

Interaction of mannose-binding lectin with primary isolates of human immunodeficiency virus type 1

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Mannose-binding lectin (MBL) is present in human serum and plays an important role in innate immunity by binding to carbohydrate on micro-organisms. Whereas the gp120/gp41 of human immunodeficiency virus type 1 (HIV-1) contains numerous *N*-linked glycosylation sites and many of these sites contain high-mannose glycans which could interact with MBL, the interaction between MBL and primary isolates (PI) of HIV-1 has not been studied. To determine if PI of HIV bind to MBL, a virus capture assay was developed in which virus was incubated in MBL-coated microtitre wells followed by detection of bound virus with an ELISA for p24 antigen. The X4 HIV-1_{MN} T cell line-adapted strain and PI of HIV (R5 and X4) bound to MBL. Binding of virus to MBL was via the carbohydrate-recognition domain of MBL since binding did not occur in the absence of Ca²⁺ and was blocked by preincubation of MBL-coated wells with soluble mannan. The interaction of virus with MBL-coated wells was also inhibited by preincubation of virus with soluble MBL, indicating that both immobilized and soluble forms of MBL bound to HIV. Although host cell glycoproteins are incorporated into the membrane of HIV, binding of virus to immobilized MBL required expression of gp120/gp41 on virus particles, suggesting the presence of either an unusually high carbohydrate density and/or a unique carbohydrate structure on gp120/gp41 that is the target of MBL. This study shows that PI of HIV bind to MBL and suggests that MBL can selectively interact with HIV *in vivo* via carbohydrate structures on gp120/gp41.

Introduction

Mannose-binding lectin (MBL) is an effector molecule of the innate immune system. MBL binds to carbohydrates on certain micro-organisms and plays a role in their clearance and destruction by activation of complement and also by direct opsonization for binding to the MBL receptor on phagocytic cells (Turner, 1998; Fraser *et al.*, 1998). In human serum, MBL is present as multimers consisting of mostly dimers to hexamers of trimeric subunits (Lu *et al.*, 1990; Lipscombe *et al.*, 1995). Median serum MBL levels are 1–2 µg/ml and levels increase during the acute response phase by as much as 3-fold (Ezekowitz *et al.*, 1988; Thiel *et al.*, 1992), although in some individuals MBL is present at much lower levels due to mutations in the MBL gene (Lipscombe *et al.*, 1995).

Human immunodeficiency virus type 1 (HIV-1) is an enveloped virus that encodes gp120/gp41, a receptor/fusion

protein complex on the virion surface. HIV-1 gp120 is extensively glycosylated, with *N*-linked carbohydrate accounting for nearly half of the molecular mass (reviewed in Fenouillet *et al.*, 1994), whereas gp41 has a lower carbohydrate to protein ratio with only four or five potential carbohydrate sites. Leonard *et al.* (1990) determined that all 24 potential carbohydrate attachment sites of the HIV-1_{IIIB} strain gp120 produced in CHO cells are utilized, including 13 sites that contain complex-type oligosaccharides and 11 sites that contain either high-mannose type or hybrid oligosaccharides. This endows gp120 with an unusually high number of high-mannose oligosaccharides for a mammalian glycoprotein (Fenouillet *et al.*, 1994). Many of these glycosylation sites were found to be conserved among HIV isolates, even though some are found in hypervariable regions of gp120 (Geyer *et al.*, 1988; Leonard *et al.*, 1990; Mizouchi *et al.*, 1990).

Several recent studies provide structural and experimental evidence that the gp120 carbohydrates on HIV and simian immunodeficiency virus (SIV) are involved in protection of virus from reactivity with neutralizing antibodies and may also

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help prevent the host from establishing effective neutralizing antibody responses. The crystal structure of gp120 showed that many of the *N*-linked sites are arrayed to cover variable regions of gp120 which would otherwise be exposed to neutralizing antibody (Kwong *et al.*, 1998). Additionally, mutation of specific glycosylation sites in SIV gp120 increased the antibody response of infected macaques and increased the susceptibility of mutated virus to neutralization by antibody (Reitter *et al.*, 1998). While these studies indicate that the carbohydrate of gp120/gp41 on virions provides protection from antibodies, they further suggest a structure that could be a prime binding site for MBL.

Despite the fact that HIV-1 appears to be an excellent target for interaction with MBL due to the extensive high-mannose glycosylation of gp120, there are no studies that have investigated the interaction of MBL with HIV primary isolates (PI). A previous study investigated the interaction of MBL with T cell line-adapted (TCLA) HIV_{IIIB} and showed that MBL bound to and neutralized this strain (Ezekowitz *et al.*, 1989). However, there are significant differences between TCLA and PI viruses. The goal of the current study was to determine if MBL binds to intact virions of HIV-1 PI and whether this binding is dependent on gp120/gp41 expression. A virus capture assay was developed to test both CCR5-tropic and CXCR4-tropic PI for binding to MBL.

Methods

■ **Mannose-binding lectin.** Human serum MBL was prepared according to the method utilized by Suankratay *et al.* (1998) using sequential affinity column chromatography. Briefly, pooled human serum was dialysed in starting buffer containing 50 mM Tris-HCl, 1 M NaCl, 20 mM CaCl₂ and 0.05% (w/v) NaN₃ (pH 7.8), clarified by centrifugation and applied to a mannan-Sepharose 4B column. The column was washed with the starting buffer and bound MBL was eluted with a buffer containing 50 mM Tris-HCl, 1 M NaCl and 20 mM EDTA (pH 7.8). The eluate was equilibrated to 50 mM CaCl₂ and reappplied to a second column. The bound MBL was recovered as in the first column, equilibrated with CaCl₂ and passed over protein G and anti-IgM columns to remove immunoglobulins. Recombinant MBL was kindly provided by Alan Ezekowitz (Harvard Medical School, Boston, MA, USA).

■ **Cells and virus.** The human primary embryonic kidney cell line (293; contributed by Andrew Rice, Baylor College of Medicine, Houston, TX, USA) and the T-lymphoblastic H9 cell lines were obtained through the AIDS Research and Reference Reagent Program (NIH, Rockville, MD, USA). The promonocytic U937 (ATCC CRL 1593) cell line was obtained from the ATCC (Manassas, VA, USA). The 293 cells were grown in DMEM with 10% foetal bovine serum (FBS) (Whittaker Bioproducts), while the other cell lines were grown in RPMI-1640 medium (Whittaker Bioproducts) with 10% FBS.

TCLA HIV-1_{MN} was obtained through the AIDS Research and Reference Reagent Program and PI of virus used in this study were prepared as previously described (Takemfan *et al.*, 1998). Briefly, PI GP (X4), TH (R5) and TA (R5) were isolated from HIV-1-infected patients and grown in phytohaemagglutinin (PHA; Sigma)-stimulated peripheral blood mononuclear cells (PBMC) in the presence of IL-2 for 7–14 days. The macrophage-tropic Ada strain of HIV-1 was obtained through the

AIDS Research and Reference Reagent Program and was passaged in primary macrophage culture as described previously (Gendelman *et al.*, 1988). In brief, Ficoll Hypaque-isolated PBMC from normal donors were incubated in tissue culture flasks and then plastic-adherent cells were cultured for 5 days in the presence of macrophage colony stimulating factor (5 ng/ml; R&D Systems). Cells were then infected and cultured for an additional 14 days.

The gp120/gp41 envelope-positive (env+) and the corresponding envelope-negative (env-) viruses were prepared by transiently transfecting 293 cells with plasmids pNL4-3 and pNL4-3(E⁻) obtained through the AIDS Research and Reference Reagent Program, contributed by Malcolm Martin (NIH) and Nathaniel Landau (Aaron Diamond AIDS Research Center, New York, NY, USA), respectively. Cells were transfected using Lipofectamine reagent (GIBCO BRL) according to the manufacturer's protocol. At 4–5 days after transfection, cell supernatants were collected, centrifuged to remove cellular debris and then purified by ultracentrifugation (140 000 g) over 20% glycerol (Saifuddin *et al.*, 1997). The amount of virus was determined by p24 antigen ELISA (Coulter) after lysis with 0.5% Triton X-100.

■ **Binding of HIV-1 to MBL.** Ninety-six-well, flat-bottom polystyrene tissue culture plates (Costar) were coated with 100 µl of either MBL (10 µg/ml in most experiments, see Results) or 1% BSA diluted in veronal-buffered saline (5 mM veronal, pH 7.5 and 0.145 M NaCl) containing 10 mM CaCl₂ (VBS-Ca). In some experiments, wells were coated with human MAb IgG1b12 (Burton *et al.*, 1994) at 10 µg/ml in VBS-Ca. After overnight incubation at room temperature, wells were blocked with BSA (1%) for 60 min at room temperature, washed with VBS-Ca and then incubated for 2–8 h with 100 µl of different isolates of HIV-1. In most experiments, virus was adjusted to 10–40 ng p24 antigen/ml by dilution with VBS-Ca. In some experiments, wells were incubated with virus diluted in VBS containing 10 mM EDTA (VBS-EDTA). The plates were washed, and bound virus was lysed with 1% Triton X-100 and detected by p24 ELISA (Coulter). The percentage of HIV bound was calculated by dividing the total input of virus (100%) into the amount of bound virus detected by ELISA. A control experiment showed that when free p24 was incubated with immobilized MBL and wells were then treated with detergent, no p24 was detected by ELISA (not shown). Experiments were also performed which showed that detection of p24 in the ELISA was not affected by MBL (not shown).

In some experiments, the MBL-coated wells were preincubated with 100 µl *Saccharomyces cerevisiae* mannan (Sigma) at 100 µg/ml in the presence of VBS-Ca before addition of virus. To determine whether MBL can interact with HIV in solution, virus was preincubated with different concentrations of MBL before addition to MBL-coated microtitre wells.

Results

Primary isolates of HIV-1 bind to immobilized MBL

A microtitre capture assay was developed for measuring binding of HIV to MBL. Wells of microtitre plates were coated with MBL and virus was incubated in the MBL-coated wells for 2 h. Binding of HIV to MBL was assessed by lysing virus with detergent and measuring released p24 core protein by ELISA. Addition of increasing amounts of either TCLA HIV-1_{MN} or a PI of HIV (GP) to MBL-coated microtitre wells resulted in increasing levels of detected p24 (Fig. 1A). In contrast, virus binding did not increase when wells were coated with BSA. The amount of virus binding was also dependent on the amount of MBL used to coat wells with virus binding detected

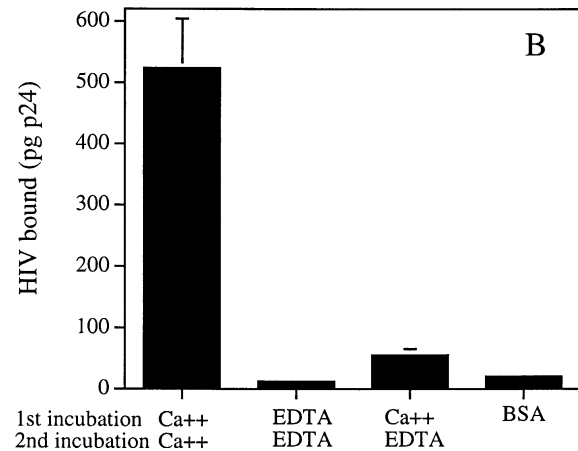
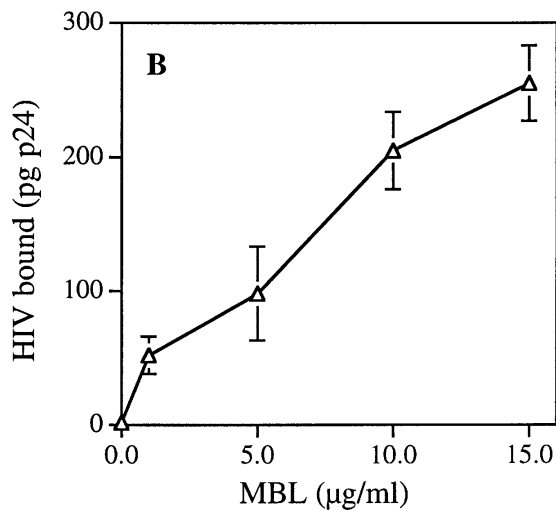
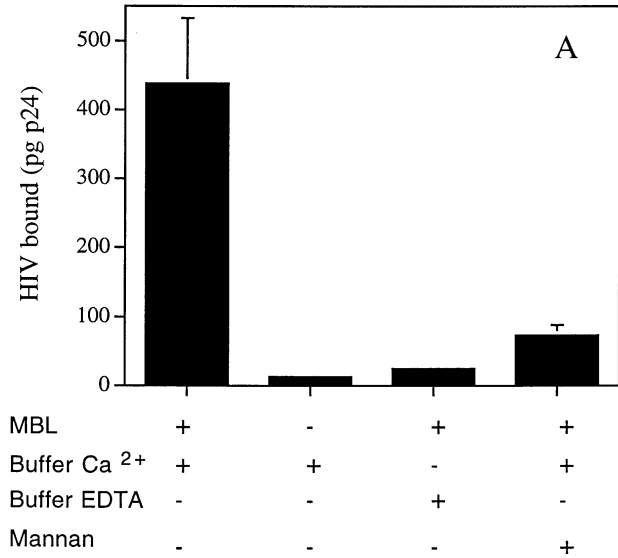
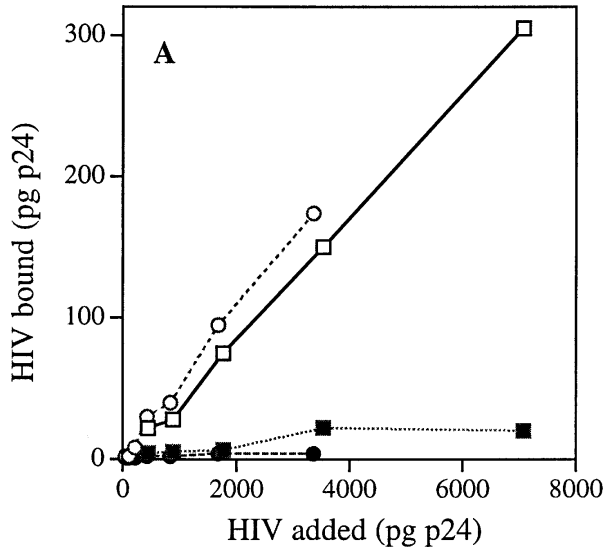


Fig. 1. Binding of HIV to MBL. (A) Microtitre wells were coated with 100 µl MBL at 15 µg/ml overnight and blocked with 1% BSA for 60 min at room temperature. After washing, the GP PI (circles) or HIV-1_{MN} (squares) (total amounts shown on the X axis) were incubated in wells in 100 µl for 2 h. The wells were washed and bound virus was detected by ELISA for p24 after detergent lysis. Open and filled symbols represent amounts of p24 bound to MBL-coated wells or BSA-coated wells, respectively. Results are expressed as the total amount of p24 detected in wells and are representative of three experiments. (B) Microtitre wells were coated with 100 µl MBL at 0, 1, 5, 10 and 15 µg/ml, washed and blocked. HIV-1_{MN} (4 ng p24) was incubated in wells for 2 h and the total amount of bound p24 was detected as in (A). Results are the mean ± SD and are representative of two experiments.

Fig. 2. Specificity of HIV-1/MBL binding. Microtitre wells were coated with serum MBL or BSA and blocked as described in the legend to Fig. 1. (A) The HIV-1_{MN} (5 ng p24) was incubated in coated wells in the presence or absence of EDTA (10 mM) as shown. Some MBL-coated wells were preincubated with mannan at 100 µg/ml for 60 min prior to addition of virus. (B) The GP PI (5 ng p24) was similarly incubated in MBL-coated wells in the presence or absence of Ca²⁺ or EDTA. After washing, the wells were further incubated with Ca²⁺ or EDTA. Results are representative of three experiments.

when wells were coated with as little as 1 µg MBL/ml (Fig. 1B). In order to provide sensitive detection of virus binding, further experiments used wells coated with 10 µg MBL/ml.

The effect of incubation time on virus binding to MBL was also determined. Increasing the time of incubation of HIV in MBL-coated wells from 2 to 8 h increased HIV-1_{MN} binding to MBL by approximately 80% but did not change binding to BSA-coated wells (not shown). Therefore, to increase detection

of virus binding, the incubation time for further experiments was increased from 2 h to 4 and 8 h.

Previous studies have shown that the binding of MBL to carbohydrates is Ca²⁺-dependent. To determine whether the binding of HIV to MBL-coated wells was also Ca²⁺-dependent, HIV-1_{MN} was incubated in wells with either 10 mM Ca²⁺ or EDTA. The amount of virus binding to MBL-coated wells was reduced to background levels in the presence of EDTA indicating that the interaction of MBL with HIV was Ca²⁺-dependent (Fig. 2A). Binding of the GP PI was also reduced to background levels in the presence of EDTA (not shown). Preincubation of MBL-coated wells with mannan at 100 µg/ml

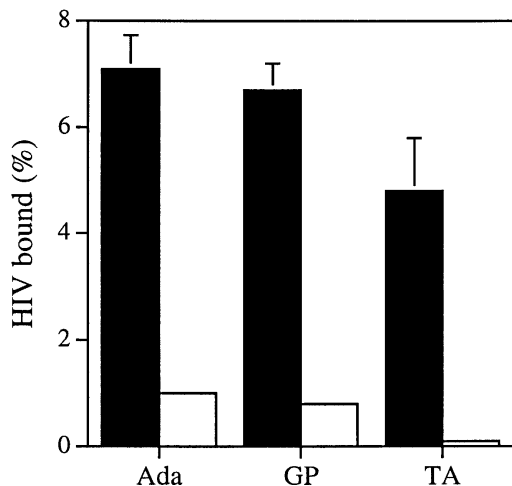


Fig. 3. Binding of HIV primary isolates to MBL. The GP and TA PI, produced in PHA-stimulated PBMC and the Ada isolate, produced in macrophages, were incubated in MBL-coated wells. Bound virus was detected as described in the legend to Fig. 1 and expressed as the percentage of the total that was incubated in wells. Filled bars and open bars represent % \pm SD bound to MBL-coated and BSA-coated wells, respectively. Results are representative of two experiments.

(the highest amount tested) also substantially reduced HIV binding (Fig. 2A), although there remained a small amount of residual binding to MBL. These experiments provide evidence that HIV bound to MBL-coated wells via its carbohydrate-recognition domain (CRD) and that binding to MBL was dependent on carbohydrates on the virus surface.

The initial binding of carbohydrates to MBL is Ca^{2+} -dependent and is prevented by EDTA. Addition of EDTA releases MBL from preformed carbohydrate-MBL complexes when the carbohydrate is covalently linked to a simple structure such as Sepharose. Interestingly, several studies found that addition of EDTA to preformed carbohydrate-MBL complexes did not release MBL when the complexes were on a complex surface such as the surface of red blood cells (Suankratay *et al.*, 1998). To determine whether the MBL-HIV interaction is disrupted by EDTA, GP virus was bound to MBL-coated wells in the presence of Ca^{2+} , wells were washed and EDTA was added. Approximately 90% of bound virus was released from the MBL-coated wells in the presence of EDTA (Fig. 2B) showing that Ca^{2+} is required for virus to remain bound to MBL.

To confirm that other PI also bind to MBL, the TA strain (R5) produced in PHA-stimulated PBMC and the Ada strain (R5) produced in macrophages were compared with the GP isolate (X4) for binding to MBL. All three virus strains bound to MBL-coated wells at similar levels (Fig. 3) indicating that binding to MBL is a common characteristic of HIV PI.

In the above experiments, HIV was shown to bind to microtitre wells coated with MBL purified from serum. In order to confirm that HIV bound to MBL and not to a serum component that co-purified with MBL, the GP PI and HIV-1_{MN}

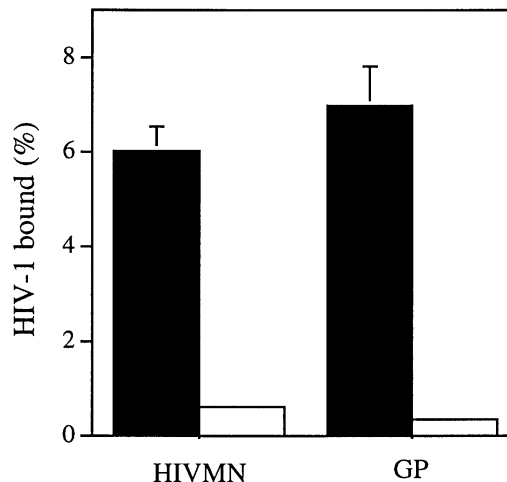


Fig. 4. Binding of HIV to recombinant MBL. Microtitre wells were coated with recombinant MBL at 10 $\mu\text{g}/\text{ml}$. Binding of GP and HIV-1_{MN} were assessed as described in the legend to Fig. 3.

Table 1. Comparison of the ability of MBL and a neutralizing MAb to capture the GP PI of HIV

Microtitre well coating (10 $\mu\text{g}/\text{ml}$)	Amount of virus captured (% \pm SD)
BSA	0.2 \pm 0.1
MBL	9.9 \pm 0.6
IgG1b12	0.5 \pm 0.2

were also tested for binding to immobilized recombinant MBL (rMBL). Both virus preparations bound to rMBL at levels similar to those that bound to serum MBL (Fig. 4). These data indicate that rMBL and serum MBL have similar abilities to bind HIV and provide additional evidence that HIV bound to MBL purified from serum.

We next compared the ability of MBL and the human MAb IgG1b12 to capture the GP PI. Neutralization experiments showed that the GP isolate was > 90% neutralized by incubation with 50 $\mu\text{g}/\text{ml}$ IgG1b12 (not shown) indicating that it binds to the GP isolate. Microtitre wells were coated with either BSA, IgG1b12 or MBL and the amount of virus captured by each of these proteins was determined. While the antibody bound 0.5% of added virus, 9.9% of the added virus bound to MBL and background binding to BSA was 0.2% of the input virus (Table 1). The level of binding of virus to MAb in these experiments agrees with our previous results (Saarloos *et al.*, 1997) and those of others (Nyambi *et al.*, 1998; Orentas & Hildreth, 1993) showing that immobilized MAbs specifically bind intact virus particles but that typically less than 1% of input virus is captured. These results show that immobilized

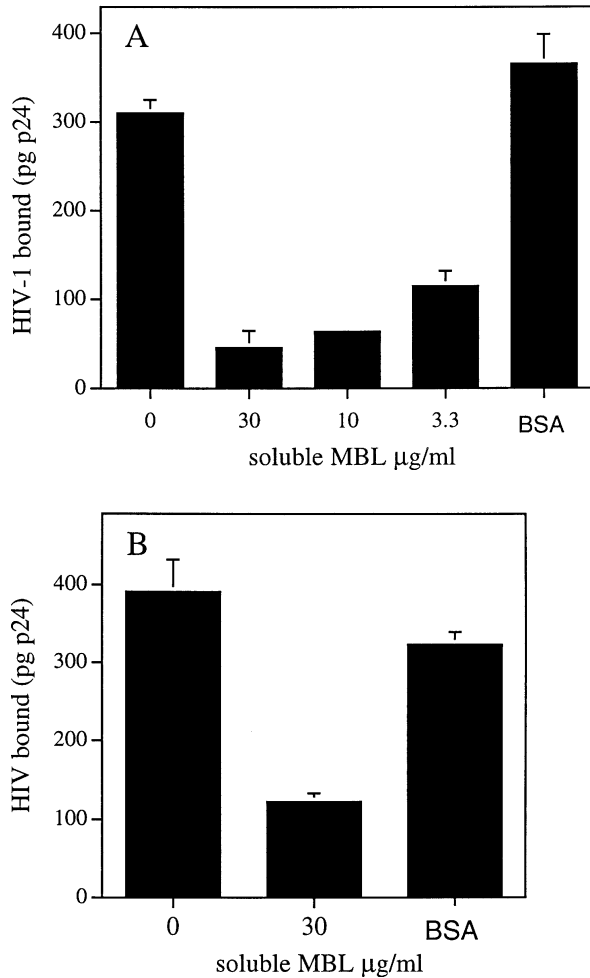


Fig. 5. Inhibition of binding of HIV to immobilized MBL by soluble MBL. Plates were coated with MBL and blocked with BSA as described in the legend to Fig. 1. GP PI (A) or HIV-1_{MN} (B) were preincubated with either soluble MBL at 0–30 $\mu\text{g/ml}$ or 200 mg/ml BSA for 60 min at room temperature prior to addition to MBL-coated wells. Bound virus was detected as described in the legend to Fig. 1 and expressed as mean pg p24 \pm SD. Results are representative of three experiments.

MBL binds a large fraction of virus compared to immobilized neutralizing antibody and indicate that MBL is capable of reacting with a large fraction of PI.

MBL interacts with HIV in solution

The above experiments show that immobilized MBL binds HIV. To determine whether MBL can bind to HIV in solution, competition experiments were performed. Virus was preincubated with increasing concentrations of MBL (up to 30 $\mu\text{g/ml}$) in solution before incubation in MBL-coated wells. As shown in Fig. 5(A), binding of the GP PI to MBL-coated wells was reduced by preincubation with soluble MBL in a dose-dependent manner with even 3.3 $\mu\text{g/ml}$ soluble MBL inhibiting HIV binding by 63%. In contrast, preincubation with BSA at 200 mg/ml did not decrease HIV binding. Similarly,

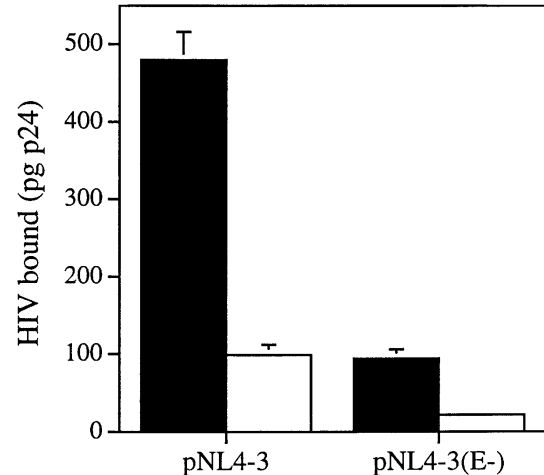


Fig. 6. HIV-1 binding to MBL is dependent on gp120/gp41. Microtitre plate wells were coated with serum MBL and then blocked as described in the legend to Fig. 1. HIV produced in 293 cells transiently transfected with either the pNL4-3 plasmid (for gp120/gp41-positive virus) or the pNL4-3(E⁻) plasmid (for gp120/gp41-negative virus) was adjusted to 40 ng p24/ml in either 10 mM CaCl₂ (filled bars) or EDTA (open bars) and added to wells. The plates were washed and bound virus was detected as described in the legend to Fig. 1. Data are expressed as mean pg p24 \pm SD. Results are representative of three experiments.

soluble MBL also significantly reduced the binding of TCLA HIV-1_{MN} to MBL-coated wells (Fig. 5B) showing that soluble MBL interacts with both cell line-derived and PI of HIV. For both the GP and HIV-1_{MN} viruses, a small amount of residual binding to immobilized MBL occurred with 30 $\mu\text{g/ml}$ soluble MBL, which was the highest concentration that could be tested in these experiments.

Dependence of the MBL–HIV interaction on virus gp120/gp41

The microtitre capture assay was next used to determine whether the interaction of HIV-1 with immobilized MBL occurred through carbohydrate on gp120/gp41, host cell-derived glycoproteins on virions or both. HIV-1 virions expressing gp120/gp41 (env⁺) or lacking gp120/gp41 (env⁻) were generated following transient transfection of 293 cells with either pNL4-3 or pNL4-3(E⁻) HIV-1 plasmids, respectively. The virus preparations were adjusted to 40 ng/ml and added to MBL-coated wells. Binding of env⁺ virions to MBL-coated wells was significantly higher than env⁻ HIV-1 (480 \pm 36 pg p24, 12% versus 99 \pm 13 pg p24, 2.4%, $P < 0.05$, *t*-test) (Fig. 6). Binding of env⁺ virions to immobilized MBL was 80% inhibited by EDTA indicating a specific interaction with the CRD of MBL. The low degree of binding of env⁻ virions to MBL was also 77% inhibited by EDTA, suggesting that some env⁻ virus particles bound to MBL, possibly through high-mannose carbohydrate residues on host cell-derived glycoproteins that were incorporated into virions. However, since expression of gp120/gp41 on virions resulted

in a 4.8-fold increase in binding to MBL, it appeared that glycosylation on gp120/gp41 was responsible for the major portion of virus binding to MBL.

Discussion

Several studies suggest a role for MBL during HIV infection. Thus, individuals homozygous for variant MBL alleles, and who therefore have low serum MBL levels, were at increased risk of HIV infection (Garred *et al.*, 1997; Pastinen *et al.*, 1998). Also, the presence of variant MBL alleles was associated with a slower progression to AIDS (Maas *et al.*, 1998), although this association has not been observed in all studies (Nielsen *et al.*, 1995). The mechanisms responsible for these associations are not known, although possibilities include a direct effect of MBL on HIV or an indirect effect due to increased susceptibility to co-infections (Garred *et al.*, 1997). However, those studies suggest that MBL may be involved in HIV disease and indicate that important information about the interaction of HIV and MBL could be gained by *in vitro* studies.

A previous *in vitro* study showed that the IIIB strain of HIV produced in H9 T cells bound MBL since incubation with MBL resulted in virus neutralization (Ezekowitz *et al.*, 1989). The current study confirms that TCLA virus strains interact with MBL since HIV-1_{MN} bound to immobilized MBL. However, since there are many differences between cell line-derived and PI virus, especially with regard to reactivity with anti-viral antibodies (Moore & Ho, 1995), it was important to determine if PI also reacted with MBL. Additionally, PI are usually produced in primary cells rather than cell lines and studies have shown that N-linked glycosylation of proteins can vary depending on the type of cell (Fukuda, 1985; Galvan *et al.*, 1998). The current study is the first to show that X4 and R5 PI produced in PHA-stimulated PBMC, as well as a macrophage-tropic virus strain produced in primary macrophages, bind MBL. Binding of virus was shown to occur to immobilized MBL as well as to MBL in solution since preincubation of virus with soluble MBL inhibited binding of virus to immobilized MBL. Binding was also shown to occur through the CRD of MBL since it was inhibited by EDTA as well as by soluble mannan.

Interestingly, this study also shows that expression of gp120/gp41 on virions substantially increased the amount of virus attachment to MBL indicating that most of the MBL/virus binding occurred via carbohydrates located on the virus-encoded envelope protein. This was the first study to compare the contribution of host cell- and virus-encoded glycoproteins to MBL binding. This observation helps to localize which carbohydrates are most important for the MBL/virus interaction since many host cell-derived glycoproteins are incorporated into virus at high levels and some are expressed on virions at amounts comparable to that of gp120 (Arthur *et al.*, 1992; Saarloos *et al.*, 1997; Saifuddin *et al.*, 1997, 1995). Our data support the possibility that the high-mannose

glycosylation sites of gp120 contribute largely to the MBL-HIV interaction.

A number of studies found that the ratio of infectious to non-infectious virus is low with less than 1 in 1000 particles being infectious. Since our studies show that MBL can capture as much as 10% of virus particles, MBL must bind at least some of the non-infectious virus. Previous studies by Ezekowitz *et al.* (1989) show that HIV is also neutralized by MBL suggesting that MBL can capture both infectious and non-infectious particles. Thus, it is not unreasonable to conclude that both infectious and non-infectious virus particles have gp120/gp41 that is glycosylated in such a way as to bind to MBL.

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