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GLYCOSYLATION INHIBITORS BLOCK THE EXPRESSION OF LAV/HTLV-III (HIV) GLYCOPROTEINS[1]

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SUMMARY The glycosylation inhibitors 2-deoxy-D-glucose (2-dGlc) and, to a lesser extent, β-hydroxynorvaline blocked the formation of syncytia in HIV (LAV/HTLV-III)-infected cells. Using monospecific polyclonal antibodies against recombinant envelope proteins gp110 and gp41 or monoclonal antibodies against env gp110, we could demonstrate a marked reduction in the immunoreactivity of these antigens in HIV-infected cells exposed to the glycosylation inhibitors. There was concomitant accumulation of core proteins p15 and p24, as shown by a solid phase radio-immunoassay, and a decreased oligosaccharide synthesis of env proteins, as monitored by the incorporation of [6-^{3}H]GlcNAc. The reverse transcriptase was not affected by the compounds. Glycosylation inhibitors may be considered for the chemotherapy of AIDS or AIDS-related complex, or chemoprophylaxis of HIV-positive individuals.

Human immunodeficient virus (HIV) was first isolated from a male homosexual with AIDS-related complex lymphadenopathy syndrome (1). It has been referred to as LAV or HTLV-III (2,3). This retrovirus possesses two env proteins which are glycosylated (4) and interact with the OKT4A antigen of T4+ T lymphocytes; the latter molecule appears to be a glycosylated polypeptide of 58,000 daltons (5). Both the viral attachment protein and fusion factor(s) as well as the virus-cell receptor(s) may play a key role in virus adsorption and penetration, and spread of the virus to adjacent cells. It has been shown that glycosylation inhibitors, viz. 2-deoxy-D-glucose and β-hydroxynorvaline, are capable of blocking cell surface-mediated phenomena, viz. fusion (6,7), attachment (8,9), penetration (10,11), and probably release of the virions. The reverse transcriptase has been considered as the most attractive target in the

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design of anti-HIV agents (12) and both nucleoside analogues (13,14) and anionic dyes (15,16) appear to be targeted at this enzyme. In an attempt to explore other possible target sites for antiviral chemotherapy against HIV infection, we evaluated the effect of glycosylation inhibitors on the expression of viral env gp110 and gp41 antigens.

MATERIALS AND METHODS

Virus strains. HTLV-III$_B$ was obtained from Dr. R.C. Gallo; LAV, from Prof. L. Montagnier and ARV-4 from Dr. J.A. Levy; they were propagated as previously described (2,17,18).

Cell types. The H9, CEM, HUT-78, and MT-4 (HTLV-I transformed) cell lines were maintained and infected with HIV as described previously (19).

Compounds. 2-Deoxy-D-glucose (2-dGlc) was obtained under contract from Pfanstiehl Chemical Laboratories, Waukegan, Illinois (USA); ß-hydroxynorvaline (Hnv) was purchased from Sigma Chemical Co., Milwaukee, Wisconsin (USA).

Syncytium formation. Quantification of polykaryocytes was done as previously described (6); H9 polykaryocytes were monitored by Coulter counting or phase contrast microscopy.

Monoclonal and polyclonal antibodies to env antigens. Monoclonal antibodies (MCA) against gp110 of HIV were prepared in mice by cloned hybridoma technology and were a generous gift of Dr. K. Shriver (Genetics Systems, Seattle, Washington, USA).

Recombinant proteins representing different portions of the Env reading frame(s) were produced in Escherichia coli expression vectors (18) by taking advantage of the Bgl-II site; antibodies to gp110 and gp41 were prepared in goats and kindly supplied by Dr. W. Kenealy.

Virus markers in cell culture. 5 x 10^6 Cells (H9, CEM, MT-4) were lysed, with ultrasound, in the presence of protease inhibitors and assayed for LAV/HTLV-III antigens using a solid phase radio-immunoassay (RIA). LAV/HTLV-III antigens were detected with five monoclonal antibodies, two of which were obtained from Dr. R.C. Gallo and were directed against HTLV-III p24, while the others (H311 and 8A5) were prepared by Dr. J. Cogniaux and were directed against LAV p15; monoclonal antibody against gp110 was provided by Dr. K. Shriver; the polyclonal antibodies pEnv 9 and pEnv 14 were gifts of Dr. W. Kenealy. Bound antibodies were evaluated by addition of [^{125}I]-F(ab')2 fragments of anti-mouse IgG or [^{125}I]-anti-goat IgG (rabbits) (du Pont de Nemours & Co., Wilmington, Delaware, USA). Uninfected cultures were run in parallel in order to determine amounts of non-specific antibody binding. Indexes of antibody binding were determined by dividing cpm in virus-infected cultures by cpm in control cultures (20).

Cell labelling. Cells (with and without inhibitors) were labelled with D-[6-^{3}H]GlcNAc at 5 µCi/ml for either 6 hours post-infection (MT-4 cells) or for 72-96 hours (persistently infected H9 or CEM cells). At the end of this time period, cells were lysed with Nonidet P-40 in the presence of protease inhibitors N-tosyl-L-lysine chloromethyl ketone (TLCK) and phenylmethylsulfonyl fluoride (PMSF) (2 mM) and the virus-specific glycoproteins were immunoprecipitated with monospecific, polyclonal antibodies (see above) and biotinylated rabbit anti-goat antiserum (Vector Labs, Burlingame, Ca, USA) followed by avidin cross-linked to sepharose beads. After extensive washing, viral glycoproteins were quantified by scintillation spectroscopy. Concurrent controls, derived from cells not treated with the glycosylation inhibitors were run in the same fashion.

Reverse transcriptase assay. The reverse transcriptase (RT) activity was determined as described elsewhere (21,22). Briefly, the culture fluids from H9/HTLV-III$_B$ producing cell lines (both control + 72 h-treated) were centrifuged for 10 min. at 300 x g. Virus particles were sedimented from the cell-free supernatant by centrifugation at 100,000 x g for 20 min. The viral pellet was treated with 0.1 % Triton-X 100 (150 µl) for 30 min. at 1°C. To test the direct effect of inhibitors on RT, viral suspensions were incubated with 5-10

mM 2-dGlc or Hnv for 60 min. The virus suspensions (10 µl) (with or without inhibitors) were incubated at 37°C for 60 min. in the presence of 50 mM Tris-HCl (pH 7.8), 5 mM DTT, 5 mM $MgCl_2$, 150 mM KCl, 30 µM poly(A), 8 µM oligo(dT) and 5 µCi [methyl-^{3}H]TPP. TCA-insoluble radioactivity was assessed by liquid scintillation spectroscopy. Control radioactivity (in the absence of inhibitor) ranged from 10^4 to 2 x 10^4 cpm.

RESULTS

Concentrations of 10 mM 2-dGlc and 0.75 mM Hnv blocked the formation of syncytia in H9 and MT-4 cells. However, in contrast to previous studies using BHK-21 cells or green monkey kidney cells infected with herpes simplex virus (7), Hnv proved toxic at concentrations > 0.75 mM for all cells used in the present study. Treatment of infected cells with MCA or polyclonal antibodies directed against gp110 and/or gp41 (pEnv 9 recognizes both gp110 and gp41, whereas pEnv 14 recognizes only gp110) revealed a marked reduction in the extent of binding of these antibodies to the envelope proteins from cells which had been treated with 5 mM 2-dGlc for 72 hours (Table 1). These studies were confirmed by comparing uninfected and infected cells labelled with [6-^{3}H]-GlcNAc and quantifying the extent of glycosylation of the env proteins following immunoprecipitation of gp110 and gp41 with either monospecific polyclonal or monoclonal antibody. The sensitivity of this assay was increased at least 10-20 fold by using biotinylated rabbit anti-goat antibody and avidin cross-linked to sepharose. When assayed by liquid scintillation spectroscopy, there was a 50 % reduction in the amount of viral glycoproteins synthesized in the presence of 5 mM 2-dGlc (Table 2). In contrast, non-glycosylated core polypeptide, p24 and p15, accumulated in the presence of Hnv (0.5 mM) (Table 3).

TABLE 1. Effect of 2-dGlc on the expression of gp110 and gp41 in cells persistently infected with LAV/HTLV-III*

	Binding of [^{125}I]-labelled monoclonal or polyclonal antibody (cpm)			
	Period of treatment			
	24 hours		72 hours	
	Control	2-dGlc 5 mM	Control	2-dGlc 5 mM
IgG (110.1), MCA				
HUT-78 cells	1107 ± 200	904 ± 106	1306 ± 190	740 ± 208
H9 cells			1206 ± 206	810 ± 158
Rabbit polyclonal				
pEnv 14			1262 ± 184	806 ± 153
pEnv 9			1306 ± 105	896 ± 196

*As assessed by solid phase radio-immunoassay (RIA) using gamma spectroscopy (20). Media changed daily with fresh inhibitor. Mean results of 3 experiments done in triplicate; mean deviation between experiments : ± 10 %.

TABLE 2. Effects of glycosylation inhibitors on the synthesis of [6-^{3}H]GlcNAc-labelled gp110 and gp41 in MT-4 and H9 cells infected with LAV/HTLV-III*

Viral glycoproteins	Exp. 1		Exp. 2	
	Control MT-4 cells	2-dGlc 5 mM	Control H9 cells	Hnv 0.75 mM
gp110 (pEnv 14)	5650	2530	5650	5050
gp41 + gp110 (pEnv 9)	4370	2603	4706	3910

*All assays done on immunoprecipitated viral glycoproteins; cells propagated in the presence of inhibitors as described in the text.

Their expression was slightly diminished in cells exposed to 2-dGlc. Hnv did not affect the expression of reverse transcriptase as based on the enzyme activities measured in purified HIV or in the supernatant fluids of HIV-infected cells (Table 3). The addition of 2-dGlc or Hnv (at 10 mM), to the reaction mixture, did not affect the *in vitro* activity of the HIV-associated reverse transcriptase (data not shown).

DISCUSSION

2-Deoxy-D-glucose and β-hydroxynorvaline are potent glycosylation inhibitors which block the synthesis of N-linked oligosaccharides of both DNA and RNA viruses. 2-Deoxy-D-glucose is incorporated into viral glycoproteins (11), polyisoprenoids and nucleotides (as a nucleotide sugar), and is also a competitive inhibitor of glycosphingolipid biosynthesis (23). β-Hydroxynorvaline, a threonine analogue, inhibits N-linked oligosaccharide synthesis subsequently to its incorporation into the polypeptide chain. Functionally, both 2-dGlc and

TABLE 3. Effect of glycosylation inhibitors on core proteins and RT in persistently infected LAV/HTLV-III H9 or HUT-78 cells*

	Control H9 cells	Hnv 0.5 mM	Control HUT-78 cells	2-dGlc 5 mM
Core proteins				
p24	680	908	860	650
p15	1080	1560	1010	850
Reverse transcriptase				
$(A)n.(dT)_{15}$	11522 ± 485	13404 ± 3208		
$(dA)n.(dT)_{15}$	4113 ± 218	2777 ± 786		

*Upon exposure time of 72-96 hours. Values expressed in cpm. Expression of core proteins assessed by solid phase RIA using gamma spectroscopy (20). HIV purification, after clarification was sedimented at 40,000 x g for 1 hour. The viral pellet was gently resuspended in PBS and layered on a preformed metrizamide gradient (5 to 40 %, w/v). Virus was purified after equilibrium centrifugation at 40,000 x g for 16 hours. LAV/HTLV-III banded at a density of 1.12 g per cc. RT assay was done as described in the text.

Hnv block fusion (6,7), virus attachment: by altering the tertiary or quarternary structure of viral attachment proteins as well as by decreasing the receptor density of glycosylated receptor molecules (8), and virus penetration. 2-deoxy-D-glucose has been used successfully in the treatment of human genital herpes infections (24). It can be used repetitively or for extended periods of time apparently without untoward side effects (8). The present study suggests that glycosylation inhibitors, because of their inhibitory effects on the expression of the HIV env glycoproteins, should be further explored as chemotherapeutic and chemoprophylactic modalities in patients with AIDS [or AIDS-related complex (ARC)] and HIV-seropositive individuals, respectively.

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REFERENCES

1. Gottlieb, M.S., Schroff, R., Schanker, H.M., Weisman, J.D., Fan, P.T., Wolf, R.A., and Saxon, A. (1981) New Engl. J. Med. 305, 1426-1431.
2. Barré-Sinoussi, F., Chermann, J.C., Rey, F., Nugeyre, M.T., Chamaret, S., Gruest, J., Dauguet, C., Axler-Blin, C., Vézinet-Brun, F., Rouzioux, C., Rozenbaum, W., and Montagnier, L. (1983) Science 220, 868-871.
3. Gallo, R., Sarin, P.S., Gelmann, E.P., Robert-Guroff, M., Richardson, E., Kalyanaraman, V.S., Mann, D., Sidhu, G.D., Stahl, R.E., Zolla-Pazner, S., Leibowitch, J., and Popovic, M. (1983) Science 220, 865-867.
4. Montagnier, L., Clavel, F., Krust, B., Chamaret, S., Rey, F., Barré-Sinoussi, F., and Chermann, J.C. (1985) Virology 144, 283-289.
5. McDougal, J.S., Kennedy, M.S., Sligh, J.M., Cort, S.P., Mawle, A., and Nicholson, J.K.A. (1986) Science 231, 382-385.
6. Gallaher, W.R., Levitan, D.B., and Blough, H.A. (1973) Virology 55, 193-201.
7. Kumarasamy, R., and Blough, H.A. (1984) Virology 138, 156-161.
8. Blough, H.A., Kumarasamy, R., Massare, M., Giuntoli, R.L., Sprecher-Goldberger, S., and Thiry, L. (1985) in Herpes Viruses and Virus Chemotherapy. Ed. R. Kono, pp. 211-214. Elsevier, Amsterdam.
9. Mohanty, J.G., and Rosenthal, K.S. (1986) Antiviral Res. 6, 137-149.
10. Spivack, J.G., Prusoff, W.H., and Tritton, T.R. (1982) Virology 123, 123-138.
11. Blough, H.A., and Ray, E.K. (1980) Pharmacol. Ther. 10, 669-681.
12. De Clercq, E. (1986) J. Med. Chem., in press.
13. Mitsuya, H., Weinhold, K.J., Furman, P.A., St. Clair, M.H., Nusinoff Lehrman, S., Gallo, R.C., Bolognesi, D., Barry, D.W., and Broder, S. (1985) Proc. Natl. Acad. Sci. USA 82, 7096-7100.
14. Mitsuya, H., and Broder, S. (1986) Proc. Natl. Acad. Sci. USA 83, 1911-1915.
15. Balzarini, J., Mitsuya, H., De Clercq, E., and Broder, S. (1986) Int. J. Cancer 37, 451-457.
16. Balzarini, J., Mitsuya, H., De Clercq, E., and Broder, S. (1986) Biochem. Biophys. Res. Commun. 136, 64-71.

17. Popovic, M., Read-Connole, E. and Gallo, R.C. (1984) Lancet ii, 1472-1473.
18. Levy, J.A., Hoffman, A.D., Kramer, S.M., Landis, J.A., Shimabukuro, J.M., and Oshiro, L.S. (1984) Science 225, 840-842.
19. Petteway, S.R. Jr., Reed, D., Reagen, K., Matthews, T., Tritch, R., Ivanoff, L., Tribe, D., Chamberlain, M., Cybulski, R., Bolognesi, D., and Kenealy, W. (1986) in Viruses and Human Cancer. Eds. R.C. Gallo, W. Hazeltine, G. Klein, and H. Zurhausen, in press. Alan R Liss, N.Y.
20. Cogniaux, J., Olislager, R., Sprecher-Goldberger, S., and Thiry, L. (1982) J. Virol. 43, 664-672.
21. Kavian, D.L. (1977) Methods Virol. 6, 143-184.
22. Hoffman, A.D., Banapour, B., and Levy, J.A. (1985) Virology 147, 326-335.
23. Suzuki, Y., and Blough, H.A. (1982) Biochim. Biophys. Acta 710, 221-229.
24. Blough, H.A., and Giuntoli, R. (1979) J. Amer. Med. Assoc. 241, 2798-2801.