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DISRUPTION OF *PLASMODIUM FALCIPARUM*-INFECTED ERYTHROCYTE CYTOADHERENCE TO HUMAN MELANOMA CELLS WITH INHIBITORS OF GLYCOPROTEIN PROCESSING

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Abstract—Adherence of *Plasmodium falciparum*-infected erythrocytes (IE) to the venular endothelium in brain and other organs is characteristic of cerebral malaria, an often fatal complication in infected individuals. It has been shown that cytoadherence may be mediated through interaction of IE with glycoproteins on host target cell surfaces, including CD36 (GPIV), intercellular adhesion molecule-1 (ICAM-1), and thrombospondin. Inhibitors of glycoprotein synthesis and processing were tested for their abilities to decrease IE adherence to C32 human melanoma cells. The α-glucosidase inhibitor, castanospermine, was effective in disrupting cytoadherence *in vitro* when incubated with C32 cells (IC_{50} = 600–700 μM). Castanospermine-6-butyrate was even more effective than the parent compound (IC_{50} = 9 μM) in disrupting cytoadherence. The mannosidase inhibitors, swainsonine and deoxymannojirimycin, had no effect on cytoadherence at concentrations up to 2 mM. No effect on cytoadherence was observed when the glucosidase and mannosidase inhibitors were incubated with IE rather than the C32 cell cultures. The level of CD36 on the C32 cell surface was decreased as measured by fluorescence-activated cell sorting (FACS) analysis with the same inhibitors which inhibited cytoadherence. Cells labeled with fluorescein isothiocyanate (FITC) OKM5 monoclonal antibody, which recognizes CD36 and disrupts cytoadherence, showed decreased fluorescence when treated with tunicamycin and castanospermine-6-butyrate but not when treated with swainsonine or deoxymannojirimycin. ICAM-1 levels, as measured by surface labeling of C32 cells with FITC CD54 monoclonal antibody, were decreased in cells treated with tunicamycin. However, incubation of cells with castanospermine-6-butyrate or deoxymannojirimycin decreased cell surface ICAM-1 levels only slightly. These findings suggest that (1) in C32 cells, levels of cell surface CD36, and not ICAM-1, change proportionally to the level of cytoadherence; (2) drugs which can affect the carbohydrate moiety of cellular glycoproteins decrease cytoadherence of IE to C32 cells; and (3) protection against the development of cerebral malaria may be possible with inhibitors of glycoprotein biosynthesis.

Sequestration of *Plasmodium falciparum*-infected erythrocytes (IE†) in brain venular endothelium is a contributing factor in the development of cerebral malaria [1], a condition in which patients exhibit neurological dysfunction, including stupor, coma, and death in advanced cases. Trophozoite- and schizont-infected erythrocytes bind to endothelial cells in venules of some of the host's organs, including brain, heart, lung, and kidney. Cytoadherence of IE to host endothelial cells may be advantageous to the parasite in avoiding clearance in the spleen and in providing a growth environment with less oxidative stress (see Ref. 2 for a review).

Cytoadherence involves complex interactions between cell surface components on the host target cell and the IE. Three glycoproteins, CD36 (GPIV) [3–7], intercellular adhesion molecule-1 (ICAM-1) [8], and thrombospondin [4, 9], on target cells have been shown to mediate or assist in the cytoadherence process. In contrast, the IE cell surface protein(s) involved in cytoadherence has not been well characterized, although a family of high molecular weight IE plasma membrane proteins, synthesized only by cytoadherent strains of *P. falciparum* is one of the most likely candidates for mediating cytoadherence [10, 11]. There is also evidence that some *P. falciparum* strains may convert band 3 of the erythrocyte cytoskeleton into a cytoadherence mediating protein [12].

Since glycoproteins are involved in the cell–cell interaction between IE and host target cells, it was logical that cytoadherence could be perturbed by inhibition of glycoprotein synthesis or processing. In fact, we have shown that treatment of C32 cells with tunicamycin, an inhibitor of the first step of glycoprotein synthesis, dramatically decreases cytoadherence of IE [13]. However, inhibitory effects of tunicamycin can be misleading as the compound is cytotoxic and may lead to inhibition of general protein synthesis as a secondary effect upon prolonged treatment [14]. Here we show that cytoadherence of IE to C32 human melanoma cells decreased when the C32 cells were incubated with α-glucosidase but not mannosidase inhibitors, and

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† Abbreviations: FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate; IE, *Plasmodium falciparum*-infected erythrocytes; and ICAM-1, intercellular adhesion molecule-1.

that this decreased cytoadherence was correlated with a reduction in the surface expression of CD36.

MATERIALS AND METHODS

Chemicals. [^{3}H]Hypoxanthine (1–10 Ci/mmol) was purchased from Dupont-New England Nuclear, Boston, MA; OKM5, monoclonal antibody (FITC conjugated), from Ortho Diagnostics, Raritan, NJ; and CD54, monoclonal antibody, clone 84H10 (FITC conjugated), from AMAC Inc., Westbrook, ME. Swainsonine, deoxynojirimycin, and deoxymannojirimycin were obtained from Sigma, St. Louis, MO. Castanospermine and the esters of castanospermine were prepared at the Marion Merrell Dow Research Institute [15].

Cell culture. Human C32 amelanotic melanoma cells (American Type Culture Collection No. CRL 1585, Rockville, MD) were maintained at 37° in a 5% CO_2 atmosphere in RPMI 1640 medium containing 25 mM HEPES, 50 μg/mL hypoxanthine and 10% fetal bovine serum (GIBCO, Grand Island, NY). C32 cells were seeded onto coverslips at about 5000 cells/cm^2 for cytoadherence assays.

P. falciparum *culture. P. falciparum* strain ITG2 (Brazil, provided by Dr. I. J. Udeinya, Walter Reed Army Institute of Research, Washington, DC) was cultured *in vitro* in human erythrocytes (type 0$^+$) at a 6% hematocrit in RPMI 1640 supplemented with 25 mM HEPES, 50 μg/mL hypoxanthine, 0.2% sodium bicarbonate, and 10% human serum (Worldwide Biologicals, Cincinnati, OH) at 37° in a 3% O_2:5% CO_2:92% N_2 atmosphere [16]. Parasitized erythrocytes were selected for cytoadherence to C32 cells every 2–3 weeks as described by Magowan *et al.* [11].

Cytoadherence assay. Binding of [^{3}H]hypoxanthine-labeled infected erythrocytes was measured as described [13]. Briefly, infected erythrocytes (2–6% parasitemia) were labeled in hypoxanthine-free culture medium containing 5 μCi/mL [^{3}H]hypoxanthine for 18–20 hr prior to the binding assay. Unincorporated [^{3}H]hypoxanthine was removed from IE with three washes in growth medium (minus serum), and the resulting washed IE were enriched for trophozoite- and schizont-form parasites with Plasmagel (Cellular Products, Inc., Buffalo, NY) [17]. The enriched cultures were diluted in growth medium (pH 7.0) to approximately 10^6 cpm of [^{3}H]hypoxanthine/mL and adjusted to 1% hematocrit using unlabeled, uninfected erythrocytes. This IE suspension (0.5 mL) was added to coverslips seeded with C32 cells in 12-well cluster plates (Corning, Wexford, PA). Binding was carried out at 37° for 60 min with shaking of the dishes at 15-min intervals to resuspend settled erythrocytes. At the end of the incubation period, unbound erythrocytes were washed from the cultures by dipping the coverslips in culture medium (minus serum). The coverslips were transferred directly to vials, and Ready Protein$^+$ (Beckman, Fullerton, CA) was added. Quadruplicate coverslips were counted for each data point. Alternatively, some coverslips were stained with Giemsa, mounted on slides, and visually scored as described previously [18].

Fluorescence-activated cell sorting. C32 cells were seeded and cultured as for cytoadherence assays, and then incubated with the indicated drugs for 72 hr. The cultures were washed with serum-free medium or phosphate-buffered saline (PBS; 137 mM NaCl, 3 mM KCl, 16 mM Na_2HPO_4, 2 mM KH_2PO_4) three times and then incubated with either FITC conjugated OKM5 (anti-CD36) or CD54 (anti-ICAM-1) monoclonal antibodies, or with an FITC conjugated IgG1 control antibody for 1 hr on ice. The cultures were washed with PBS three times and then removed from the wells by scraping with a rubber policeman. The detached cells were pelleted at 3000 *g* for 2 min at 4°, and resuspended in 0.1% paraformaldehyde. Labeled cells were analyzed on a Coulter Epics C flow cytometer equipped with an argon ion laser, at 488 nm as described previously [19]. A minimum of 10^4 cells was analyzed for each

Table 1. Inhibition of cytoadherence: Relative potency of α-glucosidase and mannosidase inhibitors

Compound*	MDL No.	Specificity†	IC_{50} (μM)
Castanospermine	NA‡	α-Glucosidase I	640
Castanospermine-6-butyrate	28574	α-Glucosidase I	9
Castanospermine-6-benzoate	43305	α-Glucosidase I	150
Deoxynojirimycin	NA	α-Glucosidases I and II	~2000§
Deoxymannojirimycin	NA	Mannosidase I	>2000
Swainsonine	NA	Mannosidase II	>2000

* C32 cells were seeded as described in Materials and Methods, incubated with various levels of the indicated compounds for 72 hr, and assayed for cytoadherence of *Plasmodium falciparum*-infected erythrocytes.

† Specificities and structures for castanospermine, deoxynojirimycin, deoxymannojirimycin, and swainsonine are reviewed in Elbein [14]. The structures of the castanospermine esters, along with their relative inhibition of cellular α-glucosidase activity, have been described [15, 20].

‡ NA, not applicable.

§ Deoxynojirimycin inhibited cytoadherence 40% at 2000 μM.

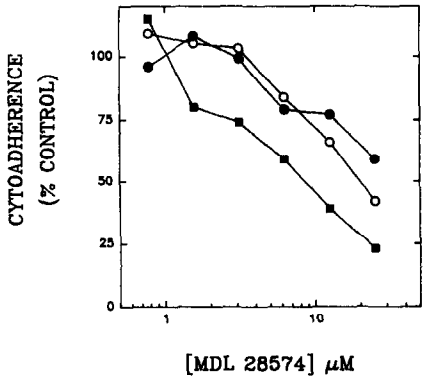

Fig. 1. Time dependence of inhibition of cytoadherence by MDL 28574. C32 cells were incubated with castanospermine-6-butyrate (MDL 28574) at the indicated concentrations for 24 hr (●), 48 hr (○), and 72 hr (■), and cytoadherence was measured by counting Giemsa stained slides as described in Materials and Methods. The control value was 810 IE bound/100 melanoma cells.

sample. Photomicrographs (phase contrast and fluorescence) of C32 cells incubated with OKM5 and CD54 monoclonal antibodies were taken on an Olympus BH2-RFL equipped for reflected light fluorescence, using an EY 455 exciter filter.

RESULTS

Decrease of cytoadherence of IE to C32 cells by α-glucosidase inhibitors. Previously, we have shown that cytoadherence of IE to C32 melanoma cells can be decreased by incubation of C32 cells with the glycoprotein synthesis inhibitor tunicamycin [13]. Incubation of the melanoma cells with the α-glucosidase inhibitor castanospermine also decreased cytoadherence (Table 1). Another α-glucosidase inhibitor, deoxynojirimycin, inhibited binding by 40% when C32 cells were incubated with a 2 mM concentration of the compound. The mannosidase inhibitors swainsonine and deoxymannojirimycin (up to 2 mM) had no effect on cytoadherence. Neither the α-glucosidase nor the mannosidase inhibitors had any effect on cytoadherence when incubated with ITG2 strain *P. falciparum*-infected erythrocytes (IE) rather than the C32 cells (data not shown). Several castanospermine esters, which have been shown to inhibit glucosidase I activity in a whole cell assay [20], were tested for their effectiveness in decreasing cytoadherence when incubated with C32 melanoma cells. One of the most effective compounds tested was the castanospermine-6-butyrate (MDL 28574) derivative which was about seventy-one times more effective than castanospermine. Relatively long-term incubations were required for these compounds to inhibit cytoadherence of IE to C32 cells. Figure 1 shows that a 72-hr incubation of C32 cells with several concentrations of MDL 28574 (the -6-butyrate derivative) was more effective than a 24- or 48-hr incubation with the same concentrations of the drug.

Decreases of OKM5 antigen (CD36) in α-glucosidase-treated C32 cells. To measure the effect glycoprotein synthesis and processing inhibitors had on the cell surface expression of CD36 and ICAM-1, FITC conjugated monoclonal antibodies directed against these proteins were used to label control and drug-treated C32 cells. Figure 2 shows representative photomicrographs (phase contrast and fluorescence) of C32 cells showing the labeling of the cells by OKM5 (anti-CD36) and CD54 (anti-ICAM-1) monoclonal antibodies. The pattern of OKM5 antibody labeling was heterogeneous for control cultures in that most cells were stained. However, the level of fluorescence for each cell was variable (Fig. 2B, some bright, some faint). Cytoadherence to C32 cells was also heterogeneous, in that some cells bound many IE, whereas other cells bound only a few or no IE. The cells were stained more uniformly and more intensely with the ICAM-1 antibody than with the CD36 antibody (Fig. 2D).

The percentage of untreated and drug-treated (tunicamycin, castanospermine, castanospermine-6-butyrate, and deoxymannojirimycin) cells that were tagged with the antibody was determined by fluorescence-activated cell sorting (FACS). A nonspecific control FITC conjugated IgG1 monoclonal antibody gave no significant signal above the intrinsic fluorescence levels for cells not incubated with any monoclonal antibody. About 74% of the untreated C32 cells were tagged with FITC OKM5 (Table 2, Fig. 3). Incubation of C32 cells with tunicamycin or castanospermine-6-butyrate (MDL 28574) decreased the level of staining with OKM5 82 and 68%, respectively. Increasing concentrations of castanospermine-6-butyrate reduced the percentage of C32 cells expressing OKM5 in a concentration-dependent manner. In contrast, deoxymannojirimycin had no effect on OKM5 binding, similar to its lack of inhibition of cytoadherence. Swainsonine, another mannosidase inhibitor, had no effect on the percentage of C32 cells expressing cell surface CD36 (data not shown). Greater than 90% of the untreated C32 cells were labeled with the CD54 antibody (Table 3, Fig. 4). Cells treated with tunicamycin (1 μg/mL) had decreased the levels of the ICAM-1 epitope on the cell surface by about 80%. However, incubation of C32 cells with the glycoprotein processing inhibitors appeared to have very little effect on the level of ICAM-1 antibody staining. Castanospermine-6-butyrate (500 μM) and deoxymannojirimycin (1 mM) each maximally decreased ICAM-1 immunoreactivity by only about 15%.

To assess the effects of the inhibitors on cell growth, incorporation of [^{3}H]thymidine by C32 cells incubated with the drugs was measured. Only tunicamycin, of the drugs used for the experiments described in Tables 2 and 3, markedly affected incorporation of [^{3}H]thymidine into DNA for treated C32 cells (tunicamycin at 1 μg/mL inhibited incorporation 85% after a 72-hr incubation).

DISCUSSION

It has been demonstrated recently that cytoadherence of IE to host target cells is mediated by one or

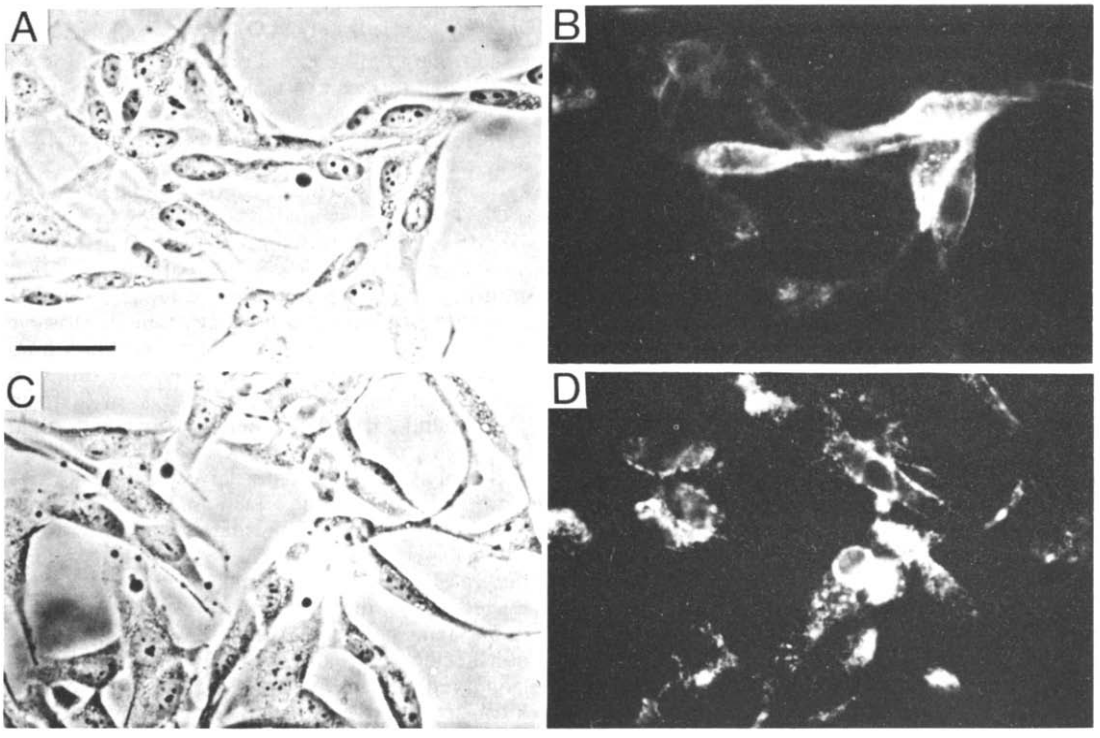

Fig. 2. Immunofluorescence staining of C32 cells with FITC conjugated OKM5 and CD54 monoclonal antibodies. Phase contrast and fluorescence photomicrographs for OKM5 (anti-CD36, A and B) and CD54 (anti-ICAM-1, C and D) are shown. All photographs were taken at the same magnification. Bar, 50 μm.

Table 2. Effects of castanospermine-6-butyrate, deoxymannojirimycin, and tunicamycin on OKM5 antigen (CD36) expression in C32 cells

Treatment	Concentration (μM)	No. cells OKM5$^-$	% Control
None		7420	(100)
Castanospermine-6-butyrate	10	6480	87.3
	50	3820	51.5
	500	2400	32.4
Deoxymannojirimycin	1000	6870	92.6
Tunicamycin	1.1	1300	17.5

C32 cells were treated with the indicated drugs for 72 hr prior to harvest, incubated with FITC conjugated OKM5 monoclonal antibody, and analyzed by flow cytometry as described in Materials and Methods (10^4 cells were counted for each sample).

more glycoproteins on the target cell surface, including CD36, ICAM-1, and thrombospondin [2]. It is possible that some combination of these glycoproteins is responsible for determining the tissue distribution of sequestration of the parasites *in vivo*. Although these glycoproteins have been identified as mediators of cell–cell interactions, little is known regarding the features of these proteins which are critical to their role in cytoadherence. Previously, others have shown that high levels of amino sugars can inhibit cytoadherence of IE to C32 cells [21], suggesting that the carbohydrate portion of the protein is important for cytoadherence. However, the high concentrations of amino sugars (50 mM) required for inhibition make it unclear whether a lectin-like interaction or a general charge interaction is affected. Here we have shown that specific interference with glycoprotein synthesis and/or processing in the target cells with α-glucosidase, but not mannosidase, inhibitors, disrupts cytoadherence of IE.

Although cytoadherence was inhibited, a relatively

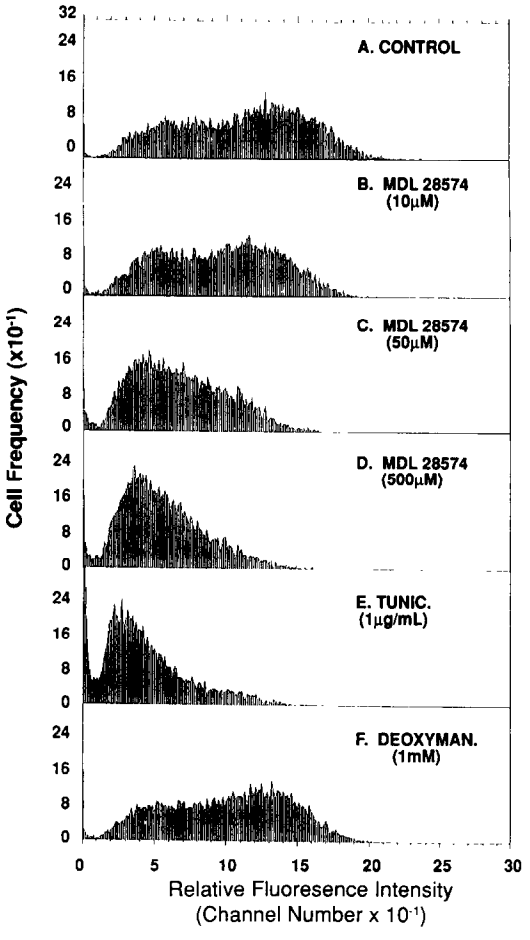

Fig. 3. Effect of glycoprotein processing inhibitors on cell surface expression of CD36 (OKM5 antigen). C32 cells were incubated for 72 hr with drugs indicated in each panel. The cultures were then incubated with monoclonal antibody, OKM5, and processed for flow cytometry as described in Materials and Methods. The abbreviations used are: MDL 28574, castanospermine-6-butyrate; tunic., tunicamycin; and deoxyman., deoxymannojirimycin.

long exposure of the target cells to the drug was required for the inhibition to reach maximum levels. This finding suggested that the glycoprotein receptors turn over slowly and that cell division may be necessary for dilution of the unmodified receptors. Primary cultures of human monocytes have also been shown to bind IE and represent an example of a non-dividing cell in which to examine cytoadherence [5]. Castanospermine and castanospermine analogs were ineffective in decreasing cytoadherence of IE to treated monocytes when the monocytes were incubated for up to a week at the concentrations shown here to be effective with C32 cells (Wright PS, Cross-Doersen DE and Bitonti AJ, unpublished results). This finding indicates that the turnover of the glycoproteins mediating cytoadherence is slow, and that cell division is probably required for the α-glucosidase inhibitors to be effective.

The glucosidase and mannosidase inhibitors had no effect on cytoadherence when incubated with IE rather than the C32 cells. Uninfected and infected erythrocytes took up radiolabeled castanospermine-6-benzoate (MDL 43305) to about the same extent as C32 cells when differences in cell volume were considered (Wright PS, Cross-Doersen DE and Bitonti AJ, unpublished observations). This suggests either that interference with *P. falciparum* glycoprotein processing has no effect on cytoadherence or that the enzymes involved in glycoprotein processing in the parasites are not affected by these compounds. Infected erythrocytes treated with a variety of glycosidases (e.g. neuraminidase, endoglycosidase F, β-galactosidase, α-galactosidase, and endo-β-galcatosidase) [22] are still able to bind to C32 cells. In fact, it has been reported that removal of sialic acid from the IE cell surface with neuraminidase actually enhances cytoadherence to C32 cells [22, 23]. In contrast, incubation of IE with a variety of proteases was quite effective in destroying the cytoadherence molecules on the IE cell surface [22]. These previous findings plus the work described here would seem to indicate that a specific carbohydrate structure on the IE cell surface is not important for cytoadherence, but that the glycoproteins, in particular CD36, on the target cell surface are important for IE sequestration.

OKM5 staining decreased in qualitatively the same manner as did cytoadherence. The presence of the OKM5 antigen correlates with the ability of human melanoma cells to bind IE [24]. Tunicamycin and α-glucosidase inhibitors, but not mannosidase inhibitors, decreased cell surface levels of CD36, the OKM5 antigen. In contrast, ICAM-1 on the cell surface was not affected qualitatively or quantitatively in the same manner as CD36 or cytoadherence. Quantitatively, cytoadherence was more sensitive to the castanospermine esters than was the cell surface level of CD36. The level of castanospermine-6-butyrate required to decrease the immunofluorescence 50% was about five times greater than the IC_{50} for cytoadherence. It may be that the determinants for cytoadherence are perturbed more easily than are the determinants for OKM5 binding to the cell surface. This may be true if two or more glycoproteins interact intracellularly to create a site for binding of IE, as has been shown in other systems for the neural adhesion molecules, L1 and N-CAM [25]. The functional cooperativity of these proteins (*cis* interactions within the same cell membrane) can be decreased in cells treated with α-glucosidase inhibitors, but not mannosidase inhibitors. Perhaps some cooperativity of target cell surface receptor proteins important for IE cytoadherence is affected by interference with glycoprotein synthesis.

Administration of castanospermine can have side effects due to the activity of the drug against the host. For instance, glycogen levels decrease and its subcellular localization is altered in the liver of rats injected with high doses of castanospermine (500–1000 mg/kg body weight) [26]. However, Ruprecht *et al.* [27] found that high castanospermine doses (400 mg/kg) are tolerable in animals when adminsitered orally rather than injected and that these levels of drug are somewhat effective against Raucher murine leukemia virus *in vivo*. We show here that MDL 28574 is about seventy times more

Table 3. Effects of castanospermine-6-butyrate, deoxymannojirimycin, and tunicamycin on CD54 antigen (ICAM-1) expression in C32 cells

Treatment	Concentration (μM)	No. cells CD54$^+$	% Control
None		9620	(100)
Castanospermine-6-butyrate	100	7450	77.4
	500	8100	84.2
Deoxymannojirimycin	1000	8150	84.7
Tunicamycin	1.1	2140	22.2

C32 cells were treated with the indicated drugs for 72 hr prior to harvest, incubated with FITC conjugated CD54 monoclonal antibody, and analyzed by flow cytometry as described in Materials and Methods (10^4 cells were counted for each sample).

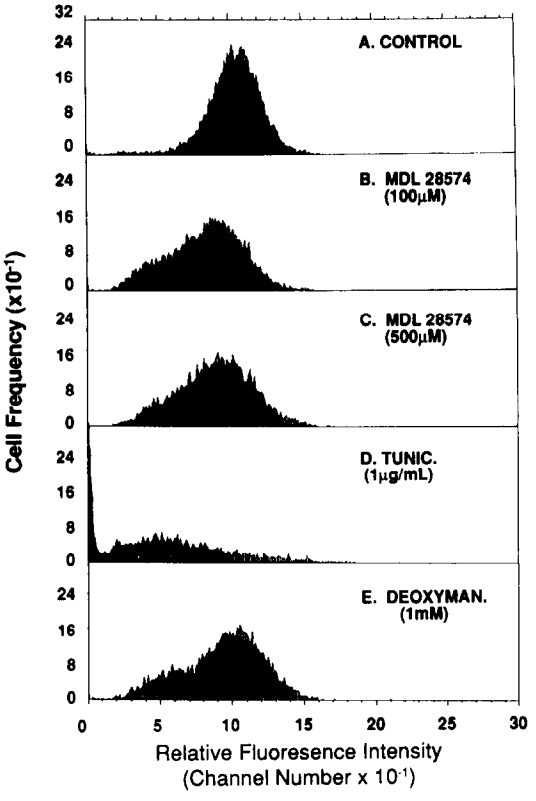

Fig. 4. Effect of glycoprotein processing inhibitors on cell surface expression of ICAM-1 (CD54 antigen). C32 cells were incubated for 72 hr with the drugs indicated in each panel. The cultures were then incubated with monoclonal antibody, CD54, and processed for flow cytometry as described in Materials and Methods. The abbreviations used are: MDL 28574, castanospermine-6-butyrate; tunic., tunicamycin; and deoxyman., deoxymannojirimycin.

effective than castanospermine in inhibiting *P. falciparum*-infected erythrocyte cytoadherence with human melanoma cells. Sunkara *et al.* [20] have shown that this derivative is also about forty times more effective *in vitro* against the human immunodeficiency virus and state that mice are able to tolerate daily doses of 200 mg/kg of the castanospermine derivative for up to 2 weeks with no noticeable side effects. The lower dose of the -6-butyrate analog required for these activities *in vitro* suggests that potentially effective doses would be within the so-called "tolerable" levels of castanospermine and MDL 28574 reported previously [20, 27]. Joubert *et al.* [28] describe the use of a 1-deoxynojirimycin derivative, another inhibitor of α-glucosidase, in humans to treat type II diabetes mellitus with no reported adverse side effects in a short-term study.

The slow response to the compounds may preclude their effectiveness as chemotherapeutic agents, but the more potent compounds may have some prophylatic applications for travelers or for individuals that have moved from a non-malarious region into an area where malarial infections are prevalent. These individuals represent one of the highest risk groups for developing cerebral malaria from *P. falciparum* infections [29], perhaps because they have little or no immunological resistance to infection. It has been shown that antibodies from some individuals who have developed immunity to *P. falciparum* can reverse cytoadherence *in vitro* and *in vivo* [23], and therefore the lack of any such antibodies may increase the potential risk for an individual developing cerebral malaria. Castanospermine analogs or other inhibitors of glycoprotein synthesis might provide a high enough degree of protection for such individuals to avoid the severe complications of cerebral malaria.

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