A HUMAN SERUM MANNOSE-BINDING
PROTEIN INHIBITS IN VITRO INFECTION
BY THE HUMAN IMMUNODEFICIENCY VIRUS

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Mammalian mannose-binding proteins (MBP)[1] were first isolated from the serum of rabbits (1), and subsequently were found in the liver and serum of humans (2–5) and rodents (5–8). Analysis of the encoded human MBP, like its two rat homologues (9), reveals that the protein is divided into three domains: a cysteine-rich NH_2-terminal domain which stabilizes the α helix of the second collagen-like domain, and a third COOH-terminal carbohydrate-binding region (10). MBP may have a role in host defense; this is suggested first by its ability to bind high mannose glycans, which are present in the cell walls of many pathogens, including some Gram-negative bacteria (11–13), mycobacteria (14), yeasts and fungi, certain parasites (15), and envelope glycoproteins of certain viruses, such as the human immunodeficiency virus (HIV) (16, 17). Second, the synthesis of human MBP appears to be stress induced, as it is an acute-phase protein (10). Third, human MBP is a member of a family of homologous lectin-like proteins (18) that includes proteins found in the coelomic fluid of sea urchins (19) and in the hemolymph of Sacraphaga perigintaa (20), which may have a role in the host defense of these organisms.

We wish to evaluate whether the high mannose oligosaccharides identified on the HIV envelope glycoprotein (gp120) were potential ligands for MBP. gp120, isolated from HIV-infected $CD4^+$ H9 lymphoblasts (18) and recombinant gp120 expressed in Chinese Hamster Ovary (CHO) cells (Gregory, T., and M. Spellman, a personal communication) has been shown to possess high mannose glycans. The importance of these high-mannose oligosaccharides in HIV target interaction is suggested by studies showing that plant lectins that recognize certain configurations of high-mannose oligosaccharides inhibit HIV infection and syncytia formation in vitro (21). These conclusions are supported by studies that show that deglycosylated forms of gp120 (22) as well as bacterially expressed recombinant gp120 bind with reduced affinity

[1] Abbreviations used in this paper: CHO, Chinese Hamster Ovary cells; MBP, mannose-binding protein; OPD, o-phenylene diamine; RT, reverse transcriptase.

to CD4 (22). In this report, we demonstrate that MBP can inhibit HIV infection of H9 lymphoblasts at physiologically relevant concentrations, and that MBP binds selectively to HIV-infected cells. Mannan-inhibitable, -saturable binding of recombinant gp120 to MBP suggests that the high mannose glycans on gp120 are indeed the ligand recognized by MBP.

Materials and Methods

Reagents

DME, FCS, glutamine, penicillin, and streptomycin were obtained from Gibco Laboratories (Grand Island, NY). Yeast mannan was obtained from Sigma Chemical Co., (St. Louis, MO) and a glycoconjugate of mannose and BSA was used as described (23). HIV antisera were obtained from seropositive patients, gp120 antiserum, anti-gp120 mAb B56, and recombinant gp120, which were generously provided by Dr. T. Gregory, Genentech Inc., San Francisco, CA. Anti-rabbit IgG coupled with rhodamine was obtained from Fisher Scientific Co. (Pittsburgh, PA), and anti-rabbit IgG coupled with alkaline phosphatase was supplied by Promega Biotec (Madison, WI). Goat anti-mouse IgG coupled with horseradish peroxidase was obtained from Bio-Rad Laboratories (Richmond, CA).

Purification of Human MBP

Human MBP was purified from human plasma from patients undergoing plasmapheresis as described (2). Briefly, the plasma was incubated with mannan-Sepharose beads in the presence of 20 mmol calcium chloride, and after extensive washing, the beads were loaded onto a column and a protein peak was eluted with 10 mmol Tris/EDTA. The protein peak was collected, recalcified, and passed down a second mannan-Sepharose column and was eluted with 50 mmol D-mannose. C-reactive protein and serum amyloid A protein were retained on the column in the presence of 50 mmol D-mannose, and only MBP was eluted. Subsequent elution of this second column with 10 mmol Tris/EDTA released these other proteins. Binding assays using ^{125}I-mannose-BSA that was radiolabeled as described (23) revealed a 3,000-fold purification. Specific binding was not observed in the absence of calcium (not shown). An aliquot of MBP was boiled in the presence of 2% SDS and 2% beta-mercaptoethanol and separated on 10% SDS PAGE (Laemmli system [24]). The gels were either fixed in 10% methanol and proteins were detected with Coomassie Brilliant Blue, or transferred to nitrocellulose by Western blotting. The procedure was performed on an Hoeffer Scientific Instruments (San Francisco, CA) apparatus according to the manufacturer's instructions. Thereafter the filter was incubated with a rabbit anti-human MBP antiserum (a gift from Dr. J. Baenziger, Washington University School of Medicine, St. Louis, MO) for 1 h, washed, and incubated with a goat anti-rabbit alkaline phosphatase-coupled second antibody (Promega Biotec). No staining was observed in the absence of the first antibody. The results shown in Fig. 1 demonstrate a major band at 32 kD and minor band 64 kD. Both these species react specifically with the antiserum. The upper band represents incompletely reduced dimers of MBP.

Cell Lines

The CD4$^+$ and T cell lines H9 and CD4$^+$ myeloid-like cell line U937 have been described in detail elsewhere (25). Cell lines were maintained in DME supplemented with 20% heat-inactivated FCS and 2 mm glutamine plus penicillin and streptomycin. Assays of free virus infectivity were performed in the same medium.

HIV Preparation

Reverse Transcriptase (RT) Activity. Magnesium-dependent activity was measured as described (26). 100% activity was equal to 1.5×10^6 cpm and background counts were 5×10^3 cpm. Values presented are means of duplicate assays with standard deviation of $\pm$ 15% and are representative of three independent experiments. Independent batches of MBP were used and gave similar results.
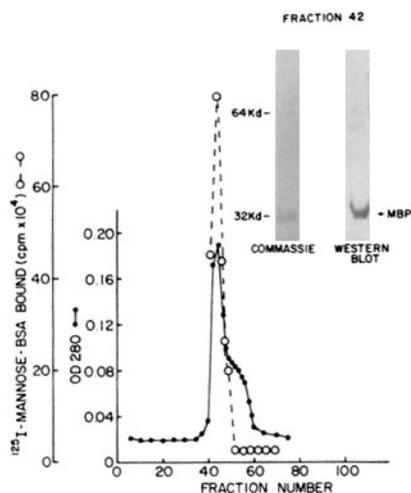
FRACTION 42

FIGURE 1.   Purification of human mannose-binding protein. The closed circles represent the protein concentration of the peak eluted with 50 mmol D-mannose from a mannan-Sepharose column (6) as determined by OD_{280}. The open circles represent calcium-dependent binding assays across the peak with ^{125}I-mannose-BSA (sp act 2×10^6 cpm/µg). Fraction 42 contained the maximum activity and protein content and was analyzed by SDS-PAGE under denaturing conditions and stained with Coomassie blue. A sample run in parallel was transferred to nitrocellulose by Western procedure and specifically stained with a rabbit anti-human MBP antiserum and a goat anti-rabbit alkaline phosphatase-coupled second antibody.

Assays of HIV Infectivity.   A HIV virus inoculum of 100 tissue culture infective dose 50s (1 $TCID_{50}$ is defined as the infective dose that infects half the wells in a 24-well tissue culture tray containing 2×10^5 cells/well) was preincubated for 60 min at 37°C with various concentrations of MBP, ranging from 50 µg/ml to 1 µg/ml, in the presence or absence of 1 mg/ml of the mannose-rich yeast mannan. Similar pretreatment with 1 µg/ml or 10 µg/ml Con A or anti-gp120 antisera was also performed. The treated and untreated virus inoculum was then added to 2×10^5 H9 cells, incubated for 1 h at 37°C, and transferred to 2 ml of fresh media in a 24-well tissue culture tray. After 7 d of culture, RT activity was measured in the supernatants as described (26). Percent neutralization was calculated as percent RT activity after various treatments over RT activity of untreated inoculum multiplied by 100. Infectivity was also assessed by the presence of HIV proteins on the cell surface, as detected by specific anti-HIV antiserum and fluorescent-labeled goat anti-human antibody. After 7 d in culture, cells were fixed and then lightly counterstained with Evans' stain and examined by fluorescence microscopy. Numerous fields of quadruplicate wells were examined and the photomicrographs shown in Fig. 3 are representative of five independent experiments.

MBP Binding to HIV-infected U937 Cells

CD4+ U937 were cultivated in the presence of HIV as described above for H9 cells. 30–50% of the population expressed gp120 on the cell surface as detected by a specific gp120 antiserum and a fluorescently labeled second antibody (not shown). To detect MBP binding to the surface of infected cells, fixed cells were incubated with 10 µg/ml of MBP in the presence of 10 mmol calcium chloride for 30 min at room temperature. The cells were then washed three times in HBSS and incubated for 30 min with a rabbit anti-human MBP antiserum, which was detected by a goat anti-rabbit antibody coupled to alkaline phosphatase. After five washes, nitroblue tetrazolium and BCIP were added according to the manufacturers instructions (Promega Biotec).

Measurement of MBP Levels in Serum

Two dilutions of 78 coded serum samples from 4 groups, healthy volunteers, patients who were seropositive for HIV but asymptomatic, patients with AIDS-related complex (ARC), and patients with AIDS were incubated with a 1:5,000 dilution of anti-MBP antiserum for 30 min and then added to a 96-well Immulon II plate (Dynatech Laboratories, Inc., Alexandria, VA) that had been precoated with 50 ng/well of MBP. After a 30-min incubation at room temperature, the wells were washed with PBS and 0.05% Triton and a goat anti-rabbit horseradish peroxidase second antibody was added. Specific binding was detected by a soluble

chromagen o-phenylene diamine (OPD) (Sigma Chemical Co.) and read against known standards in an ELISA reader. The values of MBP in serum were calculated on a Cricket graph program on a Macintosh computer. The code of the samples was broken after values had been attained and were ranked within the four groups described. In five samples, the values obtained from the two dilutions gave values that were varied by >15% and these were removed from the analysis.

Binding of Recombinant gp120 to MBP

1 μg/well of human MBP was bound to Immulon II Elisa plates in phosphate buffer, pH 9.5, overnight at 37°C. The plates were washed three times in PBS and 0.5% Triton 100. Thereafter, varying concentrations of recombinant gp120 (0.9 ng to 9 μg) that was expressed in CHO cells (gift from Dr. T. Gregory, Genentech, Inc.) were incubated with MBP in 100 μl of HBSS at room temperature for 1 h. The plates were then washed three times and a 1:100 dilution of a mouse anti-gp120 mAb was added to each well. This antibody, BC5, is directed against a domain of gp120 that does not interfere with the binding of gp120 to CD4 (Gregory, T., personal communication). After 60 min at 37°C .the plate was washed three times with HBSS and 0.5% Triton and a goat anti-mouse horseradish peroxidase-coupled second antibody (Bio-Rad Laboratories) was added. OPD was added and the reaction was read on an Elisa reader. To remove all the carbohydrate moieties, 20 μg of gp120 was incubated overnight at 37°C in 0.1% SDS and 0.55 M sodium phosphate buffer, pH 8.6, with 10 U of N-glycanase. The reaction was terminated by the addition of sodium citrate according to manufacturers instructions (Genzyme Corp., Boston, MA). Detailed analysis of gp120 so treated revealed removal of the high mannose glycans (Gregory, T., Genentech, personal communication).

Results

Human MBP Inhibits HIV Infection of H9 Cells

Reverse Transcriptase. Pretreatment of HIV with MBP inhibited in vitro infection of CD4$^+$ H9 cells in a dose-dependent manner (Fig. 2), as determined by the measurement of RT activity in the media of the cultured cells (26). Inhibition was dependent on the concentration of MBP with 25% inhibition observed at 1 μg/ml and
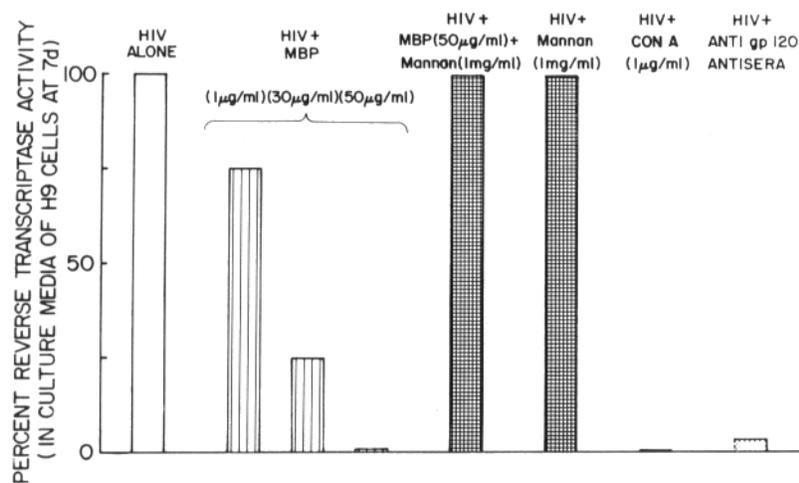

FIGURE 2. Percent RT activity measured in the tissue culture media of H9 cells. 100% activity was equal to 1.5 × 10^6 cpm and background counts were 5 × 10^3 cpm. Values presented are means of duplicate assays and representative of four independent experiments.

100% inhibition at 50 μg/ml of MBP. The results were highly reproducible with four independent batches of MBP. The inhibition of virus infection by MBP was specifically abrogated by preincubation of MBP with the mannose-rich yeast mannan. Pretreatment of the viral inoculum with Con A at 1 μg/ml and anti-gp120 antiserum also inhibited virion infectivity, as has previously been shown (25). Higher concentrations of Con A adversely affected cell viability.

HIV Surface Antigens. We next examined the H9 cells for the presence of HIV surface proteins, a hallmark of a productive infection, using a specific heteroantiserum and a fluorescently labeled second antibody. As expected, untreated virus resulted in a productive infection. Fig. 3 *A* reveals the presence of specific staining against the background red Evans' counterstain. The absence of HIV surface proteins on the cells that received HIV plus 50 μg/ml MBP is shown in Fig. 3 *C*, consistent with the 100% inhibition of RT activity. The inhibition of virus infection by MBP could be specifically abrogated by the mannose-rich yeast mannan, as evidence by the reappearance of HIV surface proteins (Fig. 3 *E*).

MBP Binds Specifically to HIV-infected Cells. H9 Lymphoblasts. Studies with recombinant vaccinia virus containing the HIV envelope gene demonstrated that Con A-mediated inhibition of HIV-induced cell fusion involved lectin binding to the viral envelope glycoprotein (21). It seemed reasonable, therefore, that human MBP inhibited HIV virus infectivity by binding to mannose residues of the viral envelope glycoprotein. To evaluate this possibility we stained HIV-infected and uninfected H9 cells with directly fluoresceinated MBP. Direct specific calcium-dependent binding was observed on the surface of HIV-infected cells (Fig. 4 *A*). The binding was specifically inhibited by the neoglycoprotein mannose-BSA (Fig. 4 *C*) and no specific fluorescence was detected on uninfected H9 cells (Fig. 4 *B*), although in some rare cells nonspecific cytoplasmic staining was observed (not shown).

U937 Cells. We next examined whether MBP would selectively recognize the HIV envelope glycoprotein expressed on the surface of nonlymphoid cells. U937 cells, a CD4$^+$ monocyte cell line, were infected with HIV, fixed with methanol, and incubated with MBP. The calcium-dependent binding of MBP to infected U937 cells was detected by a sandwich technique with anti-MBP antibody followed by a second antibody coupled to alkaline phosphatase. The reaction product catalyzed by this enzyme revealed a pattern of staining in infected HIV U937 cells (Fig. 5 *a*). No specific staining was observed in the untreated control cells (Fig. 5 *b*), and the binding of MBP to infected cells was specifically inhibited by 200 μg mannose-BSA (Fig. 5 *c*). These results suggest that MBP selectively recognizes the configurations of high mannose glycans on the gp120 expressed on the surface of HIV-infected myeloid as well as lymphoid cells.

Recombinant gp120 Binding to MBP. Our next goal was to assess direct binding of gp120 to MBP. As gp120 is difficult to radiolabel, we used an ELISA method to demonstrate specific saturable binding of gp120 to 1 μg of MBP (Fig. 6). Inhibition studies with 1 mg/ml of yeast mannan, the decreased binding of *N*-glycanase-treated gp120, and the divalent cation dependence of the interaction, strongly implicate the carbohydrate moieties on gp120 as the ligand recognized by MBP. The anti-gp120 mAb did not react with MBP in the absence of gp120. Similarly, the goat anti–mouse IgG–coupled horseradish peroxidase antibody did not bind to the MBP in the absence of gp120 and the mouse anti-gp120 mAb.
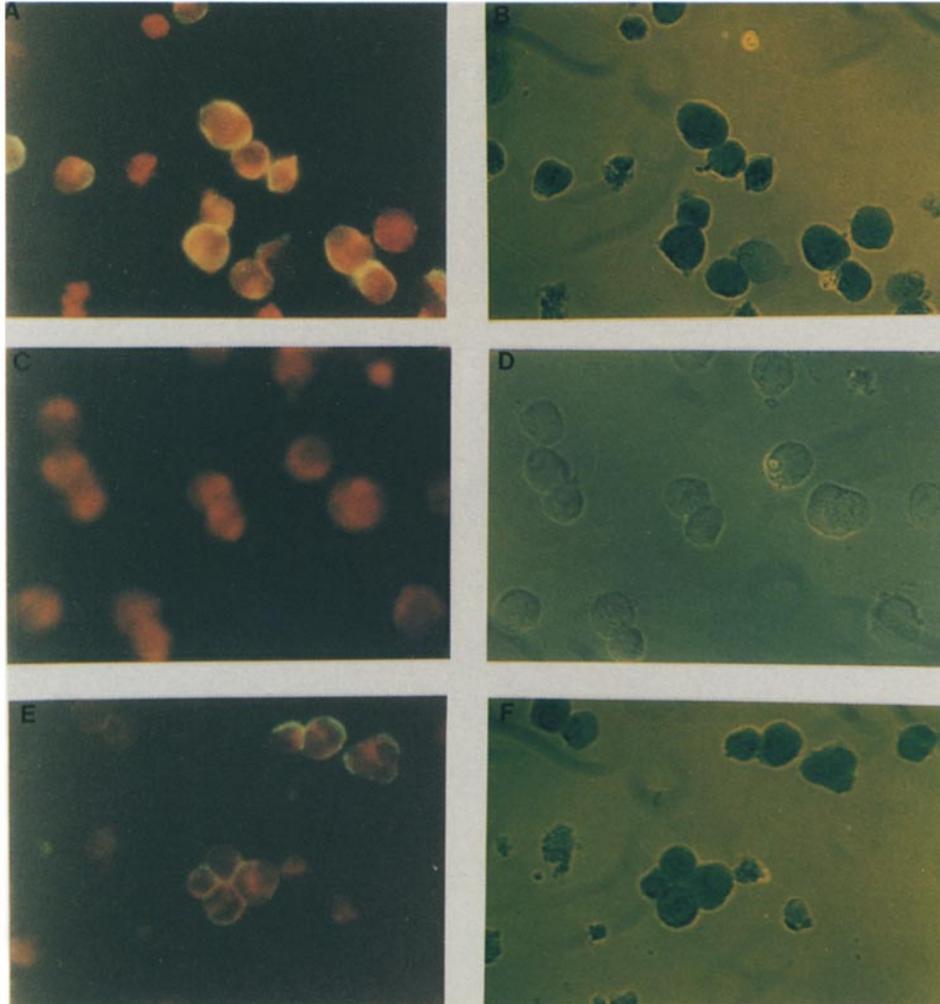
FIGURE 3.   Inhibition of HIV infection of H9 cells in vitro. Infectivity was assessed by the presence of HIV proteins as detected by specific anti-HIV antisera and a fluorescent-labeled goat and human antibody on fixed preparations after 7 d of culture. (A) The expression of HIV proteins, a hallmark of a productive infection after 7 d of culture in the presence of stock virus titer (100 $TCID_{50}$). (B) Phase-contrast micrograph of the same field. (C) After preincubation of stock virus with 50 µg/ml MBP, no HIV proteins were detected by specific immunofluorescence. (D) Phase-contrast micrograph; the cells appear healthy, viable, and do not form obvious syncytia. (E) Specific appearance of HIV proteins after addition of 1 mg/ml yeast mannan. (F) Phase-contrast micrography. Original magnification was ×400.

MBP Concentrations Active In Vitro Are within the Physiological Range.   MBP levels were measured in serum from a total of 78 randomly coded samples of HIV antibody–seronegative hospital workers, HIV antibody–seropositive patients, patients with AIDS-related complex (ARC), and patients with AIDS. The results shown in Fig. 7 demonstrate that although MBP serum levels were significantly higher in the ARC and AIDS groups compared with the seronegative and seropositive groups,
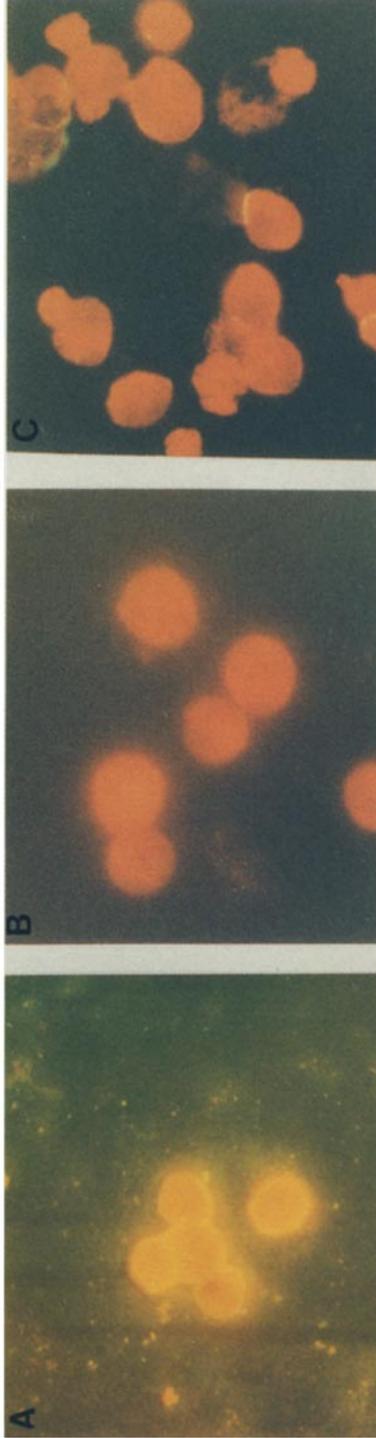
FIGURE 4.   Directly labeled fluorescein (FITC)-coupled MBP binds to HIV-infected but not uninfected H9 cells. (A) FITC-MBP bound to HIV-infected H9 cells. (B) MBP did not bind to uninfected H9 cells. (C) The binding is specifically inhibited by the addition of mannose-BSA (200 µg/ml). Cells were counterstained with Evans' stain accounting for the red background.
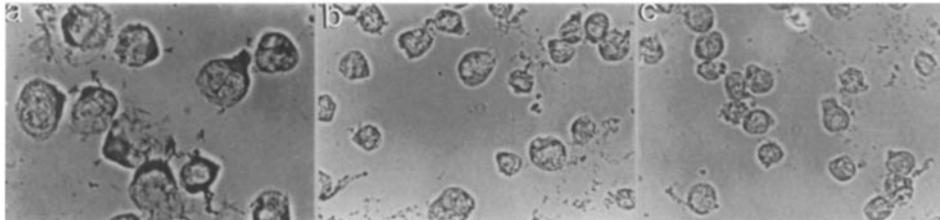
FIGURE 5.   Staining of HIV-infected and uninfected U937 cells with human MBP shows alkaline phosphatase-catalyzed reaction product in which MBP bound is detected by rabbit anti-MBP antiserum, which in turn is detected by a horseradish peroxidase goat anti-rabbit Ig. (a) Binding to HIV infected U937 cells. (b) Lack of binding to uninfected cells. (c) Mannan inhibition of MBP binding to HIV-infected cells.

34 of 37 patients had values below the effective in vitro inhibitory concentration of 50 µg/ml. As the MBP is an acute-phase reactant, we would predict that the one patient in the AIDS group who did have MBP serum levels between 50–60 µg/ml most likely had an acute infection. Review of the medical records of this patient revealed that he had an acute episode of pneumocystis pneumonia. Of the two ARC patients with high MBP levels, one had acute cytomegalovirus infection and the other appeared to be well. The medical records of the other patients were not available for review. Unfortunately, serial samples were not available and therefore no definitive statement can be made from this limited information on whether any correlation exists between lower than expected MBP serum levels and disease progression, or conversely, whether high levels confer protection in vivo.

Discussion

In this study we show that preincubation of HIV I with human mannose-binding protein results in almost a 100% inhibition of HIV infection of CD4$^+$ H9 lymphoblasts in vitro. The mannose-rich yeast mannan, which binds with high affinity to MBP, abrogates the inhibitory effects of MBP on HIV tropism of the target cells. More direct evidence that MBP selectively recognizes high mannose glycans known to be present on gp120 comes from the demonstration that recombinant gp120 binds directly to MBP. The binding saturable can be inhibited by mannan and N-glycanase
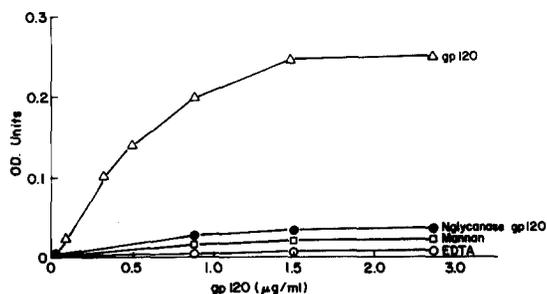

FIGURE 6.   Binding of recombinant gp120 to 1 µg/well of human MBP. (△) gp120 in increasing concentrations from 0.9 ng to 2.7 µg/ml. (●) gp120 treated with N-glycanase, which removes carbohydrate moieties. (□) gp120 plus 1 mg/ml mannan. (○) gp120 plus 10 mmol EDTA. gp120 was detected by a specific mouse anti-gp120 mAb that bound a goat anti-mouse IgG coupled to horseradish peroxidase. The color reaction was initiated by addition of OPD and H_2O_2 and read at OD_{441}. Results shown are representative of four independent experiments.
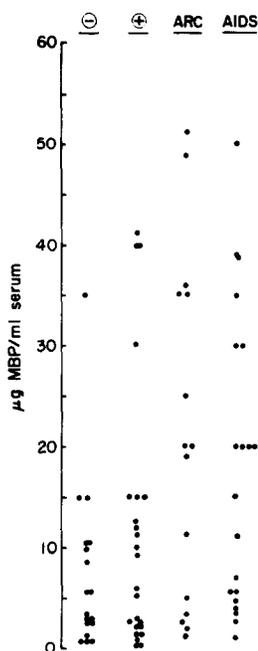
FIGURE 7.   MBP levels in serum from 18 HIV antibody–seronegative hospital workers, 24 HIV antibody–seropositive patients, 18 ARC patients, and 18 AIDS patients. MBP levels were determined by inhibition ELISA against a known standard. Points represent a mean of assays performed in triplicate ± 10% SD.

treatment of gp120 and is dependent on the presence of cations, a characteristic common to lectin-ligand interactions (18).

The precise molecular basis for MBP-mediated inhibition of HIV infection in vitro is not clear. It is possible that through its interaction with the exposed mannose chains on the envelope glycoprotein, MBP interferes with the topology of the ligand either by masking those epitopes required for adhesion to the receptor or by inducing a conformational change of the ligand. Alternatively, MBP may not affect the initial adhesion event, but may mask fusigenic domains on the virus and thereby inhibit viral fusion and entry of the virus into the cell. It would appear most likely, however, that MBP recognizes in large part exposed high mannose glycans on gp120. It is intriguing to note that the region from amino acid 397 to 439 of the HIV I gp120, which appears critical for the interaction of gp120 with CD4 (27), has two N-linked glycosylation sites, both of which have high mannose oligosaccharides (Gregory, T., and M. Spellman, a personal communication). Ligation of these sites by MBP would mask the domain on gp120 required for binding to CD4. It has been suggested by Laskey et al. (27) that this CD4 interaction site of gp120 may be found in a cleft, hence rendering it relatively antigenically inaccessible. This same tertiary conformation may explain the presence of high chains at these sites as steric accessibility of oligosaccharides is an important factor in processing of high mannose oligosaccharides to complex sugars (28, 29). The conservation of this region and the potential glycosylation sites in different isolates of HIV I as well as between HIV I and HIV 2 (30), raises the possibility that these high mannose glycans could provide conserved targets for potential therapeutic agents. A hybrid molecule containing the mannose-binding domain of MBP, coupled to a toxin, may have potential in this regard.

The lack of high affinity binding of MBP to normal cells implies that the configurations of carbohydrate normally present on cell surface glycoproteins are not recognized by MBP. This is confirmed by the failure of MBP to bind to lymphocytes, polymorphonuclear leukocytes (PMN), and monocytes in the absence of ligand (Ezekowitz, R. A. B., unpublished data), and contrasts with the mannose-binding plant lectin Con A, which has been shown to bind to lymphocytes, PMN, and monocytes (31). These differences are not surprising as these two proteins bear no structural homology to one another (32) and illustrate that MBP appears to have a more restricted specificity than Con A.

The measurement of MBP levels in AIDS patients' sera serves only to illustrate that the concentrations of MBP that inhibit HIV infection of CD4$^+$ cells in vitro are within the range found in vivo. Understanding the precise role of MBP in HIV infection in vivo is complicated by studies in which we have shown that MBP is able to opsonize mannose-rich bacteria that are then taken up by PMN and monocytes (Kuhlman, M., K. Joiner, R. A. B. Ezekowitz, submitted for publication). Therefore, while MBP may mask the CD4-binding domain of gp120, its physiological role may be to clear free virus, virally infected cells, or circulating gp120 from the circulation into monocytes and PMN. This may enhance HIV entry into monocytes via a CD4-independent pathway.

This study is the first step in examining the role of this naturally occurring lectin-like protein in HIV I infection. The selective binding of MBP to HIV-infected cells provides some insights into the specificity of this host defense molecule, and suggests that mannose-binding proteins may be part of a primitive host response to infection. Present studies are aimed at examining whether MBP will selectively recognize other viruses and virally infected cells, which may express exposed high mannose oligosaccharides as part of their viral envelope on the surface of infected cells.

Summary

In vitro infection by the human immunodeficiency virus (HIV) of CD4$^+$ H9 lymphoblasts is inhibited by a mannose-binding protein (MBP) purified from human serum. In addition, MBP is able to selectively bind to HIV-infected H9 cells and HIV-infected cells from the monocyte cell line U937. These results indicate MBP most likely recognizes high mannose glycans known to be present on gp120 in the domain that is recognized by CD4 and thereby inhibits viral entry to susceptible cells. In support of this contention, recombinant gp120 binds directly to MBP; the binding is saturable, mannan inhibitable, removed by N-glycanase treatment, and dependent on divalent cations.

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