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Selective antiviral activity of synthetic soluble L-tyrosine and L-dopa melanins against human immunodeficiency virus in vitro

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Summary

Melanins are pigments found in hair, skin, irides of the eye, and brain. Their functions in mammals include protection from exposure to sunlight, camouflage from predators, sexual recognition within species, and possible electron transfer reactants. Most natural melanins exist in an insoluble form, which is one reason there is little information on the biological properties of soluble melanins. Here, synthetic soluble melanins were obtained by chemical oxidation of L-tyrosine or spontaneous oxidation of L-β-3,4-dihydroxyphenylalanine (L-dopa). Replication of human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2) was inhibited by soluble melanin in two human lymphoblastoid cell lines (MT-2 and H9) and in phytohemagglutinin-stimulated human T cells. Effective concentrations of 0.15–10 μg/ml had no cell toxicity. Melanin blocked infection by cell-free virus and interfered with HIV-induced syncytium formation and cytopathic effects when fusion-susceptible, uninfected cells, were mixed with chronically infected cells. Melanin also impeded the HIV-1 envelope surface glycoprotein, and T cell specific monoclonal antibody leu-3a (CD4), but not leu-5b (CD2), from binding to the surface of MT-2 cells. No effect on HIV-1 reverse transcriptase activity in viral lysates was observed. These results identify a unique biological property of melanin, and suggest that soluble melanins may represent a new class of pharmacologically active substances which should be further investigated for potential therapeutic utility in the treatment of Acquired Immune Deficiency Syndrome (AIDS).

Melanin; Antiviral therapy; Acquired immune deficiency syndrome

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Introduction

Melanins are heteropolymers derived from the spontaneous polymerization of intermediates formed during the enzymatic or chemical oxidation of L-tyrosine and other phenolic molecules, or the auto-oxidation of L-dopa and similar catechols (Mason, 1948; Prota, 1980; Raper, 1928). They are pigments found throughout nature, including hair, skin, irides of the eye, and substantia nigra and locus ceruleus of the brain (Bazelton et al., 1967; Nicolaus, 1968). Their known biological functions derive from their diverse coloration, their ability to absorb ultraviolet radiation, and their electron transfer properties (Gan et al., 1976; Lerner, 1971; Menon and Haberman, 1977; Pathak et al., 1976). The various colors of melanin depend, in large part, on the initial substrate. Brown and black melanins originating from L-tyrosine and L-dopa are termed eumelanins, while yellow and red melanins that arise from eumelanins containing sulfhydryl compounds are termed pheomelanin (Prota, 1980). Black melanin synthesized by the brain is termed neuromelanin (Lerner, 1974; Lillie, 1955, 1957). Eumelanin and pheomelanin biosyntheses occur in specialized organelles of melanocytes called melanosomes (Seiji et al., 1963) which subsequently become insoluble melanin granules. Neuromelanin is found in the cytoplasm of catecholamine-producing neurons (Bazelton et al., 1967) and can be synthesized from L-dopa, dopamine, norepinephrine, epinephrine, and 5-hydroxytryptamine (Rodgers and Curzon, 1975).

Human immunodeficiency virus (HIV) is the etiologic agent of Acquired Immune Deficiency Syndrome (AIDS) (Kalyanaraman et al., 1984; Kaminski et al., 1985; Sarngadharan et al., 1984). This lentivirus primarily infects $CD4^+$ cells causing their direct or indirect destruction (Lifson et al., 1986; Siliciano et al., 1988). As a consequence of $CD4^+$ cell depletion, the host becomes susceptible to opportunistic infections and neoplasms. Several drugs have been identified which inhibit the replication of this virus in vitro (Haseltine, 1989) but only 3′-azidothymidine (AZT) has received wide acceptance for clinical use. The proven clinical efficacy of AZT is limited, however, and its use is restricted by toxicity and drug-resistant forms of the virus (Larder et al., 1989; Richman et al., 1987). Therefore, new antivirals are urgently needed. In a previous study, soluble melanins synthesized from dopamine, norepinephrine and 5-hydroxytryptamine (serotonin) were shown to have anti-HIV-1 activity in MT-2 cell cytopathic infection experiments and syncytium inhibition experiments (Montefiori et al., 1990). Here, L-tyrosine and L-dopa melanins are shown to have broad and selective anti-HIV-1 and anti-HIV-2 activities in several cell systems in vitro. In addition, their mechanism of action was examined.

Materials and Methods

Cells and viruses

The $CD4^+$ human lymphoblastoid cell lines MT-2 (obtained from D. Richman, Veterans Administration Medical Center, University of California, San Diego) and

H9 (obtained from R.C. Gallo, National Cancer Institute) were cultured at 37°C in RPMI-1640 containing 12% heat-inactivated fetal bovine serum and 50 μg of gentamicin per ml. Stocks of the HIV-1 isolates HTLV-III_B and HTLV-III_{RF} (obtained from R.C. Gallo), and the HIV-2 isolate HIV-2_{ROD} (obtained from J.-C. Chermann, Bâtiment INSERM, Marseilles) were harvested from chronically-infected H9 cultures. Virus-containing culture fluids were clarified of cells by low speed centrifugation and passed through 0.45 micron filters. Infectious virions were quantitated by microtitration on MT-2 cells using cytopathic effect (CPE) as end point for infection (Montefiori et al., 1988a); the reciprocal dilution at which 50% of wells showed CPE after 2 weeks incubation defined the infectious titer (1 $TCID_{50}$).

Melanins

L-dopa melanin was synthesized essentially as described previously (Arnow, 1938). One gram of L-dopa (Sigma Chemical Company, St. Louis, MO) was dissolved in 400 ml of 0.025 N NaOH and incubated for 2 days at room temperature with constant aeration. Aeration was accomplished with the aid of an air sparger using air that had been passed through a solution of 1 N NaOH. Melanin was precipitated from the dark brown solution by adding 2 ml of concentrated HCl. The precipitate was collected by centrifugation, dissolved in 400 ml of deionized water, and precipitated again with 1 ml of concentrated HCl. The melanin was washed this way a total of four times and then dissolved in 20 ml of 0.025N NaOH (the solution had a neutral pH at this time) and lyophilized. The final yield was 100 mg of a fine black powder which was soluble in phosphate buffered saline (PBS), pH 7.4, to at least 0.5 g/ml. When dialyzed against water, 54% of the material had a molecular weight greater than 14 000 and retained anti-HIV activity. The infrared spectrum of this melanin had absorptions at 3400 cm^{-1} and at 1650 cm^{-1}, which are attributed to the amine or aromatic hydroxy bond, and the conjugated carbonyl groups of melanin, respectively (Swan, 1963). L-tyrosine melanin (obtained by hydrogen peroxide oxidation of L-tyrosine) was purchased from Sigma Chemical Company (St. Louis, MO) and was soluble to 0.2 mg/ml in PBS.

Larger quantities of L-dopa melanin were synthesized by dissolving 10 g of L-dopa in 400 ml of 0.1 N NaOH. This was incubated at room temperature for 3 days with constant aeration as described above. At the end of 3 days incubation the black solution, which had a pH of 6.7, was lyophilized. The final yield was 10 g of a highly soluble, dark-brown powder which had anti-HIV-1 activity in MT-2 infection experiments very similar to the acid-precipitated L-dopa melanin described above.

Infection assays

Antiviral activities of synthetic melanins derived from L-tyrosine and L-dopa were measured in 96-well microdilution plates as described (Montefiori et al., 1988a). Briefly, 2-fold serial dilutions of melanins were made in triplicate in a total of 100 μl growth medium (RPMI-1640 containing 12% heat-inactivated fetal

bovine serum and 50 μg gentamicin/ml) per well. MT-2 cells (5×10^4) in 100 μl of growth medium were added to each well and incubated for 10 min. Fifty microliters of virus (5×10^4 $TCID_{50}$/50 μl) were then added to all wells except for 1 row of eight non-cytopathic control wells; these received growth medium in place of virus. Viral-induced cytopathic effect (CPE) was quantitated by vital dye (neutral red) uptake in remaining viable cells three days later. Neutral red uptake is a linear function of cell viability when measured by light absorption (540 nm), where absorption is linear between 0.025 and 0.85 readings which correspond to 2×10^4 to 25×10^4 viable cells/well (Montefiori et al., 1988a). Percent protection is defined as the difference in light absorption between test wells (cells + melanin + virus) and virus control wells (cells + virus) divided by the difference in light absorption between cell control wells (cells only) and virus control wells.

Infection of MT-2, H9 and phytohemagglutinin (PHA)-stimulated T cells were performed in flask cultures to obtain enough volume for immunofluorescence assays (IFA) and measurement of reverse transcriptase (RT) activity. For this, cells ($2–5 \times 10^6$/10 ml growth medium) were challenged for 4 h with 5×10^6 $TCID_{50}$ of HIV-1 ($HTLV\text{-}III_B$) in the presence and absence of synthetic L-tyrosine or L-dopa melanins (10 μg/ml). The cells were then washed in growth medium to remove melanin and unadsorbed virus, and incubated in fresh growth medium without melanin. Another set of experiments included cultures of MT-2 cells which were incubated with L-dopa melanin for 1 h and then washed before adding virus. Cultures were maintained for 3 days (MT-2), 5 days (H9) or 9 days (PHA-stimulated T cells), then examined by IFA and RT activity for viral antigen synthesis. IFA was performed on air-dried, acetone-methanol fixed cells as described (Montefiori and Mitchell, 1986) using serum from an HIV-1-positive individual. This serum was positive by Western immunoblot (E.I. DuPont, Wilmington, DE) for all major HIV-1 antigens. RT activity was measured by the incorporation of methyl [^{3}H]dTTP into poly(rA)·$(dT)_{15}$ template-primer (see below) as described (Poiesz et al., 1980). Peripheral blood lymphocytes (PBLs) were isolated from heparinized whole blood using the Sepracell 2-step procedure (Sepratech Corp., Oklahoma City, OK). Residual monocytes were removed by adherence to the surface of a culture flask after which B cells were removed by passage through an immunoaffinity column (T-Cell Column Kit, Beckman Instruments, Inc., Fullerton, CA). The final T cell preparation was greater than 98% T cells as shown by IFA using fluorescein-conjugated anti-leu-4 (CD3) and phycoerythrin-conjugated anti-leu-12 (CD19), both from Becton Dickinson (Mountain View, CA). Fluorescence was detected using a Nikon DIAPHOT-TMD-EF fluorescence microscope. T cells were stimulated with PHA-P (P-L Biochemicals) at 1 μg/ml for 24 h, washed with growth medium, and cultured in the presence of recombinant interleukin-2 (rIL-2, E.I. DuPont, 12.5 units/ml final) for the duration of experiments.

Measurement of antisyncytial activity

Syncytium formation was induced by mixing MT-2 cells with chronic HIV-infected H9 cells, at a 10:1 ratio, in the presence and absence of L-tyrosine or

L-dopa melanin in 96-well microdilution plates. Serial dilutions of melanins were made in triplicate. MT-2 cells (1.5×10^5) in 100 μl of growth medium were then added to each well. HIV-infected H9 cells (1.5×10^4) in 50 μl of growth medium were added to all wells except one row of 8 non-cytopathic control wells, which received uninfected H9 cells. Syncytium formation leads to, and is directly proportional to, cytopathic effect in this assay (Montefiori et al., 1988b). After incubation at 37°C for 20 h, syncytium formation was observed microscopically while viable cells were measured by vital dye uptake as described above.

Flow cytometry

The ability of melanin to block gp160 and gp120 binding to MT-2 cells was detected by flow cytometry. MT-2 cells were adjusted to 10^6 cells per 100 μl of phosphate-buffered saline containing 0.1% bovine serum albumin (PBS-BSA) and incubated for 20 min on ice with melanin or PBS. Baculovirus-derived gp160 or gp120 (MicroGeneSys, Inc., West Haven, CT) were added at 10 μg/ml to all samples and the incubations continued another 20 min. The cells were washed twice with cold PBS-BSA and then incubated on ice for 20 min with a 1:20 dilution of rabbit anti-gp120 (MicroGeneSys, Inc.,). The cells were washed again and incubated on ice for 20 min with fluorescein-conjugated, goat anti-rabbit IgG (heavy and light chains specific, Cappell, West Chester, PA). After final washes, fluorescence intensities were measured on an EPICS 753 multiparameter flow cytometer (Coulter) using an argon laser (Coherent Laser Division, Palo Alto, CA) set at 488 nm excitation light, 300 mW power, and 25A current. Data analysis was performed using the multiparameter data acquisition and display system (MDADS, Coulter, Hialeah, FL). Background emission was adjusted using cells incubated without gp160, gp120 and melanin, but otherwise stained the same.

Soluble melanins were also examined for an ability to block binding of T-cell specific monoclonal antibodies, leu-3a (CD4) and leu-5b (CD2) (Becton Dickinson, Mountain View, CA), to MT-2 cells. These experiments were performed as described above except that fluorescein-conjugated monoclonal antibodies (1:10 final dilution) were used after preincubation of cells with melanin. Background emission was adjusted using cells stained with fluorescein-conjugated, mouse IgG_1 (Becton Dickinson).

Reverse transcriptase (RT) assay

Virus was concentrated from cell-free (0.45 μm filtered) conditioned H9/HTLV-III_B culture supernatants by centrifugation at 18 000 rpm for 4 h at 20°C in a Beckman JA-20 rotor. A viral pellet obtained from 50 ml of conditioned culture fluid was dissolved in 0.5 ml of a solution containing 17 mM Tris-HCl (pH 7.8), 3 mM dithiothreitol (DTT), 55 mM KCl, 0.32% w/v Triton X-100, and 33% glycerol. This viral lysate was stored at −20°C and was used as a source of HIV-1 RT. RT reactions were performed in 100 μl reaction volumes containing 40 mM Tris-HCl (pH 7.8), 4 mM DTT, 50 mM KCl, 10 mM $MgCl_2$, 0.0325% w/v Triton X-100, 3

μM [^{3}H]dTTP (80 Ci/mmol, NEN) and poly (A)·(dT)$_{15}$ (2.5 μg/ml) template-primer after the addition of 10 μl enzyme. Melanins were added at various concentrations after adjusting water volumes so that reaction volumes remained constant. Reactions were incubated at 37°C for 1 h in a humidified environment and terminated by adding 2 ml of 10% cold trichloroacetic acid. Precipitate was collected on 0.45 μm cellulose-acetate Millipore filters which then were dissolved in 10 ml of 3a70B aqueous scintillant. Counts per minute were quantitated using a Beckman LS 6800 liquid scintillation spectrometer.

Results and Discussion

Antiviral activity in MT-2 cytopathic infection experiments

Soluble synthetic L-dopa and L-tyrosine melanins were initially screened for antiviral activity in MT-2 cell infection assays. Both melanins protected MT-2 cells from infection by cell-free virus as indicated by dramatic reductions in viral-induced cytopathic effects (Fig. 1). Representatives of the two predominant genetic and serological types of HIV (i.e., HIV-1 and HIV-2, see Clavel et al., 1986; Hahn et al., 1985; Popovic et al., 1984) were used in these experiments. Antiviral activity was strongest against the genetically diverse, HTLV-III$_B$ and HTLV-III$_{RF}$ isolates of HIV-1. Most potent activity was that of L-dopa melanin, which at 0.31–10 μg/ml provided 100% protection against HTLV-III$_B$, and at 0.62–10 μg/ml provided 100% protection against HTLV-III$_{RF}$. Effective doses that provided 50% protection (ED$_{50}$) against HTLV-III$_B$ and HTLV-III$_{RF}$, respectively, were 0.2 μg/ml for L-tyrosine melanin, and 0.2 and 0.4 μg/ml for L-dopa melanin. Both melanins were less active against the serologically and genetically diverse HIV-2 isolate, HIV-2$_{ROD}$. Here, the ED$_{50}$ was 1.5 μg/ml for L-tyrosine melanin and 3 μg/ml for L-dopa melanin. Melanin concentrations greater than 10 μg/ml were toxic to the cells

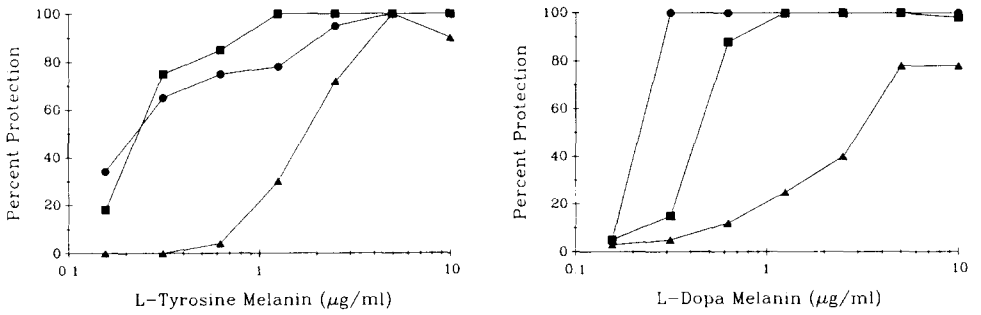

Fig. 1. Protection of MT-2 cells from HIV-1 and HIV-2 cytopathic infection. MT-2 cells were challenged with cell-free virus in 96-well microdilution plates in the presence and absence of 7 drug concentrations. Percent protection was determined by vital dye uptake of viable cells 3 days post virus challenge. For cell controls and virus controls, n=8. For each concentration of test substances, n=3. Standard deviations were less than 10% for each data point. Closed circles, HTLV-III$_B$; closed squares, HTLV-III$_{RF}$; closed triangles, HIV-2$_{ROD}$.

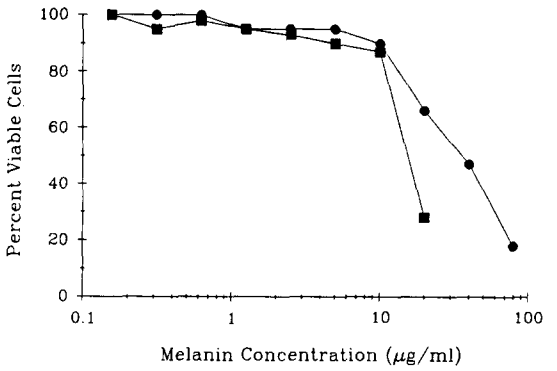

Fig. 2. Toxicity of L-tyrosine and L-dopa melanins in MT-2 cells. Two-fold dilutions of melanins were made in triplicate in wells of a 96-well microdilution plate. MT-2 cells were added (10^5 cells/well) and incubated for 3 days. Viable cells were quantitated by vital dye uptake as described in Materials and Methods. Percent viability is the percent of absorption at 540 nm of vital dye retained by cells in the absence of melanin. Closed circles, L-dopa melanin; closed squares, L-tyrosine melanin.

(Fig. 2). L-tyrosine and L-dopa at 1–100 μg/ml had no antiviral activity in these assays (data not shown). Therefore, beginning substrates for melanin synthesis were not responsible for the antiviral activities observed. It is also unlikely that intermediates in melanin biosynthesis were responsible for antiviral activity since these intermediates are highly unstable and short-lived (Mason, 1948; Prota, 1980; Raper, 1928).

Further characterization of melanin's antiviral activity using MT-2 and H9 cells, and PHA-stimulated T cells

The anti-HIV-1 (HTLV-III_B) activities of L-tyrosine and L-dopa melanins were further investigated in the $CD4^+$ human lymphoblastoid cell lines, MT-2 and H9, and in PHA-stimulated human T cells. Immunofluorescent positive cells and RT release into culture fluids were greatly reduced in all cultures in the presence of melanin (Table 1). Since melanin was present during a 4 h virus adsorption period only, the site of antiviral activity was probably an early stage in the HIV replication cycle. These results also demonstrate that the antiviral activity of melanin is not cell line specific.

In another set of experiments with MT-2 cells, we examined whether pretreatment of cells alone was sufficient for melanin to elicit antiviral activity, or whether melanin needed to be present during viral challenge or during and after viral challenge. Based on the appearance of HIV-1 antigen expression, no antiviral activity was observed when pretreated cells were washed and then challenged with virus in the absence of melanin (Fig. 3). Here, less than 2% of cells challenged with virus in the presence of melanin were expressing HIV-1 antigens three days post viral challenge, while 100% of cells pretreated only or with no treatment were antigen positive at this time. This result suggests that melanin must be present with the virus in order to exhibit anti-HIV activity. Should melanin have bound to the

TABLE 1

Inhibition of HIV-1 replication in MT-2, H9 and PHA-stimulated T cells by synthetic soluble melanins

Cells*	% IFA positive		RT activity (cpm × 10^{-4}/ml)	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
MT-2	100[s]	100[s]	581	395
MT-2 + L-tyrosine melanin	5	1	45	5
MT-2 + L-dopa melanin	5	1	11	1
H9	100	100	375	344
H9 + L-tyrosine melanin	<1	<1	5	1
PHA T cells	20	20	52	55
PHA T cells + L-tyrosine melanin	<1	<1	1	2

*Cells were challenged with HTLV-III_B in the presence and absence of melanins at 10 μg/ml. This concentration of melanins was shown by vital dye uptake to have no inhibitory effect on cell viability. Virus and melanins were removed after a 4 h adsorption period, and the cells incubated in fresh growth medium for the duration of experiments.

[s]Denotes an abundance of syncytia and the beginning of cytopathic effect.

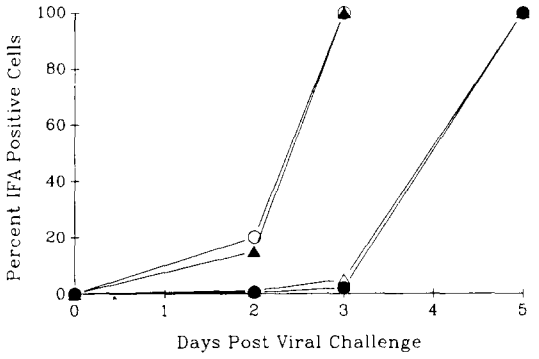

Fig. 3. Antiviral effect of L-dopa melanin when cells are pretreated and then washed prior to addition of virus. Cultures of MT-2 cells (10 ml) were preincubated in the presence or absence of L dopa melanin (10 μg/ml) for 1 h. One set of cultures then was washed twice with growth medium and resuspended in fresh growth medium minus melanin. Two milliliters of high titer HIV-1 (HTLV-III_B) were added to all cultures and the virus allowed to adsorb to cells for 2 h. The cells from all cultures were washed twice with growth medium, resuspended in fresh growth medium, and placed into fresh flasks. Melanin was added back to one set of cultures which had melanin present continuously. Infections were monitored by IFA as described in Materials and Methods. Open circles, control cultures with no melanin added; closed triangle, melanin removed from the growth medium prior to addition of virus; open triangle, melanin removed from the growth medium after virus adsorption; closed circle, melanin present continuously before, during and after virus addition. Each point represents the average of two cultures.

cells, the binding would have been of such low affinity that the melanin was readily removed by washing. It is unlikely that potent antiviral activity could be due to a low affinity attraction of melanin to the MT-2 cell surface since the gp120-CD4 binding is of high affinity (McDougal et al., 1986). Therefore, melanin's antiviral activity was most likely due to an interaction with the virus. Results of a previous

study with dopamine melanin support the notion that interaction with the virus is critical for melanin's anti-HIV activity (Montefiori et al., 1990). In addition, the ability of melanin to protect cells from infection with only a two hour viral adsorption period (Fig. 3) supports results obtained previously (Table 1) indicating that melanin blocks HIV-1 infection early in the viral replication cycle.

Syncytium inhibition

HIV-induced cytopathic effect in cell culture is often the result of syncytium formation. Syncytium formation occurs when the viral surface glycoprotein, gp120, expressed on the surface of HIV-infected cells binds to the viral receptor, CD4, on the surface of uninfected target cells (Lifson et al., 1986; Sodroski et al., 1986). The viral transmembrane glycoprotein, gp41, and possibly additional cellular surface molecules (Hildreth and Orentas, 1989; Kowalski et al., 1987) have been reported to mediate a membrane fusion process where one infected cell can fuse with multiple uninfected cells, the end result being the formation of multinucleated giant cells called syncytia. L-tyrosine and L-dopa melanins were found to block syncytium formation and subsequent cytopathic effects when uninfected MT-2 cells were mixed with H9 cells chronically infected with HTLV-III_B, HTLV-III_{RF} or HIV-2_{ROD} (Fig. 4). As in infection assays (Fig. 1), both melanins were less effective against HIV-2_{ROD} than against the two HIV-1 isolates.

Effect of soluble melanin on binding of gp160, gp120 leu-3a and leu-5b to MT-2 cells

Gp120-CD4 binding is an early event in HIV replication, and is critical to virus attachment and syncytium formation. The fact that melanin blocked HIV early (Table 1 and Fig. 3), and interfered with syncytium formation (Fig. 4), suggests that gp120-CD4 interaction may be affected. To investigate this possibility, HTLV-

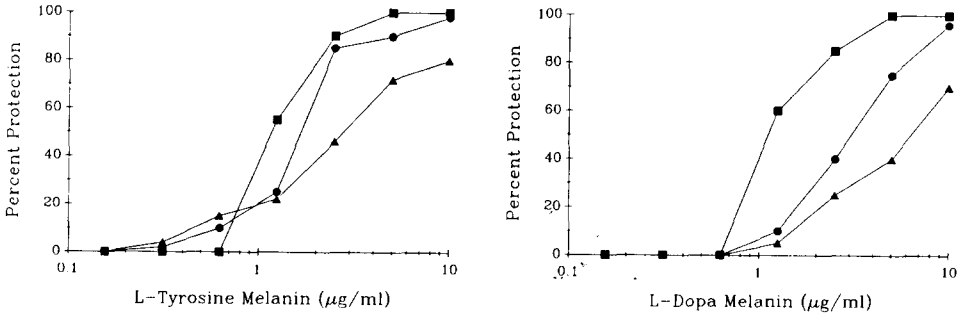

Fig. 4. Inhibition of HIV-induced syncytium formation by melanin. MT-2 cells were mixed with H9/HIV cells in the presence and absence of 7 drug concentrations in 96-well microdilution plates. Viable cells were measured by vital dye uptake 1 day later. For non-cytopathic (MT-2 + H9 cells) and cytopathic (MT-2 + HIV-infected H9 cells) controls, n=8. For melanin, n=3. Standard deviations were less than 15% for each data point. Closed circles, HTLV-III_B; closed squares, HTLV-III_{RF}; closed triangles, HIV-2_{ROD}.

III_B gp160, which also binds CD4, was incubated with MT-2 cells after the cells had been preincubated with increasing concentrations of L-tyrosine melanin. MT-2 cells were used in these experiments because they express CD4 in high abundance (Robinson et al., 1989). Unbound gp160 was then removed by a series of washes after which bound gp160 was detected by flow cytometry. Strong fluorescence intensity was detected in the absence of melanin, while fluorescence intensities decreased with increasing concentrations of melanin (Fig. 5). Complete blockage of gp160 binding was possible when cells were preincubated with melanin at 10 μg/ml.

The ability of soluble melanins to effect HTLV-III_B envelope glycoprotein

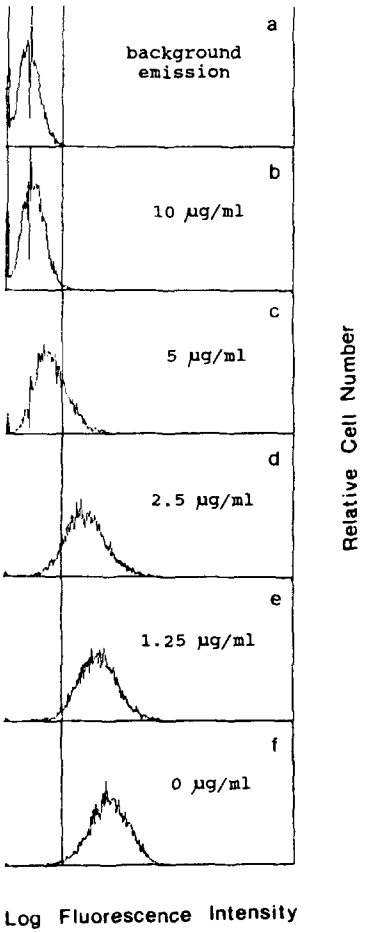

Fig. 5. Concentration-dependent inhibition of binding of gp160 to MT-2 cells by soluble melanin. The ability of various concentrations of L-tyrosine melanin to block gp160 binding to MT-2 cells was examined as described in Materials and Methods. For background emission, no gp160 or melanin were present. The limit of background emission is marked vertically on the x-axis.

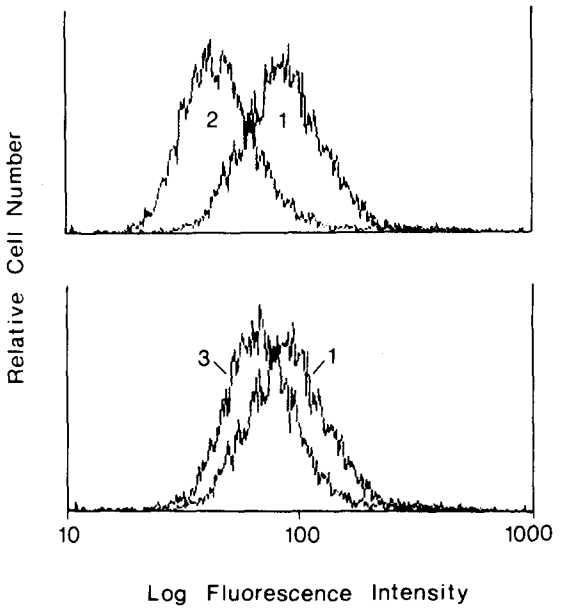

Fig. 6. Inhibition of binding of HIV-1 gp120 to MT-2 cells by soluble melanins. HIV-1 gp120 was added to cells after cells were preincubated: peak #1, PBS; peak #2, L-tyrosine melanin, 10 μg/ml or; peak #3, L-dopa melanin, 10 μg/ml. Bound gp120 was detected by flow cytometry as described in Materials and Methods. For background emission, no gp120 or melanin were present.

binding to MT-2 cells was also examined using gp120. Gp120 was allowed to bind to the surface of MT-2 cells in the presence and absence of L-tyrosine and L-dopa melanins (10 μg/ml). Unbound gp120 was then removed by a series of washes after which bound gp120 was detected by flow cytometry. The fluorescence intensity of MT-2 cells was greatly decreased in the presence of both melanins (Fig. 6, peaks 2 and 3 vs peak 1). Compared to background fluorescence, L-tyrosine and L-dopa melanins reduced relative fluorescence intensities by 95 and 59%, respectively. These results indicate that melanin either blocked gp120 from binding to MT-2 cells or decreased the avidity of binding allowing the gp120 to be washed off. When melanin was added after gp120 was allowed to bind, fluorescence intensity was similar to that seen in the absence of melanin (data not shown). Therefore, the absence of gp120 detection was not simply due to melanin preventing antiserum from binding gp120.

It is possible that soluble melanin coats the surface of MT-2 cells in a non-specific way, which may prevent many ligand interactions with MT-2 cell receptors, in addition to gp120-CD4 ligand-ligand interactions. This possibility was tested using two T-cell specific monoclonal antibodies, leu-3a (CD4) and leu-3b (CD2). Both antibodies bound in abundance to MT-2 cells in the absence of melanin as determined by fluorescence intensities (Fig. 7). Whereas melanin was found to significantly block binding of leu-3a, it had no effect on binding of leu-5b (Fig. 7). Since leu-3a also blocks HIV-1 binding to $CD4^+$ cells in vitro (McDougal et al.,

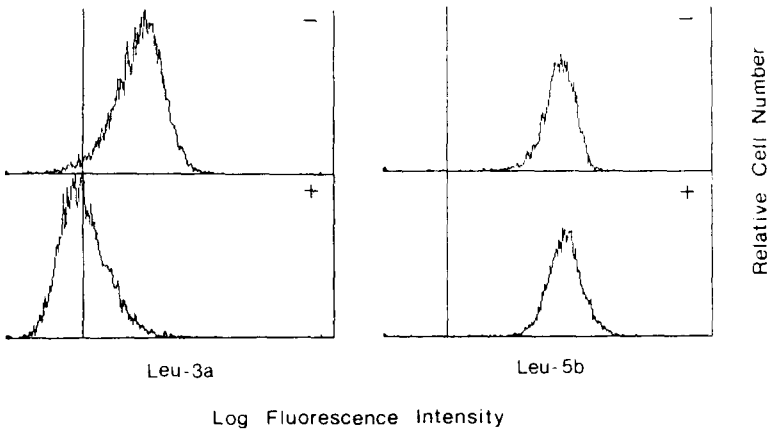

Fig. 7. Effect of soluble melanin on the ability of T-cell specific monoclonal antibodies leu-3a and leu-5b to bind MT-2 cells. MT-2 cells were preincubated with L-tyrosine melanin (10 μg/ml) on ice for 20 min prior to addition of fluorescein-conjugated monoclonal antibodies. Fluorescence intensities of washed cells were measured by flow cytometry as described in Materials and Methods. Background emission was measured on cells stained with fluorescein-conjugated, mouse IgG_1. The limit of background emission is marked vertically on the x-axis.

1986), these results are suggestive of at least some degree of melanin specificity in HIV-1 recognition. In addition, these results further suggest that a general coating of cells is not responsible for the anti-HIV activity of melanin.

A potential mechanism for melanin's interference with gp120 and gp160 binding to $CD4^+$ cells could be that it binds or chemically modifies gp120, gp160, or another ligand on MT-2 cells, thereby diminishing affinity. It is difficult to speculate on the possible molecular basis for this phenomenon since structural analyses of melanins are incomplete (Duff et al., 1988). However, eumelanins, such as those used here, have been shown to contain substituted hydroxyindoles, indolequinones, pyrroles, free carboxylic acid groups, phenolic hydroxyls, carbon-based free radicals, and uncyclized aliphatic chains (Blois, 1978; Duff et al., 1988). Melanin is also known to bind certain chloroquine and phenothiazine antibiotics, which may account for the toxicity of these antibiotics in tissues with high melanin content (Ings, 1984; Larsson and Tjalve, 1979; Lindquist, 1986). Electrostatic forces involving anionic sites on melanin, presumably carboxyl groups, appear to be important to the antibiotic affinity of melanin (Ings, 1984; Larsson and Tjalve, 1979). Similar electrostatic forces could attract melanin to gp120 or a ligand on MT-2 cells, such as CD4. Alternatively, van der Waals forces involving π electrons in aromatic indole portions of melanin and aromatic amino acids in gp120 or a ligand on MT-2 cells could conceivably form intermolecular attractions between melanin and ligand. In contrast to these relatively non-specific attractions, it is also possible that a portion of the melanin heteropolymer may structurally resemble a peptide region found on gp120, CD4, or some other protein required for HIV entry. As a result, melanin could occupy a site which directly competes with contact points for HIV, or which causes conformational changes that affect these processes distally. The fact that

L-dopa melanin did not block binding completely (Fig. 6) may also indicate that another mechanism for antiviral activity is involved.

Effect of melanins on RT activity

Both melanins were examined for an ability to inhibit the viral reverse transcriptase in triton X-100-treated HTLV-III_B lysates. Concentrations as high as 50 μg/ml (which is notably brown) failed to inhibit the enzyme, a result which makes RT inhibition an unlikely mechanism for melanin's antiviral activity. Also, RT inhibition would require that melanin get into the cell. However, when MT-2 and H9 cells were incubated for 4 and 48 h in the presence of L-tyrosine melanin at 10 μg/ml (a concentration which turns the medium light tan in color), no visual evidence of cell pigmentation was observed. Therefore, these cells do not appear capable of transporting and concentrating melanin from the medium.

Concluding remarks

The results presented here demonstrate that melanins have potent and selective anti-HIV activity in vitro. Melanin appears to exert its anti-HIV activity by inhibiting gp120-CD4 binding. However, the results do not rule out the possibility that melanin can effect a post-binding event early in the replication cycle where membrane fusion is inhibited. Since a variety of melanins occur naturally and can be synthesized, it is possible that antiviral activities and other biological properties will be discovered that are specific for different soluble melanins. In preliminary experiments, L-dopa melanin was active against simian immunodeficiency virus (SIV), isolate SIVmac251, in rhesus macaque peripheral blood lymphocytes but not against encephalomyocarditis virus in WISH cells, or vesicular stomatitis virus in HeLa cells (unpublished observations). Therefore, the antiviral action of melanin appears to be restricted to certain viruses. Should melanins have acceptable toxicological and pharmacological properties in humans, they could prove to be a new class of pharmacologically active substances with possible utility as anti-HIV therapeutics.

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