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Inhibition of human immunodeficiency virus syncytium formation and virus replication by castanospermine

(acquired immunodeficiency syndrome/glycosylation inhibitor/human immunodeficiency virus envelope glycoprotein processing/antiretroviral agent)

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ABSTRACT **Castanospermine (1,6,7,8-tetrahydroxyoctahydroindolizine) is a plant alkaloid that modifies glycosylation by inhibiting α-glucosidase I. Castanospermine is shown to inhibit syncytium formation induced by the envelope glycoprotein of the human immunodeficiency virus and to inhibit viral replication. The decrease in syncytium formation in the presence of castanospermine can be attributed to inhibition of processing of the envelope precursor protein gp160, with resultant decreased cell surface expression of the mature envelope glycoprotein gp120. In addition, castanospermine may cause defects in steps involved in membrane fusion after binding of CD4 antigen. The antiviral effects of castanospermine may be due to modifications of the envelope glycoprotein that affect the ability of the virus to enter cells after attachment to the CD4 cell receptor.**

One of the striking properties of the acquired immunodeficiency syndrome (AIDS) virus, human immunodeficiency virus (HIV), is tropism for cells displaying the CD4 surface protein, with resultant formation of multinucleated syncytia and cell death (1–5). The CD4 surface antigen has been shown to be a specific cellular receptor for HIV, and anti-CD4 monoclonal antibodies have been shown to inhibit virus–host cell binding, syncytium formation, and infectivity (5, 6). The HIV envelope consists of an external glycoprotein, gp120, that directly binds to the CD4 molecule (7) and a transmembrane protein, gp41, that anchors the envelope in the viral membrane (8). It has been proposed that HIV-induced syncytium formation depends upon cell surface expression of adequate levels of envelope and CD4, a binding reaction between the gp120 and the CD4 molecule, and a membrane fusion event (9). Interference with any of these steps should interfere with the cytotoxic consequences of HIV infection.

Castanospermine (1,6,7,8-tetrahydroxyoctahydroindolizine) is a plant alkaloid isolated from seeds of an Australian chestnut tree, *Castanospermum australe* (10). This alkaloid has been shown to be a potent inhibitor of the endoplasmic reticulum enzyme α-glucosidase I, thereby preventing removal of glucose residues during the normal processing of glycoproteins (11). The resultant proteins contain incompletely processed carbohydrate chains composed of $Glc_3Man_{7-9}(GlcNAc)_2$ (12), and this alteration has had profound effects on cell surface expression and function of some glycoproteins (13–15). In the present study, the ability of castanospermine to interfere with the functions of the envelope glycoproteins of HIV in syncytium formation and virus infection is investigated.

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MATERIALS AND METHODS

Cell Lines. The H9, C8166, Jurkat, and SupT1 cell lines are $CD4^+$ human lymphocyte lines and were cultured as described (1, 16–18). Jurkat-*tat*-III cells express constitutively the HIV tat_{III} protein (17). Chinese hamster ovary (CHO) fibroblasts constitutively expressing the HIV envelope were made by transfection of the pIIIenv3 (9) and pIII tat_{III} plasmids (17) along with a selectable marker (J.S. and M.K., unpublished data).

Virus Stock. Infectious HIV was obtained from supernatant fluid of logarithmically growing H9 cells infected with human T lymphotropic virus type IIIB (HTLV-IIIB) (provided by R. C. Gallo, National Cancer Institute).

Castanospermine. Castanospermine was isolated from the ripe seeds of *Castanospermum australe* as described (19). The drug was greater than 99% pure as judged by paper chromatography and 360-MHz NMR spectroscopy.

Transient HIV *env* Expression Assay. In the expression assay, 1×10^7 Jurkat-*tat*-III cells were transfected with 10 μg of pIIIenv3 plasmid DNA by using the DEAE-dextran technique (20). Castanospermine at specified concentrations was added immediately after transfection, and total syncytia per 10^4 cells were counted 48–72 hr later.

Cocultivation Assay. For cocultivation, 3×10^4 C8166 cells grown either in the presence or in the absence of castanospermine for 48 hr were added to 1×10^4 H9 cells chronically infected with the HTLV-IIIB isolate of HIV, likewise grown either with or without drug for 48 hr. After a 4-hr incubation, total syncytia were counted.

Virus Replication Studies. To study virus replication, 2×10^6 H9 cells in logarithmic-phase growth were placed in 4.5 ml of medium. Then 0.5 ml of either GIBCO phosphate-buffered saline (PBS) or specified concentrations of castanospermine in PBS was added. After a 30-min incubation, 5000 tissue culture 50% infectious doses ($TCID_{50}$s) of HTLV-IIIB were added, and the cells were incubated in 25-ml flasks (Falcon) for 9 days. Culture supernatant was assayed for reverse transcriptase activity and for p24 antigen, as previously described (21, 22). In addition, cell-free supernatant was stored at −70°C for subsequent determination of virus yield (see below). Radioimmunoprecipitation was performed as described (23, 29). Fixed-cell membrane fluorescence studies were performed as described (24), utilizing a 1:40 dilution of serum from an HIV-seropositive individual and a 1:80 dilution of anti-human fluorescein isothiocyanate (FITC)-conjugated IgG (heavy and light chains) (Electronucleonics, Columbia, MD). Cell cultures were fed and prop-

Abbreviations: AIDS, acquired immunodeficiency syndrome; HIV, human immunodeficiency virus; HTLV-IIIB, human T lymphotropic virus type IIIB; H9/HTLV-IIIB, HTLV-IIIB-infected H9 cells.

agated by adding 2 ml of culture suspension to 5 ml of appropriate medium on days 3, 5, and 7.

Virus Yield. Virus yield was determined by using a modification of a previously described procedure (25). Briefly, serial 10-fold dilutions of cell-free supernatant were added in triplicate to 2×10^4 C8166 cells in 96-well microtiter plates (Costar, Cambridge, MA). The $TCID_{50}$ was calculated by determining the dilution at which 50% of the cell cultures showed cytopathic effect after 6–7 days.

Radioiodination. CHO cells constitutively expressing the HIV envelope were plated as described in medium containing lipoprotein-deficient serum of newborn calf (26). Cells were incubated in the presence of castanospermine at 300 μg/ml for 48 hr and radioiodinated as described (27).

HIV–CD4 Binding Studies. To measure binding, 1×10^7 H9 cells chronically infected with HTLV-IIIB were cultured for 12 hr with castanospermine at 100 μg/ml or without it. The cells were then centrifuged, washed twice with PBS, and labeled overnight with [^{35}S]cysteine at 100 μCi/ml (1 Ci = 37 GBq) in a total volume of 2.0 ml of otherwise cysteine-free RPMI 1640 medium with or without castanospermine (100 μg/ml). The cells were centrifuged and the supernatant containing the ^{35}S-labeled virions and envelope glycoproteins was incubated with 1×10^7 CD4$^+$ SupT1 cells for 60 min at 37°C. For some experiments, SupT1 cells were first incubated with monoclonal antibodies specific for epitopes of the CD4 receptor (OKT4 and OKT4A, Ortho Diagnostics) by suspending 1×10^7 cells in 100 μl of PBS with 20 μl of monoclonal antibody for 30 min at 37°C. The SupT1 cells were harvested, washed twice with PBS, lysed, and used for immunoprecipitation as described (29).

RESULTS

Castanospermine Inhibits HIV Envelope-Induced Syncytium Formation. The exterior portion of the envelope glycoprotein of HIV is heavily glycosylated. More than 20 potential sites for N-linked glycosylation exist in the external domain of the exterior glycoprotein, gp120, and more than half of the apparent molecular weight of this protein can be attributed to sugar residues (28). For this reason it seemed possible that an inhibitor of one of the enzymes that is required for processing glycoproteins could prevent envelope glycoprotein function and affect virus infectivity. The initial experiments were conducted to determine whether treatment with castanospermine could inhibit the ability of the envelope glycoprotein to induce syncytia among CD4$^+$ cells, a process that requires binding of the gp120 protein to the CD4 surface glycoprotein followed by a fusion event (9). A system for measurement of HIV envelope-mediated syncytium formation was described previously (9). Briefly, CD4$^+$ cells that constitutively express the *tat* gene of HIV are transfected with a plasmid that contains the *art* and envelope genes of the HTLV-IIIB strain of HIV. Expression of the HIV envelope protein results in formation of large multinucleated cells within 24–48 hr after transfection.

The effect of increasing concentrations of castanospermine on syncytium formation was determined by adding the drug immediately after transfection and counting syncytia 48 hr later. The data of Fig. 1 show a dose-dependent decrease in syncytium formation with addition of castanospermine. Essentially complete inhibition was observed for doses of castanospermine of 100 μg/ml or higher. No effect on cell viability or growth rate was observed for the concentrations of castanospermine used in this experiment.

Both the envelope protein and the CD4 receptor are glycosylated cell surface proteins. To determine whether castanospermine-induced inhibition of syncytium formation is dependent upon alteration of one or both of these proteins, syncytia were formed by cocultivation of cells producing

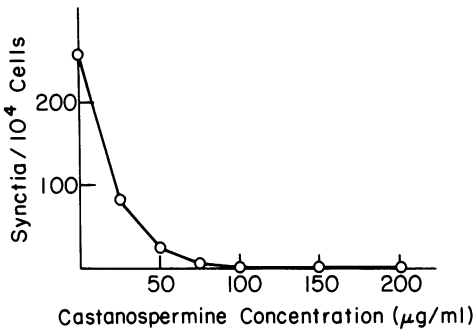

FIG. 1. Dose-dependent decrease in HIV envelope-induced syncytium formation with castanospermine. CD4$^+$ Jurkat-tat_{III} cells were transfected with a recombinant plasmid which, in the absence of added drug, resulted in HIV envelope expression and the formation of multinucleated syncytia, scored here at 48 hr.

virus and uninfected CD4$^+$ cells individually pretreated with castanospermine prior to cocultivation. For this experiment, the H9 cell line that constitutively produces the HTLV-IIIB strain of HIV (H9/HTLV-IIIB) and the C8166 CD4$^+$ human T-cell line were used. The two cell lines were mixed and syncytia were counted after 4 hr. Pretreatment of the H9/HTLV-IIIB cell lines with castanospermine resulted in a marked decrease in the number and size of syncytia formed (Table 1). By contrast, no inhibitory effect on syncytium formation was seen when the CD4$^+$ cells were pretreated with castanospermine prior to incubation with the HTLV-IIIB-infected cells. Similarly, reduction in the ability of CHO fibroblasts constitutively expressing the HIV envelope to induce syncytia when cocultivated with CD4$^+$ lymphocytes was observed upon pretreatment of the CHO cells with castanospermine (data not shown).

Treatment with castanospermine is anticipated to result in an envelope protein exhibiting modified glycosylation, as the drug inhibits an enzyme that trims the sugar residues of the nascent glycoprotein. The size of the viral envelope glycoprotein produced in cells treated with castanospermine was determined by labeling of HIV-infected cells with [^{35}S]methionine and immunoprecipitation of cell-associated proteins with serum from an HIV-seropositive individual. The apparent molecular weight of the envelope glycoprotein precipitated from castanospermine-treated cells was compared to that precipitated from untreated HIV-infected cultures. Inspection of the autoradiogram of the denaturing polyacrylamide gel (Fig. 2*A*) reveals that treatment with castanospermine results in an increase in the apparent molecular weight of both the precursor of the glycoprotein (gp160) and the cleaved exterior glycoprotein (gp120). Additionally, there is an increase in the relative abundance of the precursor glycoprotein to the cleaved exterior glycoprotein compared with the untreated control, indicating a castanospermine-induced inhibition of envelope glycoprotein processing. Sim-

Table 1. Castanospermine inhibits HIV-induced syncytium formation by alteration of the envelope glycoprotein rather than by alteration of the CD4 molecule

	Syncytia per 10^4 H9/HTLV-IIIB cells	
	C8166	C8166 + Cas
H9/HTLV-IIIB	350 ± 71	400 ± 70
H9/HTLV-IIIB + Cas	24 ± 24	12 ± 12

CD4$^+$ C8166 cells were grown in the presence or absence of castanospermine (Cas) at 100 μg/ml, as were chronically HIV-infected H9 cells (H9/HTLV-IIIB). Cells were mixed, and syncytia were counted at 4 hr. Results are presented as mean ± SD.

ilar results were obtained after castanospermine treatment of CHO cells constitutively expressing the HIV envelope (data not shown). The effect of treatment with concentrations of castanospermine that affected HIV envelope processing on the size of the CD4 protein was determined by using anti-CD4 monoclonal antibodies. No change was observed in the electrophoretic mobility of the CD4 protein precipitated from the castanospermine-treated C8166 cell culture compared with an untreated control culture (not shown).

To determine if treatment with castanospermine altered the concentration of virus-encoded proteins on the cell surface, CHO cells constitutively expressing the HIV envelope gene product were cultured in the presence or absence of castanospermine and then radioiodinated (Fig. 2*B*). In the absence of castanospermine, cell surface expression of gp120 was readily apparent (lane 2). However, in the presence of castanospermine, surface expression of the cleaved exterior glycoprotein was dramatically reduced (lane 3).

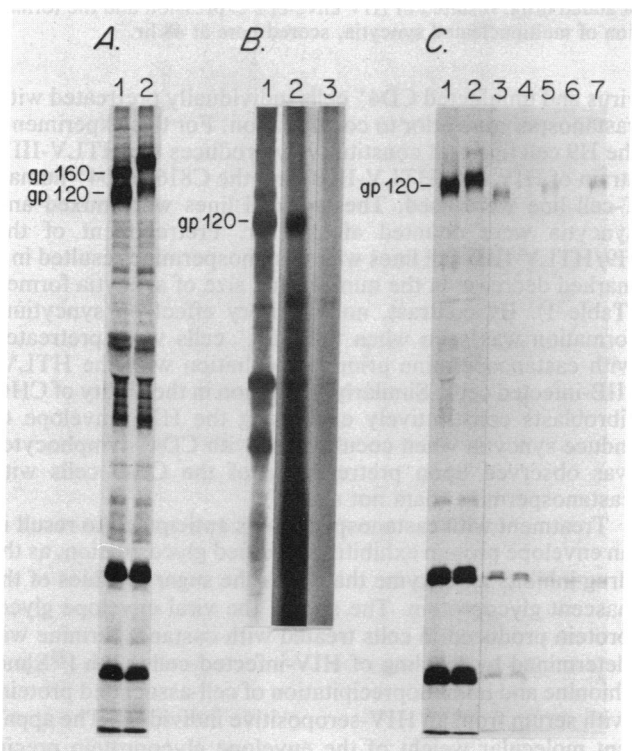

FIG. 2. (*A*) Effects of castanospermine on the *cell-associated* HIV envelope gene product. Chronically HIV-infected H9 cells, either untreated or treated with castanospermine at 100 μg/ml, were metabolically labeled and HIV-specific proteins were immunoprecipitated. Lane 1, untreated cells, cell lysate; lane 2, castanospermine-treated cells, cell lysate. (*B*) Effect of castanospermine on cell surface expression of the mature HIV exterior glycoprotein. Lane 1, immunoprecipitation of HIV envelope-expressing CHO cells; lane 2, radioiodination of HIV envelope-expressing CHO cells; lane 3, radioiodination of HIV envelope-expressing CHO cells grown in the presence of castanospermine at 300 μg/ml for 48 hr. (*C*) Effects of castanospermine on *virion* envelope and envelope–CD4 binding. Metabolically labeled cell-free supernatant from the same experiment described for *A* was immunoprecipitated with serum from an HIV-seropositive subject. Lane 1, untreated cell culture, supernatant. Lane 2, castanospermine-treated culture, supernatant. These labeled supernatants were also incubated with $CD4^+$ SupT1 cells, which in some cases had been pretreated with anti-CD4 monoclonal antibodies. SupT1 cells were then washed and lysed, and the lysate was radioimmunoprecipitated with serum from an HIV-seropositive individual. Sources of the labeled cell-free supernatants and monoclonal antibodies were as follows: lane 3, untreated cells, no antibody; lane 4, untreated cells, OKT4A antibody; lane 5, castanospermine-treated cells, no antibody; lane 6, castanospermine-treated cells, OKT4A antibody; lane 7, castanospermine-treated cells, OKT4 antibody. While minor experimental variation was seen, no reproducible difference in envelope–CD4 binding was detected with castanospermine treatment.

Evidently castanospermine inhibits HIV envelope-mediated syncytium formation through its effects on the envelope glycoprotein, which include a modification of glycosylation, a decrease in processing of the envelope precursor, and a decrease in cell surface expression of the mature exterior glycoprotein.

Castanospermine Inhibits Virus Replication. Inhibition by castanospermine of the function of the envelope glycoprotein in syncytium formation suggested that the drug might also inhibit virus replication. Accordingly, $CD4^+$ H9 cells were treated with castanospermine shortly before infection with the HTLV-IIIB strain of virus. The drug remained in the culture fluid for the remainder of the experiment.

Treatment of the cultures with castanospermine prior to infection resulted in a marked reduction of virus infection by all criteria examined. Doses of castanospermine of 50 μg/ml and above dramatically reduced the formation of virus-induced syncytia (data not shown) and eliminated the decrease in cell number accompanying virus infection of untreated cultures (Fig. 3*A*). A marked dose-dependent decrease in percentage of cells exhibiting HIV-specific membrane immunofluorescence was also observed (Fig. 3*B*). A dose-dependent decrease in extracellular virus was observed as measured by assay of extracellular reverse transcriptase (Fig. 3*C*) and by measurement of the p24 proteins using a radioimmune competition assay (not shown). Virus-specific proteins were barely detectable after 9 days in supernatants of cell cultures treated with concentrations of castanospermine of 200 μg/ml.

Treatment with castanospermine also resulted in a marked decrease in the production of infectious virus (Fig. 3*D*). The titer of infectious virus produced at a concentration of 200 μg/ml was reduced by approximately six orders of magnitude. Similarly, a marked decrease in cell-associated virus-encoded proteins was observed 9 days after infection, as judged by immunoprecipitation of radiolabeled cell extracts by using serum from an HIV-seropositive individual (Fig. 4).

Properties of Virus Produced by Cells Treated with Castanospermine. To investigate the properties of virions produced by castanospermine-treated cells, radiolabeled virus was harvested from the supernatants of matched castanospermine-treated and untreated H9 cells chronically infected with HTLV-IIIB (Fig. 2*C*, lanes 1 and 2). For these experiments, long-term cultures of infected H9 cells were treated for less than 48 hr with castanospermine. Immunoprecipitation of virus-encoded proteins by using HIV-specific antisera revealed that the virus produced from castanospermine-treated cultures contained a glycoprotein that corresponds to the cleaved cell-associated exterior glycoprotein (gp120) but migrates more slowly. The electrophoretic mobility of the protein corresponds to that observed for the modified gp120 associated with castanospermine-treated cells (Fig. 2*A*, lane 2). Interestingly, despite the castanospermine-induced decrease in cellular expression of cleaved exterior glycoprotein, the amounts of *virion* exterior glycoprotein were the same in the presence or absence of castanospermine (Fig. 2*C*, lanes 1 and 2). No protein corresponding to the modified gp160 precursor was detected in the virus. Moreover, no protein that migrated at the position of the unmodified gp120 was evident in this experiment, indicating that the α-glucosidase I activity is decreased below levels of detection at the concentration of castanospermine used.

The ability of the virus produced by cells treated with castanospermine to bind to $CD4^+$ cells was examined (Fig. 2*C*, lanes 3–7). Radiolabeled virus was incubated with $CD4^+$ SupT1 cells, a human T-cell line expressing high levels of the

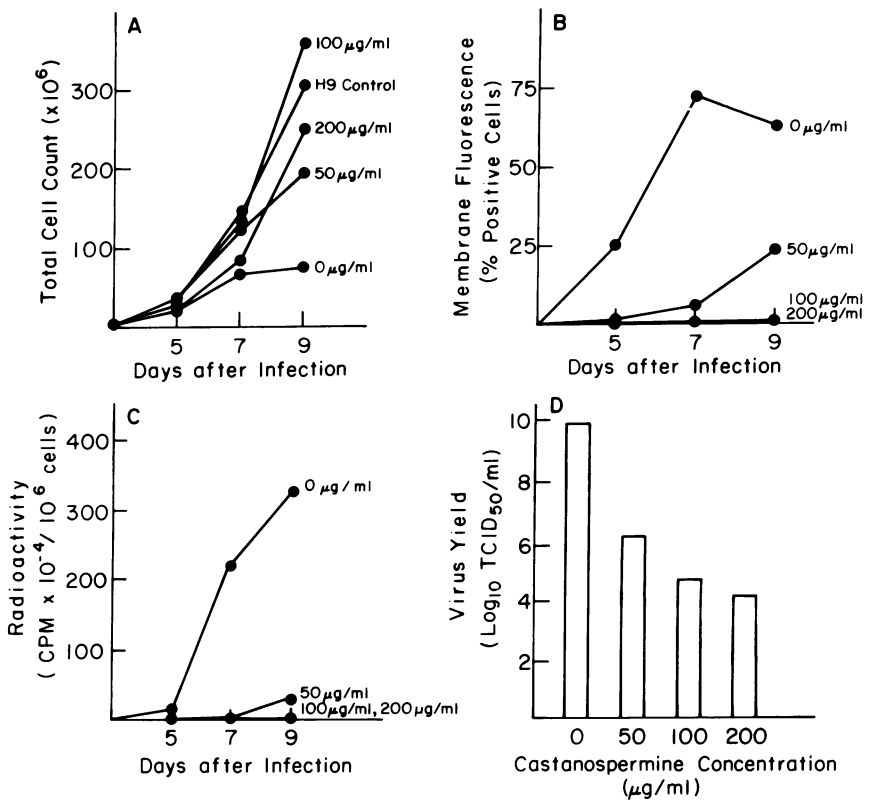

FIG. 3. Castanospermine inhibits replication of HIV. H9 cells were infected with HIV in the presence of castanospermine at various concentrations and followed for 9 days. Cultures were assayed for total viable cell count (*A*), percent of cells expressing HIV antigens as assayed by fixed-cell membrane immunofluorescence with serum from an HIV-seropositive subject (*B*), reverse transcriptase activity in the cell-free supernatant (*C*), and amount of infectious virus present on day 9 in the cell-free supernatant (*D*). Results shown are from a representative experiment.

CD4 glycoprotein. Cells were then washed, and virus-specific proteins bound to the CD4$^+$ cells were immunoprecipitated by using serum from an HIV-seropositive individual, followed by analysis of the proteins on denaturing polyacrylamide gels. Labeled virions produced in the absence of drug were shown to bind to SupT1 cells, as evidenced by the presence of gp120 in the immunoprecipitate of the lysed cells (lane 3). The specificity of this binding reaction was demonstrated by inhibition of binding by the OKT4A monoclonal antibody (lane 4), an antibody that has previously been shown to interfere with the binding of virus to the CD4 receptor. No reduction in the ability of virus produced from castanospermine-treated cells to bind to the CD4$^+$ cells, as compared with that from control cultures, was observed (lane 5). Again, the binding was shown to be specific, as it was inhibited by OKT4A but not OKT4 monoclonal antibody (lanes 6 and 7, respectively). The apparent molecular weight of the bound exterior glycoprotein of virus produced from castanospermine-treated cells is higher than that of the untreated virus. These observations indicate that the exterior envelope glycoprotein modified by castanospermine still binds to the CD4 receptor.

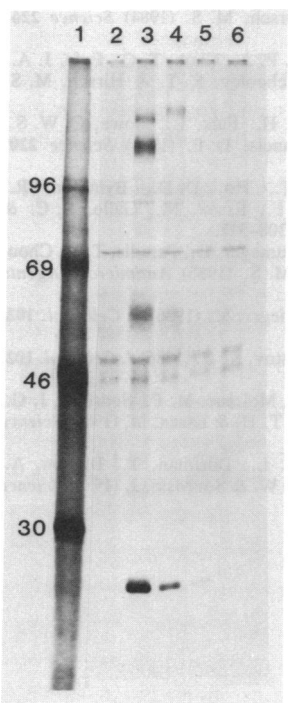

FIG. 4. Castanospermine inhibits HIV replication as assayed by viral protein production. Radiolabeled H9 cellular lysates on day 9 after HIV infection were immunoprecipitated by using serum from an HIV-seropositive subject. Lane 1, molecular weight markers (× 10^{-3}); lane 2, uninfected H9 cells; lane 3, untreated HIV-infected H9 cells, demonstrating the envelope precursor protein (gp160) and the cleaved exterior glycoprotein (gp120); lanes 4, 5, and 6, HIV-infected H9 cells grown in the presence of castanospermine at 50, 100, and 200 μg/ml, respectively. With castanospermine at 50 μg/ml, the envelope precursor protein migrates as if it had a higher molecular weight, and the amount of cleaved exterior glycoprotein is reduced. At 200 μg/ml there is no evidence of HIV envelope proteins or p24 antigen.

The infectivity of the virions prepared from castanospermine-treated chronically HIV-infected H9 cells was compared to that of chronically infected cultures not treated with drug. After a 24-hr incubation, there was no detectable difference in reverse transcriptase activity or p24 antigen concentration in the castanospermine-treated cultures compared with untreated cultures, indicating that the amount of viral particles in each culture supernatant was approximately equal. Cell-free supernatants from these cultures were then assayed for virus yield by adding serial 1:10 dilutions of supernatant to C8166 cells and monitoring daily for cytopathic effect. Yield of infectious virus in supernatant from castanospermine-treated cultures was approximately 1/10th of that in untreated supernatant. Thus, castanospermine decreases the infectivity of the virions without reducing the amount of envelope glycoprotein on the virion surface and without preventing CD4 binding of the modified envelope glycoprotein.

DISCUSSION

The results presented here demonstrate that castanospermine can inhibit both envelope glycoprotein-mediated syncytium formation and HIV infection of CD4$^+$ cells.

In acute HIV infection of susceptible CD4$^+$ lymphocytes, the presence of castanospermine dramatically inhibits the spread of virus. At concentrations of castanospermine that inhibited virus replication and affected the mobility of HIV

envelope products on $NaDodSO_4$/polyacrylamide gels, no cell toxicity was observed.

Studies of chronically HIV-infected cells treated for a 24-hr period with castanospermine yield insight into the mechanism by which the drug inhibits virus spread. Treatment of these chronically infected cells with castanospermine does not reduce virus production as measured by reverse transcriptase or p24 antigen determination. Radioimmunoprecipitation analysis of concentrated virion preparations from chronically infected cultures grown for less than 48 hr in the presence or absence of castanospermine reveals no differences in the level of virus-associated envelope glycoprotein, despite reduction of cell surface expression of envelope proteins after castanospermine treatment. The castanospermine-modified exterior glycoprotein present on the virus can bind to the CD4 receptor. Nonetheless, the infectivity of the virions produced in the presence of castanospermine is reduced as measured by the virus yield assay. These observations suggest that the reduction of virion infectivity accompanying castanospermine treatment may be due to inhibition of post-CD4 binding steps in virus entry, possibly membrane fusion.

Treatment of HIV envelope-expressing lymphoid or fibroblast cells with castanospermine results in a decreased ability of those cells to fuse to $CD4^+$ lymphocytes to form syncytia. Castanospermine treatment affects the apparent molecular weight of the cell-associated exterior envelope glycoprotein and its precursor without a detectable reduction in the total amount of envelope glycoprotein synthesis. The drug also affects the processing of the precursor glycoprotein in these cells, resulting in an increase in the ratio of uncleaved to cleaved envelope glycoprotein. Castanospermine led to a dramatic decrease in cell surface expression of the mature exterior glycoprotein in the fibroblast cells examined. A portion of the inhibition of syncytium formation may be attributed to this decreased rate of cleavage of the envelope glycoprotein precursor in the castanospermine-treated cells, as the cleavage reaction and surface gp120 expression are important for the function of the envelope protein in CD4 binding and probably also in membrane fusion (29). Inhibition of post-CD4 binding steps by castanospermine, proposed to explain the observed decrease in virus infectivity, may also play a role in the decrease in syncytium formation. Finally, reduction in cell fusion might contribute to the effects of castanospermine on virus spread, as cell-to-cell transmission of the virus would be decreased.

In summary, the observations reported here suggest that the profound inhibitory effects of castanospermine on HIV infection can be attributed to reduction of the virion infectivity and to a reduction in cell-to-cell spread of virus infection by inhibition of cell fusion events. Decreased efficiency of fusion of the modified cleaved exterior glycoprotein as well as the decrease in the processing of the cell-associated glycoprotein may contribute to reduced cell-to-cell fusion. These observations suggest that castanospermine as well as other drugs that modify the glycosylation state of the HIV envelope glycoprotein may be of use in the treatment of HIV infections.

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