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Inhibition of Human Immunodeficiency Virus-1 Infection by Human Conglutinin-like Protein: *In vitro* Studies

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The lectin-like protein analogous to bovine conglutinin was purified from human serum. The carbohydrate-binding ability of conglutinin-like protein was inhibited by D-mannose, *N*-acetylglucosamine and L-fucose as well as by mannan-containing oligosaccharides. By applying a lectin-based ELISA system it was demonstrated that conglutinin-like protein binds to human immunodeficiency virus-1 (HIV-1) glycoprotein 120 (gp120) via its carbohydrate binding site. *In vitro* experiments with T-lymphoblastoid CEM cells revealed that conglutinin-like protein abolishes infection by HIV-1; a 50% cytoprotective concentration of 23.9 μg/ml was measured. These findings demonstrate that human conglutinin-like protein binds to HIV-gp120 and inhibits, under the described *in vitro* conditions, CEM cell infection.

Key words: HIV-1 — Lectin — Conglutinin-like protein — gp120 — Mannan

The major envelope glycoprotein of the human immunodeficiency virus type 1 (HIV-1)[6] (gp120) plays a key role during the initial phase of virus infection.[1,2] gp120, which consists of more than 20 potential N-glycosylation sites, is heavily glycosylated.[3,4]

In recent years a series of mannose-specific plant and animal lectins has been described which block HIV infection *in vitro*.[5-8] Besides these lectins a mannose-binding protein from human serum has been identified which inhibits HIV infection.[9] This 32-kDa protein which binds to the carbohydrate side chains of both N-linked and O-linked glycoproteins[10] was shown to bind to HIV-gp120 and thereby to inhibit viral entry. Besides this mannose-specific, lectin-like protein, a second mannose-binding protein analogous to bovine conglutinin,[9,11] termed human conglutinin-like protein, exists in human serum. In addition to mannose, conglutinin binds terminal *N*-acetylglucosamine and fucose residues.[11-14] Binding is calcium-dependent; for optimal activity 2 m*M* $CaCl_2$ is required.[14] At present the function of human conglutinin-like protein is not known. Here we report that purified human conglutinin-like protein binds to HIV-gp120 and blocks virus infection *in vitro*.

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[6] Abbreviations: HIV, human immunodeficiency virus; AI, antiviral index; AIDS, acquired immunodeficiency syndrome; ELISA, enzyme-linked immunoassay; HTLV, human T-lymphotropic virus; IC_{50}, 50% cytoprotective concentration; RT, reverse transcriptase; TC_{50}, 50% cytotoxic concentration; EC_{50}, 50% effective antiviral concentration.

MATERIALS AND METHODS

Zymosan (from *Saccharomyces cerevisiae*), mannose (cross-linked to agarose; M-6400) and collagenase (C-0773) were obtained from Sigma, St. Louis, MO (USA); rabbit antibodies against sheep erythrocytes from Behringwerke, Marburg (Germany); all carbohydrates from BioCarb Chemicals, Lund (Sweden); alkaline phosphatase, digoxigenin-labeled lectin from *Galanthus nivalis* and anti-digoxigenin peroxidase were obtained from Boehringer Mannheim, Mannheim (Germany).

Purified gp120 was prepared from HIV-IIIB-infected H9 cells as described.[6] The lectin from *Narcissus pseudonarcissus* (NPL) was purified as described.[15] Mannans from yeast cells (*Candida albicans*) of the following structures were prepared as described[16]: M-(1→3)-[M-(1→2)]$_4$-M and [M-(1→2)]$_5$-M linked to [M-(1→6)M]$_2$ (=Man-A) and [M-(1→2)]$_3$-M-(1→3)-M-(1→2)-M and [M-(1→3)]$_2$-[M-(1→2)]$_2$-M linked to [M-(1→6)M]$_2$ (=Man-B).

Antiretrovirus assays The methodology of the anti-HIV assays has been described previously.[17] Human T lymphoblastoid CEM cells [acutely infected] (starting concentration: 1×10^5 cells/ml) were suspended in medium and infected with HIV-1 (strain $HTLV_{IIIB}$[18]) at 50 50%-cell culture infective dose [$CCID_{50}$] (1 $CCID_{50}$ being the dose infective for 50% of the cell culture) per ml of cell suspension. For the inhibition studies the 100-μl virus suspensions, used to infect the cells, were preincubated

(12 h; 4°C) with the indicated amount of conglutinin-like protein. Cultures (1 ml) were incubated for five days. The number of viable cells was monitored by the MTT colorimetric assay system[19] and evaluated with an ELISA reader.[20] After incubation for five days the uninfected CEM cells had undergone 2.16 doubling steps[21] and the infected cells underwent 0.13 doubling step. To test for virus release, cells were removed and supernatant fluids were assayed for reverse transcriptase (RT) activity.[22] The activity of this enzyme after incubation of the infected cells for 5 days was 9.3×10^5 cpm/ml culture fluid. The percentage of cells expressing p17 and p24 *gag* proteins of HIV-1 was determined by indirect immunofluorescence microscopy with the use of mouse monoclonal antibodies to HIV-1 p17 and p24. Positive cells were visualized by treatment with fluorescein-labeled goat anti-mouse IgG[17]; the reactivity of the antibodies with HIV-1-infected cells was in the range of 70–85%.

The 50% effective antiviral concentration (EC_{50}) was estimated from six different test concentrations by logarithmic regression[23] and represents that concentration which displays a 50% cytoprotective effect on HIV-infected CEM cells.

Buffers Phosphate-buffered saline (PBS): 1.5 m*M* KH_2PO_4, 8.1 m*M* Na_2HPO_4, 137 m*M* NaCl, 2.7 m*M* KCl, 15 m*M* NaN_3 (pH 7.4). Veronal-buffered saline (VBS): 4 m*M* Na-barbital, 145 m*M* NaCl, 15 m*M* NaN_3, 0.05% [v/v] Tween 20 (pH 7.4). Me^{2+}-VBS was VBS containing 2 m*M* $CaCl_2$ and 1 m*M* $MgCl_2$. EDTA-VBS was VBS containing 10 m*M* EDTA.

Isolation and purification of conglutinin-like protein Human conglutinin-like protein was prepared as described.[9, 24] Briefly, pooled frozen normal human serum (a batch of 250 ml) was thawed and heated at 56°C for 45 min. The serum was supplemented with 2 m*M* $CaCl_2$ and incubated with Zymosan (1 mg/ml). After washing with PBS, bound protein was eluted with EDTA-VBS. The resulting eluate (15 ml) was supplemented with $CaCl_2$ to give a final concentration of unchelated Ca^{2+} of 2 m*M*. This material was applied to a mannan-agarose column (2×10 cm) which was equilibrated with Me^{2+}-VBS. The protein bound to the column was eluted in a volume of 45 ml with EDTA-VBS; the protein content was 12.7 mg. This fraction was dialyzed against EDTA-VBS which was saturated to 70% with respect to ammonium sulfate. The precipitated material was collected by centrifugation and suspended in 7 ml of 10 m*M* Tris/HCl buffer (pH 7.4; 2 m*M* EDTA, 200 m*M* NaCl). This fraction was applied to a Sepharose 6B-CL column (1×50 cm) which was equilibrated with this Tris buffer. Fractions of 1.0 ml were collected and analyzed. One peak of protein (V_e/V_0 range: 1.08–1.18,[25] corresponding to an Mr of approximately 600,000 to 700,000) was identified, which coincided with the peak of conglutinin-like protein agglutination activity. The specific activity of the pooled fraction (no. 43–45) was 83.3 titer units/mg of protein (33.7 titer units/ml).

The analysis of the fraction by polyacrylamide gel electrophoresis under denaturing conditions (and without dithiothreitol treatment [unreduced sample]) revealed an Mr of 330,000 to 340,000 (Fig. 1, lane a). After treatment of the fraction with dithiothreitol (reduced sample) two major protein bands, one band with an Mr of 100,000 and a second one of Mr 67,000 were obtained (lane b). After treatment of the reduced sample with collagenase (collagenase-treated, reduced and alkylated sample) the band at Mr 67,000 disappeared, while the Mr 100,000 band split into a second species of Mr 90,000 (lane c). Based on published data[9] which are in agreement with the data presented here, the human conglutinin-like protein behaves under native conditions as a dimer of Mr 600,000 to 700,000, composed of two Mr 330,000 to 340,000 protein species (denaturing conditions).

Conglutinin reaction The agglutination activity of the conglutinin-like protein was determined according to Baatrup *et al.*[14] Briefly, sheep erythrocytes were fixed with glutardialdehyde and coated with antibodies (rabbit antibodies against sheep erythrocytes) and complement (human serum); this preparation is termed EAC. One hundred μl of Me^{2+}-VBS (containing either no other component or supplemented with various concentrations of carbohydrates) was mixed with 100 μl of conglutinin-like protein in a microplate well. After 10 min, 100 μl of a 2% suspension of EAC (in Me^{2+}-VBS) was added;

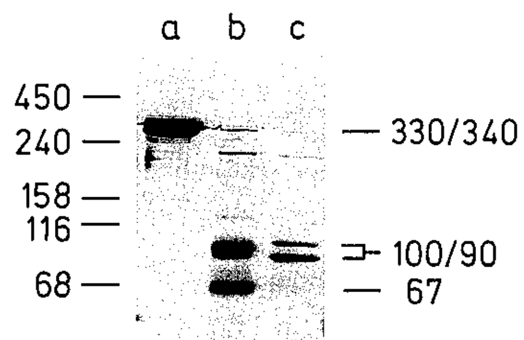

Fig. 1. Polyacrylamide gel electrophoresis of conglutinin-like protein. Final fraction, obtained after gel chromatography, was electrophoresed on 5–20% polyacrylamide gradient gel under denaturing conditions. As described under "Materials and Methods" three preparations of the fraction were analyzed; (i) unreduced sample (lane a) (ii) reduced sample (lane b) and (iii) collagenase-treated, reduced and alkylated sample (lane c). Molecular weight standards were ferritin (450 kDa), catalase (240 kDa), aldolase (158 kDa), β-galactosidase (116 kDa) and bovine serum albumin (68 kDa).

agglutination was read after 2 h of incubation (room temperature). The reciprocal of the greatest dilution at which agglutination occurred was taken as the titer in the original preparation containing the conglutinin-like protein.

For hapten inhibition experiments, varying amounts of sugars were added to the reaction mixture (300 μl) consisting of 100 μl of conglutinin-like protein and 20 μg of Man-A in Me^{2+}-VBS. After 2 h of incubation (room temperature) the reaction mixture was centrifuged and the precipitates were analyzed for protein.

Gel electrophoresis Conglutinin-like protein was size-separated by 5–20% polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate.[9,26] The conglutinin-like protein sample mixed with sample buffer (3% sodium dodecyl sulfate, 10% glycerol, 8 *M* urea) was either applied directly to the gel [unreduced sample], or was reduced (using 30 m*M* dithiothreitol) and alkylated (with 65 m*M* iodoacetic acid) prior to gel electrophoresis as described.[9] In one experiment conglutinin-like protein, adsorbed on mannan-agarose beads, was digested with collagenase[9] and then transferred to sample buffer; after reduction and alkylation the sample was analyzed [collagenase-treated, reduced and alkylated sample].

gp120 binding ELISA A detailed description was given earlier.[16,27] Microtiter plates were coated with the mannose-specific *Narcissus pseudonarcissus* lectin. Free gp120 (1 to 300 ng/assay) was dissolved in 50 μl of "dilution buffer" (3% bovine serum albumin in PBS, supplemented with 2 m*M* $CaCl_2$, 1 m*M* $MgCl_2$ and 0.02% NaN_3) and preincubated with 50 μl of conglutinin-like protein (0 to 17 μg) for 12 h at 4°C. This material was added to the wells and incubated for 24 h (4°C) in a humid atmosphere. After a washing step, digoxigenin-labeled lectin from *Galanthus nivalis* (10 μg/ml Me^{2+}-PBS) was added to each well and incubated (1 h; 20°C). Finally, after washing of the plates with Me^{2+}-PBS, anti-digoxigenin/peroxidase (0.15 U/ml PBS) was added and the immunocomplexes were detected and quantified by addition of 4 m*M* *o*-phenylenediamine as a substrate. Finally, the reaction was stopped with 0.1 *M* H_2SO_4 and the analysis was performed at 490 nm in an ELISA reader. Background staining (assays without gp120) was subtracted from the readings. The relationship between free gp120 and optical density was linear in a semi-logarithmic plot between 0.03 and 1,000 ng per 50 μl reaction volume.[16,27,28]

Other methods Protein was determined as described,[27] using bovine serum albumin as a standard.

RESULTS

Conglutinin-like protein preparation Enrichment of conglutinin-like protein was achieved by two affinity purification steps (first, batchwise adsorption on Zymosan and second, affinity chromatography on a mannan-agarose column) and finally by gel permeation as described under "Materials and Methods." This sample was used for the experiments.

Inhibition of binding activity of conglutinin-like protein by sugars Agglutination activity of conglutinin-like protein was determined in the microtiter assay system, using EAC-erythrocytes as indicator cells. Among the carbohydrates examined, the binding of conglutinin-like protein to the complement-coated EAC-erythrocytes was strongly inhibited by *N*-acetylglucosamine, and to a

Table I. Inhibition of Binding of Conglutinin-like Protein to Coated Sheep Erythrocytes (EAC)

Compound added (m*M*)	Concentration		Agglutination activity (titer/mg protein)
	(m*M*)	(μg/300 μl)	
None			83.3
EDTA	20		<5
N-Acetylglucosamine	200		23.5
L-Fucose	200		37.8
D-Mannose	200		49.3
N-Acetylmannosamine	200		74.1
Glucose	200		85.2
Galactose	200		82.7
N-Acetylgalactosamine	200		78.4
Glucose 6-phosphate	200		85.6
Mannose 6-phosphate	200		85.1
Man-A		50	<5
Man-B		50	<5

The final concentrations of the carbohydrates in the assays are given.

Table II. Inhibition of Conglutinin-like Protein-Man-A Mannan Precipitation by Oligosaccharides

Oligosaccharide	Concentration (μM) for 50% inhibition
Man(α1-2)Man	75
Man(α1-3)Man	>200
Man(β1-4)GlcNAc	34
Man(α1-3)Man(β1-4)GlcNAc	>200
Man(α1-2)Man(α1-3)Man(β1-4)GlcNAc	21
Man(α1-2)Man(α1-3)Man(α1-6)┐ Man(β1-4)GlcNAc Man(α1-2)Man(α1-3)┘	17
Man(α1-2)Man(α1-6)Man(α1-6)┐ Man(α1-3)┘ Man(β1-4)GlcNAc Man(α1-2)Man(α1-2)Man(α1-3)┘	8

lesser extent by L-fucose and D-mannose (Table I). None of the other monosaccharides tested displayed pronounced inhibitory activity at the concentration of 200 mM. However, the two mannans from yeast cells, Man-A and Man-B, at a concentration of 50 μg/ml completely blocked the activity of conglutinin-like protein. Likewise, 20 mM EDTA in the assay system nullified the agglutination activity of conglutinin-like protein.

To determine the carbohydrate binding specificity of conglutinin-like protein, hapten inhibition studies were conducted using Man-A as the precipitating polysaccharide. Man-A was selected for this purpose because of its high binding ability to conglutinin-like protein (Table I). Concentrations of sugars required for 50% inhibition were obtained from complete inhibition curves. Among the disaccharides tested, Man (β 1-4) GlcNAc was strongly inhibitory [50% inhibition at 34 μM], while Man(α1-2)Man was a less potent inhibitor [75 μM]; Man(α1-3)Man was inactive (Table II). The trisaccharide Man(α1-3)Man(β1-4)GlcNAc was inactive, while Man(α1-2)Man(α1-3)Man(β1-4)GlcNAc was highly inhibitory [21 μM]. Two oligosaccharides were tested for inhibitory potency; the high-mannose type oligosaccharide Man_8-GlcNAc was more potent [50% inhibition at 8 μM] than Man_6-GlcNAc [17 μM] (Table II).

Binding of conglutinin-like protein to HIV-gp120 An ELISA system was applied to demonstrate that conglutinin-like protein binds to HIV-gp120. The mannose-specific lectin from *Narcissus pseudonarcissus*[29] was coated onto the solid phase. Addition of gp120 to the wells resulted in gp120/lectin complex formation.[16,27] This complex was finally detected and quantified by a

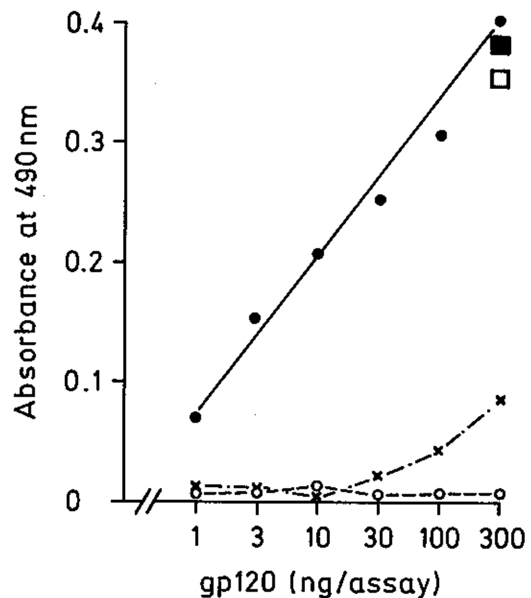

Fig. 2. Inhibition of binding of HIV-gp120 to the mannose specific lectin from *Narcissus pseudonarcissus* by conglutinin-like protein. The ELISA system used for this study is described under "Materials and Methods." Different concentrations of gp120 (1–300 ng/assay [50 μl]) were pre-incubated with 0 (●), 1.7 (×) or 17 μg/assay of conglutinin-like protein (○) and then assayed in the ELISA system. The absorbance values reflect the amount of free [not conglutinin-complexed] gp120 bound to the lectin. In two series of experiments 17 μg of conglutinin-like protein was incubated with 0.55 μg of *Candida* mannan, Man-A (■) or Man-B (□), (5 h, 4°C) prior to the addition to gp120.

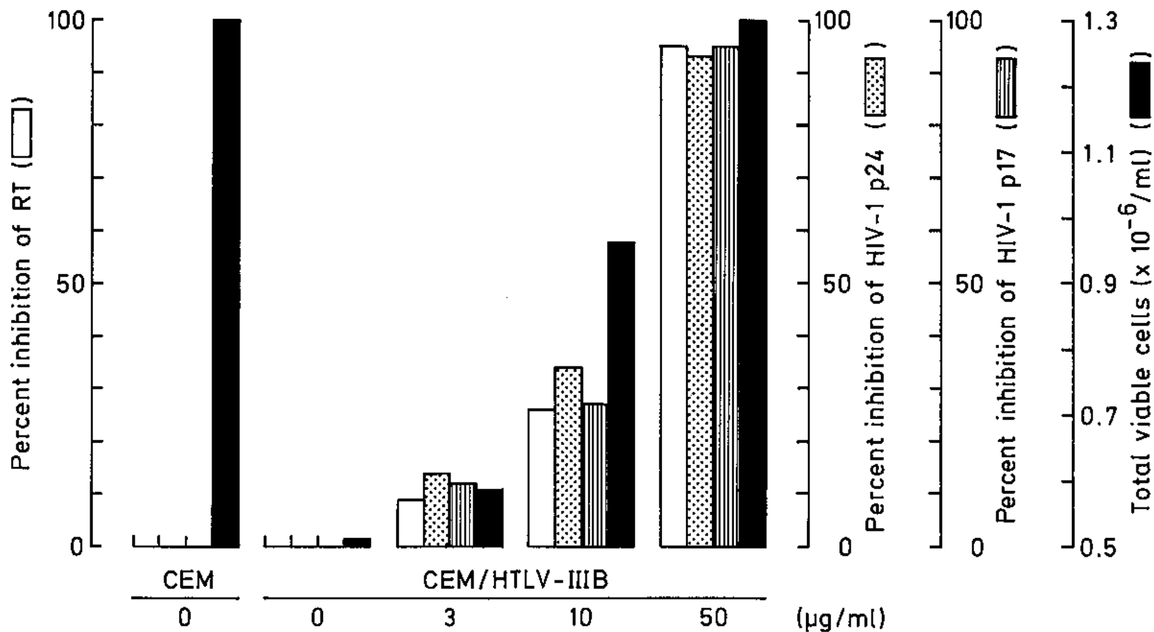

Fig. 3. Inhibition of HIV-1 (HTLV-IIIB) production by CEM cells in the presence of conglutinin-like protein. The antiretrovirus assay was performed as decribed in the text. The final concentrations of conglutinin-like protein are given. After incubation of the HIV-infected cells for five days, the RT activity in the culture medium (the data are given as percent inhibition of RT activity, compared to the infected controls; open bars), the inhibition of viral p24 (stippled bars) and p17 (hatched bars) expression of infected cells were estimated, and the number of viable cells was determined (solid bars). As a control, the growth of uninfected CEM cells is given. The means of five parallel experiments are shown.

second digoxigenin-labeled mannose-specific lectin. Addition of 300 ng [=2.5 pmol] of free gp120 to the assays resulted in an absorbance value of 0.41 at 490 nm (Fig. 2). Preincubation of gp120 with purified conglutinin-like protein resulted in a dose-dependent decrease of binding of gp120 to the lectin. At the stoichiometric concentration of conglutinin-like protein, 1.7 μg/assay [=2.5 pmol], a reduction of the absorbance to 23% was measured; at a 10-fold surplus of conglutinin-like protein, 17 μg/assay [=25 pmol], only background levels (assays in the absence of gp120) were determined (Fig. 2).

Incubation of conglutinin-like protein with mannan preparation from *Candida albicans,* Man-A or Man-B, prior to the addition to free gp120 abolished the binding ability of conglutinin-like protein to gp120 (Fig. 2). For these experiments 0.55 μg [=250 pmol] of mannan was added to 17 μg [=25 pmol] of conglutinin-like protein. From these data we conclude that conglutinin-like protein binds to mannose-containing structures, present on both HIV-gp120 and yeast glycoproteins.

Anti-HIV activity of conglutinin-like protein Prior to testing for anti-HIV activity the purified conglutinin-like protein fraction was dialyzed against PBS lacking NaN_3. This fraction was determined to be not toxic to cells at the highest test concentration (150 μg/ml). At concentrations between 10 and 50 μg/ml of conglutinin-like protein, the RT activity in the culture supernatant of infected CEM cells (as a measure of the presence of HIV-1) was reduced to approximately 50% (Fig. 3). The values for inhibition of p24- and p17-positive cells, for RT inhibition and for viable cell counts nearly match at a given concentration of conglutinin-like protein. The EC_{50} value for conglutinin-like protein was determined to be 23.9 μg/ml. At concentrations above 50 μg/ml complete inhibition of HIV production was observed.

DISCUSSION

Two human serum lectins have been described which recognize specifically mannose and *N*-acetylglucosamine, (i) the serum mannan-binding protein and (ii) the serum protein analogous to bovine conglutinin. The serum mannan-binding protein with an Mr of ~700,000 consists of approximately 20 indentical subunits of Mr 31,000.[7] It has been suggested that this protein plays a role in host defense by binding to pathogens, such as bacteria, yeast, fungi and envelope glycoproteins of cer-

tain viruses including HIV.[30] Under *in vitro* conditions the mannan-binding protein inhibits infection of lymphoblasts by HIV via binding to cell surface structures of HIV-infected cells.[30] Now we propose a similar function for the human conglutinin-like protein.

The second mannan-binding serum lectin, conglutinin, is a polypeptide of Mr ~650,000, which contains one collagenase-sensitive subunit of Mr 67,000 as shown in this and previous work.[9] Our finding that the ability of human conglutinin-like protein to agglutinate complement-coated EAC-erythrocytes was sensitively inhibited by *N*-acetylglucosamine, L-fucose, D-mannose and EDTA is in accordance with published data.[11-14] In the present study we demonstrate that the function of the conglutinin-like protein is abolished by oligosaccharides carrying terminal α1-2-linked mannose residues. In addition we report that human conglutinin-like protein binds in a Ca^{2+}-dependent manner to HIV-gp120 via the mannan-binding site of this serum protein. This interesting finding appears to be a general feature of this protein, since also bovine conglutinin was found to react with gp120.[24] Moreover, the human protein prevents infection of the CEM lymphoblasts by HIV-1. The anti-HIV activity of conglutinin-like protein (EC_{50}: 23.0 μg/ml) is as strong as that displayed by the human serum mannose-binding protein.[30] However, in contrast to the mannose-binding protein, the concentration of the conglutinin-like protein in human serum is much lower.[9] Based on the presented finding that conglutinin-like protein is inhibitory against HIV infection *in vitro*, we may anticipate a potential *in vivo* effect. Two possible mechanisms of *in vivo* action of conglutinin-like protein can be considered: conglutinin-like protein may either coat the HIV envelope and promote its attachment to $CD4^-$ target cells by cross-linking[31, 32] or it may mask the virus envelope and prevent binding as demonstrated in this work.

In conclusion, this study shows that the mannose-binding serum protein, conglutinin-like protein, binds to gp120 and — very likely — thereby prevents infection of cells *in vitro*. Future studies are needed to examine whether this finding can be generalized to *in vivo* situations.

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