with the gp120 of HIVSF2. (d) Binding of SP-D or SP-D/MBLneck + CRD was markedly reduced to HIVSF2deglyc compared to the glycosylated form of HIVSF2 at all concentrations tested (P < 0.05 < 0.0007). Results are the mean ± SEM of five independent experiments using biotinylated collectins. ▲, RhMBL; ■, RHSP-D; ▼, SP-D/MBLneck + CRD.

![Graph](image)

**Fig. 5.** Increased binding of dodecameric or multimeric forms of SP-D/MBLneck + CRD as compared to the binding of trimeric SP-D/MBLneck + CRD or RhSP-D. The binding to gp120 of HIVBAL was tested using ELISA as in Fig. 2, with the modification that bound collectins were detected using mAbs directed against either the MBL or SP-D CR domain. Results are the mean ± SEM of five experiments. The binding of either concentration of high molecular mass multimers or dodecamers of SP-D/MBLneck + CRD was significantly greater than the binding of wild-type SP-D or trimers of SP-D/MBLneck + CRD. This was true using Student’s t-test or ANOVA analysis followed by Tukey’s test.
Inhibition was variable at 0.5 and 1 µg SP-D ml⁻¹. MBL did not cause inhibition of viral growth at the same concentrations (e.g. 0.5–5 µg ml⁻¹). However, SP-D/MBLneck + CRD significantly inhibited replication of HIV in U937 cells (Fig. 6, Tables 1 and 2). The degree of inhibition caused by SP-D/MBLneck + CRD was greater than that of SP-D at 1 µg ml⁻¹ in both experiments (involving four replicate cultures for each assay condition per experiment). Overall the experiments suggest greater viral inhibition was achieved with SP-D/MBLneck + CRD than with wild-type SP-D, although this was not seen at all concentrations tested. Inhibition by SP-D/MBLneck + CRD became more pronounced after increased time in culture as shown in Table 2 in which experiments were carried out to 11 days in culture. Even at day 11 no consistent effect of MBL was obtained. Hence replacement of the N-terminus and collagen domain of MBL with those of SP-D significantly increased the neutralizing activity of MBL (as we have observed for IAV previously).

**DISCUSSION**

HIV undergoes rapid mutation allowing it to evade adaptive host defence responses. Nevertheless, some subjects are resistant to infection with HIV or to progression of HIV-related disease after infection. Recent studies indicate that innate host defence mechanisms contribute to susceptibility or resistance to HIV infection. In this paper, SP-D is demonstrated to have significant HIV-binding and -inhibitory activities that exceed those of MBL. Recent discoveries that SP-D is expressed in various mucosal locations, like the oral cavity and the female genital tract (Leth-Larsen et al., 2004; Madsen et al., 2000), indicate that SP-D may play a role during sexual or vertical transmission of HIV. Levels in BAL fluids are between 135 and 880 ng ml⁻¹ with concentrations in situ in the lung being considerably higher than this (Wright, 1997). Hence, levels of SP-D in BAL fluids should inhibit virus replication based on our findings. This is potentially important since HIV can be isolated from BAL fluid and alveolar macrophages appear to be an important site of HIV replication in advanced HIV disease (Hoshino et al., 2002; Nakata et al., 1997). SP-D levels in blood (e.g. 158 and 3711 ng ml⁻¹; Husby et al., 2002; Leth-Larsen et al., 2005) are sufficient to bind to, and in some cases to inhibit replication of, HIV. Levels in blood and multimerization of SP-D are determined strongly by genetic factors. Hence, the genotype of SP-D could influence susceptibility or progression of HIV infection. This hypothesis is supported by our finding of a differential ability of high and low molecular mass native SP-D to bind to gp120.

**SP-D inhibits infectivity of HIV at significantly lower concentrations than MBL**

To determine if the increased ability of SP-D to bind to HIV gp120 is associated with a greater ability to inhibit infectivity of the virus, we utilized a model of infection of the highly susceptible undifferentiated U937 cells. Results of an HIV neutralization experiments are shown in Fig. 6. A replicate experiment is shown in Table 1. SP-D caused significant inhibition of HIV replication at concentrations of ≥2 µg ml⁻¹ in both experiments at 3 or 6 days after infection. Although inhibition by SP-D was only partial in this model at the concentrations tested, the overall results were similar to the binding results regarding the comparative activity of SP-D, MBL and SP-D/MBLneck + CRD.

**Effect of SP-D, MBL and SP-D/MBLneck + CRD on replication of HIV in U937 cells.** HIV LAI was pre-incubated with the indicated collectins followed by addition to U937 cells and culturing for either 3 (a) or 6 (b) days. The results shown represent the mean ± SEM p24 antigen concentration in the supernatant of four replicate cultures per concentration of collectin. Significant reductions in the concentration of p24 antigen compared to control cultures not treated with collectins are indicated by * (P<0.05) or ** (P<0.01). The dotted line marks the control level for comparison. The # symbol indicates cases where SP-D/MBLneck + CRD caused significantly greater reductions in p24 concentration than SP-D (P<0.05). Data from a replicate experiment and from this experiment (experiment 1) after 11 days in culture are shown in Tables 1 and 2. White bars, SP-D; black bars, SP-D/MBL; grey bars, MBL.

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HIV undergoes rapid mutation allowing it to evade adaptive host defence responses. Nevertheless, some subjects are resistant to infection with HIV or to progression of HIV-related disease after infection. Recent studies indicate that innate host defence mechanisms contribute to susceptibility or resistance to HIV infection. In this paper, SP-D is demonstrated to have significant HIV-binding and -inhibitory activities that exceed those of MBL. Recent discoveries that SP-D is expressed in various mucosal locations, like the oral cavity and the female genital tract (Leth-Larsen et al., 2004; Madsen et al., 2000), indicate that SP-D may play a role during sexual or vertical transmission of HIV. Levels in BAL fluids are between 135 and 880 ng ml⁻¹ with concentrations in situ in the lung being considerably higher than this (Wright, 1997). Hence, levels of SP-D in BAL fluids should inhibit virus replication based on our findings. This is potentially important since HIV can be isolated from BAL fluid and alveolar macrophages appear to be an important site of HIV replication in advanced HIV disease (Hoshino et al., 2002; Nakata et al., 1997). SP-D levels in blood (e.g. 158 and 3711 ng ml⁻¹; Husby et al., 2002; Leth-Larsen et al., 2005) are sufficient to bind to, and in some cases to inhibit replication of, HIV. Levels in blood and multimerization of SP-D are determined strongly by genetic factors. Hence, the genotype of SP-D could influence susceptibility or progression of HIV infection. This hypothesis is supported by our finding of a differential ability of high and low molecular mass native SP-D to bind to gp120.

**DISCUSSION**

HIV undergoes rapid mutation allowing it to evade adaptive host defence responses. Nevertheless, some subjects are resistant to infection with HIV or to progression of HIV-related disease after infection. Recent studies indicate that innate host defence mechanisms contribute to susceptibility or resistance to HIV infection. In this paper, SP-D is demonstrated to have significant HIV-binding and -inhibitory activities that exceed those of MBL. Recent discoveries that SP-D is expressed in various mucosal locations, like the oral cavity and the female genital tract (Leth-Larsen et al., 2004; Madsen et al., 2000), indicate that SP-D may play a role during sexual or vertical transmission of HIV. Levels in BAL fluids are between 135 and 880 ng ml⁻¹ with concentrations in situ in the lung being considerably higher than this (Wright, 1997). Hence, levels of SP-D in BAL fluids should inhibit virus replication based on our findings. This is potentially important since HIV can be isolated from BAL fluid and alveolar macrophages appear to be an important site of HIV replication in advanced HIV disease (Hoshino et al., 2002; Nakata et al., 1997). SP-D levels in blood (e.g. 158 and 3711 ng ml⁻¹; Husby et al., 2002; Leth-Larsen et al., 2005) are sufficient to bind to, and in some cases to inhibit replication of, HIV. Levels in blood and multimerization of SP-D are determined strongly by genetic factors. Hence, the genotype of SP-D could influence susceptibility or progression of HIV infection. This hypothesis is supported by our finding of a differential ability of high and low molecular mass native SP-D to bind to gp120.
For viral neutralization assays, we chose to test concentrations of SP-D similar to those found in blood or unconcentrated BAL fluids. At these concentrations inhibition of infectivity of HIV was only partial, whereas similar concentrations fully inhibit infectivity of IAV (Hartshorn et al., 2000). The reasons for this discrepancy are unclear, although it is possible that SP-D-bound HIV can still enter and infect U937 cells to a reduced extent. The rationale for choosing U937 cells was to avoid known effects of SP-D on activation and proliferation of peripheral blood lymphocytes, which could be confounding (Borron et al., 1998). In addition, the most likely site of HIV replication in the lung or other mucosal sites would be macrophages so that macrophage-like cells should be more relevant for our studies. It will, of course, also be of great interest to study the effects of SP-D on HIV infection of lymphocytic cells. In any case our current results show that SP-D can exert suppressive effects on HIV replication in macrophage-like cells. This may be significant in vivo because alveolar macrophages may be an important reservoir of infection in advanced HIV disease (Hoshino et al., 2002). It will also be important to determine if SP-D can inhibit replication of primary isolates of HIV and macrophage-tropic strains since results may differ from those obtained with HIV_LAI (Saifuddin et al., 2000).

The mechanism of binding of SP-D to HIV gp120 (i.e. calcium dependent attachment to N-linked sugars) resembles its binding to various other microbial ligands, including IAV (for which SP-D plays a key role in host defence; Hartshorn et al., 1994; Hawgood et al., 2004; LeVine et al., 2001; Reading et al., 1997). Notably, SP-D shows a higher apparent affinity for HIV gp120 than MBL. Using the SP-D/MBLneck+CRD chimera we demonstrated that the greater binding activity of SP-D is not the result of differences in the binding affinity of the CRD domain of SP-D, since the SP-D/MBLneck+CRD chimera also bound more strongly than MBL.

### Table 1. Effect of collectins on replication of HIV in U937 cells, experiment 2

Viral growth was assessed by ELISA assay for HIV p24 antigen. Levels of p24 antigen in culture supernatants were measured at either day 3 or 6 after infection. The viral inoculum was pre-incubated with the indicated collectins and levels of the collectins in the cell culture media were maintained throughout the experiment. Each assay point was measured in quadruplicate. Results are expressed as the mean ± SEM % of the control p24 antigen level (the control being the amount of p24 in cultures maintained in the absence of collectins). Values that were significantly reduced compared to the control are highlighted in bold.

<table>
<thead>
<tr>
<th>Time after infection (days)</th>
<th>Collectin</th>
<th>Collectin concn (µg ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0·5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>SP-D</td>
<td>83 ± 1·5*</td>
</tr>
<tr>
<td>6</td>
<td>SP-D</td>
<td>85 ± 5</td>
</tr>
<tr>
<td>6</td>
<td>SP-D/MBL</td>
<td>77 ± 6†</td>
</tr>
<tr>
<td>6</td>
<td>MBL</td>
<td>116 ± 2</td>
</tr>
</tbody>
</table>

*P < 0·01 compared with the control.
†P < 0·05 compared with the control.
‡P < 0·05 comparing SP-D/MBL vs SP-D.

### Table 2. Comparison of the effects of MBL and SP-D/MBLneck+CRD on replication of HIV in U937 cells after 11 days in culture

The experiments were performed as in Table 1 except that the cells were maintained in culture for 11 days after infection.

<table>
<thead>
<tr>
<th>Time after infection (days)</th>
<th>Collectin</th>
<th>Experiment no.</th>
<th>Collectin concn (µg ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0·5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>11</td>
<td>SP-D/MBL</td>
<td>1</td>
<td>32 ± 2*</td>
</tr>
<tr>
<td>11</td>
<td>SP-D/MBL</td>
<td>2</td>
<td>60 ± 16†</td>
</tr>
<tr>
<td>11</td>
<td>MBL</td>
<td>1</td>
<td>101 ± 16</td>
</tr>
<tr>
<td>11</td>
<td>MBL</td>
<td>2</td>
<td>86 ± 10</td>
</tr>
</tbody>
</table>

*P < 0·01 compared with the control.
†P < 0·05 compared with the control.
Very similar results were obtained in studies of influenza viruses (White et al., 2000).

The greater binding activity of SP-D compared to MBL must then result from differences in the N-terminal and collagen domains of the molecule, or associated differences in the number or spatial distribution of CRDs. The greatly reduced binding of the trimeric form of SP-D/MBL\(_{\text{neck} + \text{CRD}}\) indicates that cooperative binding interactions between trimeric CRD globular domains in dodecameric molecules are necessary for the increased binding activity of SP-D/MBL\(_{\text{neck} + \text{CRD}}\). Presumably the N-terminal and collagen domains of SP-D allow greater cooperativity of binding between globular CRD trimeric heads on an individual molecule than those of MBL. Studies with influenza virus and bacteria indicate that the N-terminal and collagen domains of SP-D confer much greater viral and bacterial aggregating activity than those of MBL (Hartshorn et al., 1996; White et al., 2000, 2001). The finding in this paper of the reduced binding to gp120 by predominantly trimeric low molecular mass native SP-D is, therefore, consistent with prior results.

We used two different methodologies to assess relative binding of the collectins (i.e. biotinylation of the collectins, and detection with anti-SP-D or anti-MBL antibodies) with similar results. Furthermore, the relative binding paralleled findings with influenza virus. Hence, the overall conclusion that SP-D and SP-D/MBL\(_{\text{neck} + \text{CRD}}\) have stronger binding to HIV gp120 than MBL seems well supported. The relative binding activity of these collectins also correlated with their ability to inhibit replication of HIV.

Previous studies have demonstrated that MBL inhibits HIV replication in culture, although the concentrations needed to achieve neutralization were relatively high (e.g. partial inhibition at 20–30 μg ml\(^{-1}\)) (Ezekowitz et al., 1989; Hart et al., 2003). Hence, the failure to demonstrate inhibition of HIV replication by MBL at the doses used in our study is consistent with other observations. The ability of MBL to fix complement on HIV-infected cells could be important to its in vivo activity (Haurum et al., 1993). Also, the collectins could promote uptake of viruses by phagocytic cells, which could be important in vivo aside from neutralizing activity (Hartshorn et al., 1996b, 1997, 1998; Ying et al., 2004). Recent studies have demonstrated that various components of the innate immune system have important interactions with the adaptive immune system (Borron et al., 2002; Brinker et al., 2001). Furthermore, SP-D has important roles in regulating oxidant and phospholipid metabolism in the lung, and the clearance of apoptotic cells (Clark et al., 2002; Wert et al., 2000). It is possible that the ability of SP-D to bind to HIV could affect the presentation of HIV antigens to dendritic cells, or that its other immunoregulatory and homeostatic activities could affect adaptive immune responses in the context of HIV infection. Future studies are needed to address whether SP-D modulates phagocyte uptake of HIV. Such an effect could contribute to containment of HIV in vivo or even facilitate infection (e.g. as in the case of DC-Sign; Spear et al., 2003).

ACKNOWLEDGEMENTS

We would like to gratefully acknowledge Dr R. Alan B. Ezekowitz and Dr Kazue Takahashi for providing RhMBL and the mAB directed against MBL, and Dr Jeffrey Whitsett (Cincinnati Children’s Hospital Medical Center, Cincinnati, OH, USA) for providing SP-A. This research was supported by NIH grants HL69031 (K. L. H.), and HL29594 and 44015 (E. C.). We also gratefully acknowledge Dr Indresh Srivastava at the Chiron Corporation for providing the non-glycosylated form of gp120.

REFERENCES


