Brief Definitive Report

ROLE OF ENVELOPE GLYCOPROTEIN CARBOHYDRATE IN HUMAN IMMUNODEFICIENCY VIRUS (HIV) INFECTIVITY AND VIRUS-INDUCED CELL FUSION

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Human immunodeficiency virus (HIV; known previously as HTLV-III/LAV) is the etiologic agent for the acquired immunodeficiency syndrome (AIDS) and a spectrum of related disorders (1, 2). The virus is tropic and cytopathic for T lymphocytes that express the differentiation antigen CD4 (T4/Leu-3) on their cell surface (1–4). Both the binding of virus to susceptible target cells and the cell fusion that is a characteristic manifestation of HIV-induced cytopathology involve specific interactions between the viral envelope glycoprotein and cell surface CD4 (3–7).

The protein backbone of the HIV envelope glycoprotein has an M_r ~88,000 (8). The molecule is heavily glycosylated and exists in several forms. It appears to be synthesized as a 160,000 precursor, which is cleaved into gp120 and gp41 (transmembrane) portions (8). To investigate the possible role of the carbohydrate component of the viral glycoprotein in the processes of virion infectivity and virally induced cell fusion, we tested the effects of a panel of lectins of different binding specificities on in vitro assays of these processes. Our results suggest that mannose-containing carbohydrate moieties on the viral envelope glycoprotein are involved, either directly or indirectly, in envelope interactions with cell surface CD4.

Materials and Methods

Cell Lines. The $CD4^+$ T cell lines VB (6, 7) and H9 (2) have been described in detail elsewhere. Cell lines were maintained in RPMI 1640 supplemented with 10% heat-inactivated FCS and 2 mM l-glutamine. Assays of free virus infectivity and virally induced fusion (see below) were conducted in the same medium.

HIV Preparation. For use in assays of virion infectivity (see below) we used the reference isolate of LAV (1), maintained in chronically infected CEM-CCRF cells. Supernatants from these chronically infected cultures were filtered through 0.22-μm filters before use as a source of infectious virus.

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Reverse Transcriptase (RT) Assay. Magnesium-dependent RT activity, typical of HIV, was measured as described previously (9).

Assay of HIV-induced Cell Fusion. A cell-mixing syncytium-induction assay was used to evaluate virally induced cell fusion. To 5×10^4 HIV ($HTLV\text{-}III_b$)-infected H9 cells seeded in the flat-bottom wells (~0.28 cm^2) of 96-well microtiter plates, 5×10^4 uninfected VB cells were added, to yield a final volume of 100 μl. Under these conditions, syncytia arise over a period of 2–4 h through cell fusion involving interactions between the HIV envelope glycoprotein and CD4 (6, 7). Mixed cells were cultured at 37°C in humidified 5% CO_2, and observed at specific time points by light microscopy at × 200 for the presence of multinucleated giant cells. Syncytia were evaluated according to a semiquantitative scoring system based on the following criteria: the absence of multinucleated giant cells was scored as negative (−). Wells with rare but unequivocal multinucleated giant cells (greater than four included nuclei within a common cell membrane) were scored as 1. Wells with more frequent giant cells, but in which the majority of × 200 fields did not contain giant cells were scored as 2. Wells with giant cells in most but not all × 200 fields were scored as 3, and wells with syncytia in all × 200 fields were scored as 4. Cells were treated with varying concentrations of lectins or competitive inhibitors of lectin binding, and the effects on subsequent giant cell formation were evaluated. For some experiments (see below), either the H9 cells or the VB cells were pretreated with lectin and then washed extensively with lectin-free medium before mixing. In other experiments, the lectins were present in the mixed cell culture for the duration of the experiment.

Lectins and Competitors. Con A, lentil lectin, and lectins from garden pea, sweet pea, soy bean, fava bean, *Bandieraea simplicifolia*, and wheat germ were purchased from Sigma Chemical Co. (St. Louis, MO). PHA was purchased from Burroughs-Wellcome, Ltd., Beckenham, United Kingdom. α-methyl-D-mannoside and tunicamycin were purchased from Sigma Chemical Co. Con A from Pharmacia Fine Chemicals (Uppsala, Sweden) and from E-Y Laboratories (Burlingame, CA) were also tested.

Assay of Free Virus Infectivity. For each experimental group, 4×10^6 VB cells were incubated at 37°C for 1 h in 500 μl containing 10^5 cpm RT activity virus equivalent. To evaluate the effects of Con A on free virus infectivity, the virus preparation was pretreated with either Con A (50 μg/ml), Con A and α-methyl-D-mannoside (0.1 M), or medium for 30 min before addition to VB cells. A 1-h incubation was then performed with the treated virus inoculum to allow adsorption, and the cells were resuspended to a density of 10^6 cells/ml with lectin or inhibitor added to the desired final concentration. Cells were then cultured for 1 wk and observed daily for the presence of virus-induced cell fusion, with samples of cell-free culture supernatants taken on days 4 and 7 for assay of RT activity. Cells were resuspended to their original density, with fresh lectin and/or inhibitor added, at the time of supernatant harvest.

Recombinant Vaccinia Virus. A recombinant vaccinia virus (VSC-25), which contains the gene for the envelope glycoprotein of HIV, was generated as described elsewhere (10). Infection of VB cells with VSC-25 results in the specific cell surface expression of HIV envelope glycoprotein, leading to CD4-dependent cell fusion (7, 10). No other HIV-determined proteins are expressed. Infection of VB cells with wild type vaccinia virus does not induce cell fusion. To document cell surface expression of the HIV envelope glycoprotein in these studies, we used indirect immunofluorescence analysis, using serum from a patient with circulating HIV envelope–reactive antibody. The serum was extensively absorbed on VB cells infected with wild type vaccinia virus before use in immunofluorescence assays in order to remove nonspecific or vaccinia-specific reactivities. For fusion assays and immunofluorescence analysis (7), VB cells were infected as described (7), washed, then cultured for 7 h in the presence or absence of tunicamycin (20 μg/ml).

Results

Lectins with specificity for D-mannose inhibited HIV-induced cell fusion (Table I). In numerous experiments, lectins having other specificities had little or no effect on virus-induced fusion, with the exception of PHA, which accelerated

TABLE I

Inhibition of HIV-induced Cell Fusion by Mannose-specific Lectins

Lectin source	Supplier*	Carbohydrate-binding specificity	Concentration in assay (μg/ml)	Syncytium score‡
None		None	None	4
Con A (jack bean)	Sigma, Pharmacia	α-D-man	100	–
		α-D-glc	10	–
			1	4
Lens culinaris (lentil)	Sigma, E-Y	α-D-man	100	–
Pisum sativum (garden pea)	Sigma, E-Y	α-D-man	100	1
Lathyrus odoratus (sweet pea)	Sigma	α-D-man	100	2
Vicia faba (fava bean)	Sigma	D-man	100	3
Bandeiraea simplicifolia BS-II	Sigma	α-D-gal α-D-galNAc	100	4
PHA (red kidney bean)	Wellcome	Unknown	20	4
Glycine max (soybean)	Sigma	D-galNAc	100	4
Helix pomatia (snail)	Sigma	D-galNAc	100	4
Dolichos biflorus (horse gram)	Sigma	α-D-galNAc	100	4
Triticum vulgaris (wheat germ)	Pharmacia	$(D\text{-glcNAc})_2$ NeuNAc	100	4

HIV-infected H9 cells were mixed with uninfected VB cells in the continuous presence of lectins at the indicated concentrations.

* Lectins were purchased from: Sigma Chemical Co., St. Louis, MO; Pharmacia Fine Chemicals, Uppsala, Sweden; E-Y Laboratories, San Mateo, CA; Wellcome, Ltd., Beckenham, United Kingdom. When more than one supplier is designated for a single lectin, preparations from both were tested, with similar results. Lectin carbohydrate specificities are as described by suppliers.

‡ Syncytia were scored at 4 h, as described in the text.

TABLE II

Con A Inhibits HIV-induced Cell Fusion by Binding to Infected Cells

Population pretreated	Pretreatment	Competitor added	Untreated population added	Syncytium score
None	None	None	$H9_{HIV}$, VB	4
VB	Con A	None	$H9_{HIV}$	4
$H9_{HIV}$	Con A	None	VB	–
VB and $H9_{HIV}$	Con A	None	None	–
$H9_{HIV}$	Con A	α-methyl-D-mannoside	VB	4

For pretreatment studies, 10^6 cells of the indicated population were treated with Con A at 50 μg/ml in the presence or absence of 0.1 M α-methyl-D-mannoside for 1 h at 37°C then washed before mixing with an equal number of cells of the other population. Mixed cells were then cultured in the continued presence or absence of 0.1 M α-methyl-D-mannoside, and syncytia were scored at 4 h.

the fusion process slightly. Con A inhibited syncytium formation in a dose-dependent fashion, and the inhibition could be specifically competed with α-methyl-D-mannoside (Table II). Concentrations of Con A that inhibited cell fusion did not decrease the viability of cultured cells over the period of the assay, as assessed by trypan blue exclusion (data not shown).

Pretreatment of the uninfected VB cells with 50 μg/ml of Con A, followed by extensive washing before mixing with infected H9 cells in the absence of additional Con A, did not affect subsequent cell fusion. However, identical Con A pretreatment of the infected H9 cells before mixing with untreated VB cells completely blocked cell fusion (Table II). The effect of Con A on cell fusion was reversible, as infected H9 cells treated with 50 μg/ml Con A for 1 h then washed extensively underwent fusion if 0.1 M α-methyl-D-mannoside was added in the absence of additional Con A (Table II).

The possibility existed that Con A inhibited HIV-induced cell fusion by binding

TABLE III

Con A Inhibits HIV Envelope Glycoprotein–induced Cell Fusion in a Recombinant Vaccinia Virus System

Infection	Treatment	Syncytium score
None	None	–
VSC-STD	None	–
VSC-25	None	4
VSC-25	Con A	–
VSC-25	Con A α-methyl-D-mannoside	4
VSC-25	Tunicamycin	–

VB cells were either sham-infected with wild type vaccinia virus (VSC-STD) or VSC-25, a recombinant vaccinia virus that contains the gene for the HIV envelope glycoprotein, as described (7, 10). Cells were then cultured in the presence or absence of Con A (50 μg/ml) with or without 0.1 M α-methyl-D-mannoside. For tunicamycin treatment, cells were placed in 20 μg/ml tunicamycin immediately after the virus adsorption incubation (7, 10), and were cultured in the continued presence of the drug.

TABLE IV

Con A Inhibits HIV Virion Infectivity for VB Target Cells

HIV inoculation	Con A	α-Methyl-D-mannoside	Day 4		Day 7	
			Syncytia	RT activity	Syncytia	RT activity
–	–	–	–	NT	–	2,996
–	+	–	–	NT	–	2,708
+	–	–	4	391,490	4	181,620
+	+	–	–	15,475	–	3,160
+	+	+	4	178,853	4	423,713

For each experimental group, 4×10^6 VB cells were incubated at 37°C for 1 h in 500 μl containing 10^5 cpm RT activity virus equivalent. To evaluate the effects of Con A on free virus infectivity, the virus preparation was pretreated with either Con A (50 μg/ml), Con A and α-methyl-D-mannoside (0.1 M), or culture medium for 30 min at 37°C before addition to VB cells. After a 1-h incubation with the treated virus inoculum to allow adsorption, the cells were resuspended to a density of 10^6 cells/ml, with lectin or inhibitor added to the desired final concentration. Cells were then cultured for 1 wk, observed daily for the presence of virus-induced cell fusion, with samples of cell-free culture supernatants taken on days 4 and 7 for assay of RT activity. Cells were resuspended to their original density, with fresh lectin and/or inhibitor added, at the time of supernatant harvest. The RT activity detected on day 4 in the HIV inoculated, Con A–treated cells probably reflects residual virus from the initial inoculation.

to mannose residues on cell surface viral envelope glycoprotein, preventing the envelope/CD4 interactions required for cell fusion (6, 7). To evaluate this possibility, we used a recombinant vaccinia virus containing the envelope gene of HIV (7, 10). Infection of VB cells with the recombinant virus induced CD4-dependent cell fusion, and this fusion was inhibited by Con A (Table III). The Con A–mediated inhibition could be competed with α-methyl-D-mannoside (Table III). Although tunicamycin treatment completely inhibited fusion of the vaccinia recombinant–infected cells (Table III), this treatment also moderately decreased cell surface expression of HIV envelope glycoprotein, as detected by immunofluorescence analysis (data not shown).

Con A pretreatment of an infectious virus preparation, with the continued presence of lectin during subsequent culture, blocked the in vitro infectivity of HIV virions, as assessed by the development of cytopathology and RT activity in supernatants of the infected cultures (Table IV). The Con A–mediated inhibition could be specifically competed by α-methyl-D-mannoside (Table IV).

Discussion

The envelope glycoprotein of HIV is heavily glycosylated, and some of the glycosylation sites are conserved between different isolates of HIV, suggesting that glycosylation may play an important role in the biological activity of the virus (11). Our results, showing inhibition of HIV infectivity and cell fusion by lectins with specificity for D-mannose, are consistent with this hypothesis. Pretreatment studies suggested that Con A blocked fusion through an interaction with viral envelope glycoprotein displayed on the surface of infected cells.

Additional studies using a recombinant vaccinia virus system, in which the envelope glycoprotein of HIV is expressed on the surface of infected cells in the absence of synthesis of any other HIV proteins, indicate that Con A inhibition of HIV envelope glycoprotein–mediated cell fusion involves interactions between Con A and the viral envelope glycoprotein. Tunicamycin, which prevents glycosylation by inhibiting the formation of a key intermediate in lipid linked oligosaccharide assembly (12), blocked HIV envelope–induced fusion in the recombinant vaccinia virus system, providing additional evidence for the role of the carbohydrate portion of the HIV envelope glycoprotein in inducing cell fusion. However, interpretation of findings in the tunicamycin blocking studies is complicated by the fact that the drug decreased the amount of HIV envelope displayed on the surface of recombinant vaccinia virus-infected cells.

Mannose-binding lectins thus appear to inhibit the processes of AIDS virus–induced cell fusion and in vitro infectivity through interactions with the viral envelope glycoprotein. The molecular basis for these effects is unknown. Con A may bind to carbohydrate residues involved in envelope glycoprotein/CD4 interactions, may interfere with protein/protein interactions through steric hindrance, or alternatively, may cause a conformational change in the structure of the envelope molecule upon binding. Regardless of the precise mechanism, these findings, implying an important role for HIV envelope glycoprotein carbohydrate in virion infectivity and virus-induced cell fusion are relevant to efforts to develop a protective vaccine against the AIDS virus, and suggest that an optimal viral subunit vaccine should incorporate appropriately processed (glycosylated) viral protein antigen.

Summary

Human immunodeficiency virus (HIV) envelope glycoprotein interactions with cell surface CD4 are involved in both virion infectivity and virally mediated cell fusion. D-mannose-specific lectins such as Con A specifically blocked virion infectivity and cell fusion. Studies with a recombinant vaccinia virus containing the HIV envelope gene demonstrated that Con A–mediated inhibition of HIV-induced fusion involved lectin binding to the viral envelope glycoprotein. These results indicate the importance of envelope glycosylation in the pathobiology of HIV infection, and suggest potential mechanisms for interfering with HIV infectivity and cytopathology.

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References

1. Barré-Sinoussi, F., J. C. Chermann, F. Rey, M. T. Nugeyre, S. Chamaret, J. Gruest, C. Dauget, C. Axler-Blin, F. Vezinet-Brun, C. Rouzioux, W. Rozenbaum, and L. Montagnier. 1983. Isolation of a T lymphotropic retrovirus from a patient at risk for AIDS. *Science* (*Wash. DC*). 220:868.
2. Popovic, M., M. G. Sarngadharan, E. Read, and R. C. Gallo. 1984. Detection, isolation and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and Pre-AIDS. *Science* (*Wash. DC*). 224:497.
3. Dalgliesh, A. G., P. C. L. Beverly, P. R. Clapham, D. H. Crawford, M. P. Greaves, and R. A. Weiss. 1984. The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. *Nature* (*Lond.*). 312:763.
4. Klatzmann, D., F. Barré-Sinoussi, M. T. Nugeyre, C. Dauget, E. Vilmer, C. Griscelli, F. Brun-Vezinet, C. Rouzioux, J. C. Gluckman, J. C. Chermann, and L. Montagnier. 1984. Selective tropism of LAV for helper T lymphocytes. *Science* (*Wash. DC*). 225:59.
5. McDougal, J. S., M. S. Kennedy, J. M. Sligh, S. P. Cort, A. Mawle, J. K. A. Nicholson. 1986. Binding of HTLV-III/LAV to $T4^+$ T cells by a complex of the 110K viral protein and the T4 molecule. *Science* (*Wash. DC*). 231:382.
6. Lifson, J. D., G. R. Reyes, M. S. McGrath, B. S. Stein, and E. G. Engleman. 1986. AIDS retrovirus induced cytopathology: Giant cell formation and involvement of CD4 antigen. *Science* (*Wash. DC*). 232:1123.
7. Lifson, J. D., M. B. Feinberg, G. R. Reyes, L. R. Rabin, B. Banapour, S. Chakrabarti, B. Moss, F. Wong-Staal, K. Steimer, and E. G. Engleman. 1986. Induction of CD4 dependent cell fusion by the HTLV-III/LAV envelope glycoprotein. *Nature* (*Lond.*). 323:1.
8. Allan, J. S., J. E. Coligan, F. Barin, M. F. McLane, J. G. Sodroski, C. A. Rosen, W. A. Haseltine, T. H. Lee, and M. Essex. 1985.Major glycoprotein antigens that induce antibodies in AIDS patients are encoded by HTLV-III. *Science* (*Wash. DC*). 228:1091.
9. Lifson, J. D., D. T. Sasaki, and E. G. Engleman. 1986. Utility of formaldehyde fixation for flow cytometry and inactivation of the AIDS associated retrovirus. *J. Immunol. Meth.* 86:143.
10. Chakrabarti, S., M. Robert-Guroff, F. Wong-Staal, R. C. Gallo, and B. Moss. 1986. Expression of the HTLV-III envelope gene by a recombinant vaccinia virus. *Nature* (*Lond.*). 320:535.
11. Alizon, M., S. Wain-Hobson, L. Montagnier, and P. Sonigo. 1986. Genetic variability of the AIDS virus: nucleotide sequence analysis of two isolates from African patients. *Cell.* 46:63.
12. Hubbard, S. C., and R. J. Ivatt. 1981. Synthesis and processing of asparagine linked oligosaccharides. *Ann. Rev. Biochem.* 50:555.