Retroviral Transfection of Madin-Darby Canine Kidney Cells with Human MDR1 Results in a Major Increase in Globotriasylceramide and 10^5- to 10^6-Fold Increased Cell Sensitivity to Verocytotoxin

ROLE OF P-GLYCOPROTEIN IN GLYCOLIPID SYNTHESIS*

(Received for publication, November 10, 1999)

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Retroviral infection of the Madin-Darby canine kidney (MDCK) renal cell line with human MDR1 cDNA, encoding the P-glycoprotein (P-gp) multidrug resistance efflux pump, induces a major accumulation of the glycosphingolipid (GSL), globotriasylceramide (Galα1-4Galβ1-4glucosylceramide-Gb3), the receptor for the E. coli-derived verotoxin (VT), to effect a -million-fold increase in cell sensitivity to VT. The shorter chain fatty acid isomers of Gb3 (primarily C16 and C18) are elevated and VT is internalized to the endoplasmic reticulum/nuclear envelope as we have reported for other hypersensitive cell lines. P-gp (but not MRP) inhibitors, e.g. ketoconazole or cyclosporin A (CsA) prevented the increased Gb3 and VT sensitivity, concomitant with increased vinblastine sensitivity. Gb3 synthase was not significantly elevated in MDR1-MDCK cells and was not affected by CsA. In MDR1-MDCK cells, synthesis of fluorescent N-[7-(4-nitrobenzo-2-oxa-1,3-diazone)]-aminocaproyl (NBD)-lactosylceramide (LacCer) and NBD-Gb3 via NBD-glucosylceramide (GlcCer) from exogenous NBD-C6-ceramide, was prevented by CsA. We therefore propose that P-gp can mediate GlcCer translocation across the bilayer, from the cytosolic face of the Golgi to the lumen, to provide increased substrate for the lumenal synthesis of LacCer and subsequently Gb3. These results provide a molecular mechanism for the observed increased sensitivity of multidrug-resistant tumors to VT and emphasize the potential of verotoxin as an antineoplastic. Two strains (I and II) of MDCK cells, which differ in their glycolipid profile, have been described. The original MDR1-MDCK parental cell was not specified, but the MDR1-MDCK GSL phenotype and glycolipid synthase activities indicate MDCK-I cells. However, the partial drug resistance of MDCK-I cells precludes their being the parental cell. We speculate that the retroviral transfection per se, or the subsequent selection for drug resistance, selected a subpopulation of MDCK-I cells in the parental MDCK-II cell culture and that drug resistance in MDR1-MDCK cells is thus a result of both MDR1 expression and a second, previously unrecognized, component, likely the high level of GlcCer synthesis in these cells.

Verotoxin (VT) is an Escherichia coli elaborated subunit toxin associated with the (primarily pediatric) microangiopathic hemolytic uremic syndrome (1, 2). VT was shown to be the active component in an E. coli extract, which showed anticeancer activity in several in vitro and in vivo models of neoplasia (3). Ovarian carcinoma cell lines show particular sensitivity to verotoxin in vitro. Multidrug-resistant (MDR) cell lines, expressing the MDR1 P-glycoprotein (P-gp) drug efflux pump, derived from these cells showed approximately 1000-fold increased in sensitivity to verotoxin in vitro (3). The increase in sensitivity was found to correlate with the increased synthesis of Gb3 isoforms containing shorter fatty acids (4). Analysis of the neutral glycolipid content of ovarian tumors showed that Gb3 was elevated for most tumors, but the elevation was markedly greater for metastases and multidrug-resistant tumors (5). Such tumors showed a noticeable increase in Gb3 species that migrated more slowly on TLC, consistent with shorter chain fatty acid isoforms. Similarly, human astrocytoma cell lines, which show high sensitivity to verotoxin in vitro (6) and as nude mice xenografts in vivo (7), showed increased content of short chain fatty acid containing Gb3. The presence of the short chain fatty acid Gb3 species was correlated with altered intracellular trafficking of verotoxin within the sensitive cells, such that toxin was targeted to the endoplasmic reticulum, nuclear envelope, and nucleus, as opposed to the Golgi in cells deficient in such receptor isoforms (4).

In order to determine whether the known (glycolipid translocase activity of P-gp itself (8) might play a role in glycolipid biosynthesis or trafficking within cells, we have compared the verotoxin sensitivity and glycolipid biosynthesis of MDCK cells in vitro before and after transfection with the MDR1 gene. Our studies show MDR1 plays an important role in glycolipid biosynthesis. In addition, our results cast doubt on the parental origin of the MDR1-MDCK transfectants and show that the drug resistance of these cells is due in part to an additional, non-MDR1 mechanism.

MATERIALS AND METHODS

VT1 and VT1 B subunit (9) were purified by affinity chromatography as described (10). VTIB was conjugated with Texas Red (Molecular Probes) as described by the manufacturer.

* This work was supported by Canadian Medical Research Council Grant MT13073. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: VT, verotoxin; GlcCer, glucosylceramide; LacCer, lactosylceramide; NBD, N-[7-(4-nitrobenzo-2-oxa-1,3-diazone)]-aminocaproyl; MRP, multidrug resistance-associated protein; MDCK, Madin-Darby canine kidney; MDR, multidrug resistance; P-gp, P-glycoprotein; FITC, fluorescein isothiocyanate; CsA, cyclosporin A; ER, endoplasmic reticulum; PBS, phosphate-buffered saline; GSL, glycosphingolipid.

2 Glycosphingolipids are abbreviated according to IUPAC-IUB nomenclature (56).
Cell Culture—MDCK cells retrovirally infected with the human MDR1 cDNA were a gift from Dr. M. Gottesman (National Institutes of Health, Bethesda, MD) (11). Wild type MDCK cells (which we have termed MDCK-wt in this study) were from ATCC, MDCK-I cells were kindly provided by Dr. G. van Meer (University of Amsterdam, Amsterdam, the Netherlands). Cells were maintained in α-minimal essential medium supplemented with 10% fetal bovine serum and 40 μg/ml gentamycin (MDR1-MDCK medium also contained 80 ng/ml colchicine).

For P-gp inhibition studies, cells were grown in medium supplemented with 4 μg cyclosporin A (CsA) or 4 μg ketoconazole for 6 days.

Glycolipid Extraction—10^6 cells were scraped from the culture dish, centrifuged, and resuspended with 20 volumes of chloroform/methanol (2:1) as described previously (12). The cell pellet was resuspended in water and extracted with 20 volumes chloroform/methanol (1:1). The extract was dried, redissolved in chloroform/methanol (2:1), and partitioned against water. The lower phase was dried, redissolved in chloroform/methanol (98:2) and applied on a silica column. The column was washed extensively with chloroform, and the glycolipid fraction was eluted with acetone/methanol (9:1). Fatty acid analysis of purified Gb3 was performed by high performance liquid chromatography analysis of the fatty acid methyl esters as previously (4, 13).

VT Overlay—Verotoxin binding glycolipids were detected by VT overlay as described (14) using human renal neutral glycolipid fraction as standard.

Cytotoxicity—Log phase cells were incubated in the presence of increasing concentrations of verotoxin or vinblastine for a period of 3 days. Surviving adherent cells were stained with crystal violet, which was subsequently solubilized in 10% acetic acid for spectrophotometric quantitation. In some assays, cyclosporin A (4 μM) was added 3 days prior to VT or vinblastine and maintained during the cytotoxicity assay, to inhibit P-gp function.

Anti-P-glycoprotein/VT1B Cell Labeling—MDR1-MDCK cells were grown to confluence ± 4 μM CsA. After 30 min in the absence of serum, cells were treated with a liposome preparation containing a fluorescent ceramide analog, N-[7-(4-nitrobenzo-2-oxa-1,3-diazole)-ε-aminocaproyl (16) (CεCerNBD) (Molecular Probes) and 77 mol% dimyristoylphosphatidylcholine (final [lipid] = 30 μM in α-minimal essential medium) and incubated at 4 °C for 90 min to allow diffusion into the cells (17). Subsequently, cells were washed and incubated in serum-free media ± CsA for 4 h at 37 °C to allow cellular metabolism of CεCerNBD (8). Cells were then washed, trypsinized, and counted, and glycolipids were extracted, separated by TLC, and viewed under UV illumination. A separate plate was also visualized by VT1 TLC overlay.

Glycolipid Glycosyltransferase Activity—5 × 10^5 cells were washed, scraped, and 1 ml of PBS, and pelleted. The cell pellet was resuspended in 0.5 ml of ddH_2O, exposed to two freeze-thaw cycles, and sonicated for 30 s. The lysate was spun briefly to pellet membrane debris, and the supernatant (microsomal fraction) was analyzed for protein content by BCA assay (Pierce); the final protein concentration was adjusted to 2.5 mg/ml. In glass tubes, 20 μg of lipid substrate (LacCer, Gb3, or Gb4) and 10 μl of Triton X-100 in CHCl_3 (10 mg/ml) were dried under N_2. To this mixture, 20 μl of 10 mM MgCl_2, 0.2 μCi of UDP-[14C]GlcCer (Gb3, synthase assay) or 0.5 μCi of UDP-[3H]-N-acetyl-galactosamine (Gb3, and Gb4, synthase assays) (NEN Life Science Products), 60 μl of microsomal extract (150 μg of protein), ddH_2O to 100 μl final reaction volume. This mixture was vortexed briefly and incubated at 37 °C for 90 min, with shaking. Subsequently, 0.3 ml of PBS and 2 ml of 2:1 CHCl_3/CH_3OH were added, vortexed, and allowed to partition overnight. The lower phase was collected and run on TLC as described above; the plate was sprayed with enhancing reagent (EN’HANCE, NEN Life Science Products) and exposed to x-ray film in a phosphomaging cassette for 6 days.

Anti-P-glycoprotein/VT1B Cell Labeling—MDR1-MDCK cells were seeded on 12-mm diameter glass coverslips in a 24-well plate. For cell surface Gb3 localization, cells were exposed to 1 μg/ml Texas Red-labeled VT1B (or FTC-VP18) (18) in PBS for 1 h at 4 °C. (For internalization, cells were then incubated at 37 °C for 1 h and fixed). Containing for human P-gp was performed using the MRK-16 monoclonal antibody (5 μg/ml; Kamiya), followed by FITC-conjugated goat anti-mouse secondary antibody, also at 4 °C for 4 h. After washing, coverslips were mounted, and examined using a Polyvar fluorescent microscope under incident UV illumination.

For immunostaining of intracellular P-gp, cells were grown on glass coverslips to ~50% confluence, then washed, fixed in 2% formalin, quenched with 50 mM NH_4Cl, and permeabilized for 15 min with 0.1% Triton X-100, 1% bovine serum albumin in PBS. Antibodies to P-gp (MRK-16) or the 58-kDa Golgi protein (19) (1:50; Sigma), and after washing a secondary Texas Red-conjugated goat anti-mouse antibody were added to the cells in permeabilization buffer. Cells were washed, fixed again, mounted, and examined.

Results

Effect of MDR1 on the Neutral Glycolipid Content and Verotoxin Sensitivity—The glycosphingolipids (GSL) from MDCK-wt cells and MDR1-MDCK cells are distinct (Fig. 1, i). In MDCK-wt cells, Forsmann glycolipid (Gb3) is the major species detected, as reported by others for high passage cultures (20, 21). Low levels of ceramide tetra-, tri-, di-, and monohexosides are detected. No Gb3 was detected in MDCK-wt cells by VT TLC overlay (Fig. 1, ii). In contrast, MDR1-MDCK cells show a major concentration of Gb3, and increased levels of ceramide di- and monohexosides. While glycolipids normally migrate on TLC as doublets as a result of heterogeneity within the lipid moiety, it is primarily the lower species of each GSL that is enhanced in the MDR1-MDCK cells (Fig. 1). No Gb4 or Forsmann antigen are detected in the transfected cells.

Two variants of MDCK cells with differing glycolipid profiles have been described (21). In the original report (11), the subtype of MDR1 cell used for transfection was not specified but, according to the glycolipid profile in Fig. 1, the MDCK-wt cells are MDCK-II, which express Forsmann glycolipid but low levels of globoseries GSL (21). We therefore also compared the glycolipid profile of MDR1-MDCK cells with that of MDCK-I cells (Fig. 1) that do not express Gb3 or Forsmann but do express Gb4 (3). MDR1-I cells showed high GlcCer expression (unlike MDCK-wt cells) and low levels of LacCer and Gb3 (Fig. 1, a). As in MDCK-MDRI cells, the GlcCer and LacCer of MDCK-I cells was primarily the species migrating more slowly on TLC. MDCK-I Gb3 resolved as a doublet and only the slower species was dramatically elevated in the MDR1-MDCK cells (Fig. 1b). The fatty acid profiles of the elevated Gb3, LacCer, and GlcCer in MDR1-MDCK cells (Table I) were similar; the major species were C16 and C18 (GlcCer contained C18:1 in addition) fatty acids, but very long chain species were present.

The increased Gb3 content of the MDR1-transfected cells results in a 10^3- to 10^5-fold increase in sensitivity to VT as compared with either MDCK-I or MDCK-wt cells (Fig. 2a). The P-gp inhibitor, CsA (or ketoconazole; data not shown) caused a marked reduction in the Gb3 (and LacCer and GlcCer) content of MDR1-MDCK cells (Fig. 1) and a reduction in VT cytotoxicity to that of untransfected MDCK cells, coincident with inducing increased sensitivity to vinblastine (Fig. 2b). Despite the expression of endogenous MDR activity (22), the overall glycolipid profile of untransfected MDCK cells was not affected by CsA (data not shown). Although the multidrug resistance-associ-
ated protein (MRP) has also been shown to act as a lipid translocase (23), probenecid, a specific inhibitor of MRP (24) had no effect on Gb3 synthesis (Fig. 1), VT, or vinblastine sensitivity (data not shown).

As expected, MDR1-MDCK cells are resistant to vinblastine and this is abrogated in the presence of CsA (Fig. 2b). However, MDCK-wt cells are significantly more susceptible to vinblastine than MDCK-I cells, and this sensitivity was not increased by CsA (or probenecid; data not shown) (Fig. 2b). The vinblastine sensitivity of CsA-treated MDR1-MDCK cells is increased to the equivalent of that of MDCK-I cells.

**Surface and Subcellular P-gp and Gb3**—Double labeling of MDR1-MDCK cells with Texas red-conjugated VT1B and anti-P-gp at 4 °C (Fig. 3, a and b) showed significant surface VT1B staining. Surface staining by VT1B and anti-P-gp were in part, coincident. Plasma membrane VT staining was lost following cyclosporin A treatment (Fig. 3c) as expected. Less expected was the similar loss of surface P-gp (Fig. 3d). Fluorescent-VT1B incubated with MDR1-MDCK cells and allowed to internalize at 37 °C, accumulated around the nucleus (Fig. 3e) consistent with an ER/nuclear membrane location. This pattern was distinct from that of the Golgi, as labeled with antibodies against the 58-kDa Golgi marker protein (19) (f) or anti-P-gp (g) at room temperature. Several labeled Golgi are arrowed.

**Glycolipid Glycosyltransferase Activity**—Gb3, Gb4 and Forssman synthase activities were assayed in the extracts of MDR1-MDCK, MDCK-I, and MDCK-wt cells. Gb3 synthase was detected in all cell lines (slightly less in MDCK-I cells) (Fig. 5a). Radiolabeled LacCer was seen for MDCK-I cells only, likely due to their high endogenous GlcCer content. The MDR1-MDCK Gb3 synthase assay was unaffected by CsA pretreatment of cells or the direct addition of CsA in the assay, indicating that P-gp does not play any direct role in Gb3 synthesis. The ex-

| Fatty acid methyl ester | Glycolipid | Glycolipid | Glycolipid |
|------------------------|-----------|-----------|-----------|
|                        | Gb3       | LacCer    | GlcCer    |
| 14:0                   | 8.39      | 6.91      | 2.49      |
| 16:0                   | 29.24     | 25.95     | 16.50     |
| 16:1 n-7               | 8.30      | 4.55      | 1.05      |
| 18:0                   | 27.23     | 28.56     | 32.01     |
| 18:1 n-9               | 6.92      | 9.18      | 23.65     |
| 18:2 n-6               | 0.73      | 9.99      | 3.62      |
| 20:0                   | 1.35      | 2.77      | 0.73      |
| 22:0                   | 4.06      | 1.26      | 12.19     |
| 20:4 n-6               | 1.26      | 1.62      | 0.82      |
| 23:0                   | ND*       | ND*       | 4.23      |
| 24:0                   | 6.13      | 5.65      | 1.67      |
| 24:1                   | 6.40      | 3.56      | 1.04      |
| 100.00                 | 100.00    | 100.00    |

*a* ND, not detected.

**FIG. 2.** VT and vinblastine cytotoxicity to MDCK-wt, MDCK-I, and MDR1-MDCK cells. VT cytotoxicity (upper panel) and vinblastine sensitivity (lower panel) were monitored 3 days after addition, with or without CsA to inhibit P-gp.

**FIG. 3.** TRITC-VT1B and anti-P-gp labeling of MDCK-MDR1 transfectants. Cell surface double labeling with Texas Red-VT1B (a and c) and anti-P-gp (MRK16 monoclonal antibody (Ref. 43)) detected using FITC-conjugated antispecies antibodies (b and d), at 4 °C, prior to (a and b) or following (c and d) 72 h of culture in the presence of 4 μM cyclosporin A, e, internalization of FITC-VT1B by MDR1-MDCK cells at 37 °C, f, and g, permeabilized MDR1-MDCK cells labeled with anti-58-kDa Golgi marker protein (19) (f) or anti-P-gp (g) at room temperature. Several labeled Golgi are arrowed.

**TABLE I**

| Fatty acid methyl ester | Glycolipid | Glycolipid | Glycolipid |
|------------------------|-----------|-----------|-----------|
|                        | Gb3       | LacCer    | GlcCer    |
| 14:0                   | 8.39      | 6.91      | 2.49      |
| 16:0                   | 29.24     | 25.95     | 16.50     |
| 16:1 n-7               | 8.30      | 4.55      | 1.05      |
| 18:0                   | 27.23     | 28.56     | 32.01     |
| 18:1 n-9               | 6.92      | 9.18      | 23.65     |
| 18:2 n-6               | 0.73      | 9.99      | 3.62      |
| 20:0                   | 1.35      | 2.77      | 0.73      |
| 22:0                   | 4.06      | 1.26      | 12.19     |
| 20:4 n-6               | 1.26      | 1.62      | 0.82      |
| 23:0                   | ND*       | ND*       | 4.23      |
| 24:0                   | 6.13      | 5.65      | 1.67      |
| 24:1                   | 6.40      | 3.56      | 1.04      |
| 100.00                 | 100.00    | 100.00    |

*a* ND, not detected.
FIG. 4. Anabolism of exogenous NBD-ceramide by MDR1-MDCK cells ± CsA. MDR1-MDCK cells, ± 48 h of pretreatment with 4 μM CsA, were treated with NBD-ceramide for 4 h at 37 °C ± CsA. Glycolipids were extracted and separated by TLC (equivalent of 2 × 10⁶ cells/lane). Panel a, viewed under UV illumination; panel b, VT overlay; panel c, orcinol stain. Normal glycolipid standards as indicated, were run in the last two lanes. The position of the NBD-labeled glycolipids are indicated on the left.

FIG. 5. Glycolipid glycosyltransferase activity of MDCK cell variants. The activity of Gb₃, Gb₄, and Forssman synthases were assayed in the cell extract as described in the experimental procedures using LacCer, Gb₃, and Gb₄ as substrates respectively. The radiolabeled products were separated by TLC and visualized by autoradiography. Panel a, Ga₃ synthase was assayed in the extract from MDCK-wt (lane 1), MDCK-I (lane 2), untreated MDRI-MDCK cells (lane 3), CsA-treated MDRI-MDCK cells (lane 4), and untreated MDRI-MDCK cell extract to which CsA was added (lane 5). The more slowly migrating [¹⁴C]Gal-labeled species detected on the TLC (below Gb₄) were LacCer-dependent but were not characterized further. Panel b, Gb₄, and Forssman synthases were assayed in the extract from MDCK-wt (lanes 1 and 2), MDCK-I (lanes 3 and 4), and MDRI-MDCK cells (lanes 5 and 6).

DISCUSSION

Role of MDR1 in Glycolipid Biosynthesis?—We have proposed that verotoxin may provide a new approach to cancer treatment (5–7) and have observed that drug resistant tumor cells are hypersensitive to VT (3, 4). The present studies indicate that this may be based on altered expression of the verotoxin glycolipid receptor, Gb₃.

The first glycosylation step in GSL biosynthesis occurs on the cytosolic face of the Golgi apparatus (25). The second enzyme, lactosylceramide synthase, the product of which is the precursor for most GSLs, is within the lumen of the Golgi (26, 27). Thus a mechanism for the translocation of the substrate, glucosylceramide, for this enzyme from the cytosolic face to the lumen of the Golgi must exist. We propose that P-gp acts at this point. The subsequent steps in the globo-series biosynthesis occur in a stepwise fashion via Golgi-located luminal transmembrane enzymes (27). Earlier studies had shown, in polarized epithelial cells, that P-gp acts as a lipid translocase of limited specificity (8). P-gp could flip glucosylceramide containing a short fatty acid derivatized with the NBD fluorescent marker from one side of the membrane to the other. Such a “flippase” activity has also been postulated as a mechanism by which P-gp can pump cytotoxic drugs out of resistant cells (28).

Other studies in drug-resistant cancer have indicated increased GlcCer synthesis as a marker of MDR in vitro and in vivo (29, 30). It is proposed (31) that utilization of ceramide in GlcCer biosynthesis prevents ceramide-induced apoptosis. This would explain the partial drug resistance of MDCK-I as opposed to MDCK-wt cells (Fig. 4). Transfection of cells with glucosylceramide synthase can generate a modest drug resistant phenotype (32) and inhibitors of GSL biosynthesis have been shown to reverse the MDR phenotype (29). Stimulation of GSL synthesis by P-gp may provide a further route for depletion of ceramide pools to effect drug resistance.

The glycolipid species that accumulated in MDR1-MDCK cells were primarily the more slowly migrating bands on TLC (corresponding to shorter fatty acid chain containing species (Table I) for GlcCer, LacCer, and Gb₃. We therefore speculate that P-gp may be involved in the translocation of glucosylceramide from the cytosolic leaflet of the Golgi bilayer to the lumen but that this translocase activity may be biased toward short fatty acid containing species. This bias could result from a lower energy barrier to flip a short, as opposed to a long, acyl chain. This increased luminal short chain fatty acid containing GlcCer provides the substrate for LacCer synthesis, which in turn, is converted to Gb₃. Thus, P-gp overexpression results in a preferential accumulation of GSLs in this pathway, which contain shorter fatty acid isomers. The translocation of short chain fatty acid containing GlcCer across the Golgi membrane may disturb the equilibrium of the GlcCer synthetic reaction and thus stimulate the synthesis of this GlcCer isomer. Alternatively, luminal translocation may protect GlcCer from cytosolic degradation. Inhibition of P-gp with cyclosporin A (or ketoconazole; data not shown) results in reversal of the Gb₃, LacCer, and GlcCer accumulation to levels typical of MDCK-wt cells.

The untransfected MDCK-wt cells contained a more slowly migrating species corresponding to Forssman glycolipid, not present in MDR1-MDCK cells. The accumulation of Gb₃, but not Gb₄ or Forssman in MDR1-MDCK cells, together with our finding that treatment of MDR1-MDCK cells with P-gp inhibitors, while preventing the accumulation of globo-series glycolipids, did not induce Forssman, and that we were only able to detect Gb₃ synthase in the “parental” MDCK-wt cells, initially suggested that MDR1 may have a more complex effect on GSL biosynthesis. However, since two variants of MDCK cells (33) with differing GSLs have been described (21), we investigated whether the MDR1-MDCK cells might have, in fact, been derived from the other (Forssman negative) MDCK variant. Our initial results were consistent with this hypothesis: MDCK-I cells contain Gb₃, but no Gb₄, and synthesize, and express low levels of Gb₁, but no Gb₃, or Forssman. In terms of sensitivity to VT, MDCK-wt and MDCK-I cells are equally resistant (Fig. 2) and the effect of transfection with MDR1 is equally dramatic no matter which is the parental cell. However, MDCK-I cells contain high levels of lower band GlcCer, and thus the accumulation of short fatty acid chain LacCer and Gb₃ in MDR1-MDCK cells may be a consequence of the parental phenotype in this case, rather than a property of P-gp as we propose above.

The MDCK variant used in the original transfection was not investigated (11), but the cell line that has been used as the
parent (MDCK-wt) is, from our glycolipid analysis, MDCK-II (21). If this is indeed the parent, it is possible that the retroviral transfection was more efficient in a small subpopulation of MDCK-I cells (Gb_3-positive and therefore perhaps more sensitive to transfection; Ref. 34) present in the MDCK-wt cell line used. Alternatively, the drug resistance used to select transformants was MDCK-I cell selective, perhaps due, for example, to a functional relationship between Gb_3 and MDR1, as suggested by the cell surface colocalization of these species. The synthesis of high levels of GlcCer might also contribute to this resistance, as proposed by Cabot (35). The relative resistance of MDCK-I, as opposed to MDCK-wt cells, to vinblastine (Fig. 4) would appear to preclude MDCK-I cells as being the parental cell, since vinblastine in this concentration range was used in screening in the original selection of MDR1-MDCK after transfection (11) and would not have eliminated untransfected parental cells if they were MDCK-I. This would support our contention that the “MDCK-I phenotype” was selected from the MDCK-wt (MDCK-II) during the original transfection/MDR1 selection. This would have significant implications since the drug-resistant phenotype of the MDRI-MDCK cells would comprise two components: first, MDR1 expression, and second, due to the selection of the “MDCK-I phenotype,” which might compromise the interpretation of data obtained from the comparison of these cell lines (36–38).

MDCK-I appears to be the first cell line that contains Gb_3 but is insensitive to VT; MDCK-I cells have been previously shown to be insensitive to VT (39). Sensitivity was induced when the cells were pretreated with sodium butyrate (39), a procedure known to up-regulate MDR1 (40, 41), which correlated with the induction of intracellular trafficking of the toxin to the ER and nuclear membrane (42), in manner similar to our observations in astrocyoma and ovarian carcinoma cell lines (4).

P-gp is located appropriately to carry out GlcCer translocation for GSL biosynthesis, since P-gp can function in both the Golgi (43–45) and the nuclear envelope (46), in addition to the plasma membrane. P-gp in MDRI-MDCK cells is, for the most part, localized in the Golgi, consistent with the role we propose. A role for P-gp in the translocation of GlcCer to the Golgi lumen is further supported by our metabolic studies with NBD-ceramide. This fluorescent glycolipid precursor was converted to GlcCer, LacCer, and Gb_3 in MDRI-MDCK cells. The fact that the level of NBD-Gb_3 was not significantly higher than that of NBD-LacCer (cf. endogenous Gb_3 and LacCer) supports our conclusion from the Gb_3 synthase assay that MDR1 does not per se increase the activity of this α-galactosyltransferase. In the presence of CsA, abalone only to glucosylceramide was seen, suggesting that further elongation of the carbohydrate by the Golgi-located transferases of the globoseries, requires the MDR1 pump action to translocate the NBD-GlcCer from its cytosolic site of synthesis to the Golgi lumen. Thus, the NBD-GlcCer is, and in this context, behaves as, an analogue of short chain fatty acid GlcCer. It is noteworthy in this context, that MRP, another drug efflux pump, was able to translocate NBD-GlcCer but, unlike MDR1, not a C6 fatty acid glucosylceramide species (23).

As expected, VT1 bound to MDRI-MDCK cells, but not after CsA treatment. Our observation that the surface expression of P-gp was also lost was not expected. CsA is known to up-regulate the overall expression of P-gp (47). The significance of partial colocalization of P-gp and Gb_3 on MDRI-MDCK cells is under investigation.

Our present studies provide a molecular explanation for the increased levels of short chain fatty acid containing Gb_3 species in multidrug-resistant tumors (4, 5, 48). Fatty acid analysis of the Gb_3 species elevated in MDRI-MDCK cells showed a restriction to C16 and C18 saturated fatty acids. These same fatty acid isoforms were elevated in MDR variants of ovarian carcinoma cell lines (4), which were 1000-fold hypersensitive to VT and targeted VT to the ER nucleus as opposed to the Golgi. In MDRI-MDCK cells, VT1B internalization was consistent with ER nucleus, consistent, in turn with the Gb_3 fatty acid content of these cells.

We suggest P-gp acts to supply increased substrate for the Golgi luminal synthesis of GSLs. Thus, the pattern of GSL synthesis prior to P-gp expression, may determine the P-gp-dependent phenotype. In tumor cells lacking the globo-series pathway, another glycolipid series might be amplified. The fact that CsA did not have a general inhibitory effect on the GSLs of MDCK-wt or MDCK-I cells and that GM_3 synthesis was not increased in MDRI-MDCK cells, indicate that P-gp is not the only mechanism for GlcCer translocation to the Golgi lumen. Different translocators may be required (to give different GlcCer (+LacCer) pools) for the other GSL pathways, which branch from LacCer. This complements Lannert’s studies, which indicate GlcCer can translocate to the lumen of different regions of the Golgi for the synthesis of different glycolipid series (27). While the basis of the selective use of the increased LacCer for Gb_3 synthesis is unknown, this may be related to the selective retention of Gb_3 in cells treated with fumonisin B (49). Ketoconazole has a protective effect for other Gb_3-containing cell lines. The CD50 for VT1 is increased 6-fold for Vero cells, 5-fold for HeLa cells, and 11-fold for SF539 astrocytoma cells in the presence of this P-gp inhibitor (data not shown), suggesting that P-gp also plays a role in the Gb_3 synthesis of these cells.

It is of interest to note that the nephron is a primary site for the localization of P-gp in normal cells (50). Gb_3 is a major glycolipid of the human kidney (51) but is not expressed in the adult glomerulus (52). The kidney is also the major site of verotoxin-induced pathology in HUS (53).

Several studies have indicated the importance of lipids in the action of P-gp (54). Whatever its basis, the up-regulation of short fatty acid chain containing Gb_3 species in MDRI-MDCK cells exactly complements the increased efficacy of these species to mediate verotoxin-induced cytopathology in vitro (4) and in vivo (7), and thereby render multidrug-resistant tumor cells hypersensitive to VT. This effect imbues VT with cytotoxic characteristics ideal to complement current chemotherapy of Gb_3-positive tumors. Our finding of up-regulation of these Gb_3 isoforms in multiple drug-resistant cell lines (4) and in drug-resistant human tumors (5) strongly supports the clinical utility of this approach.

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J. Biol. Chem. 2000, 275:6246-6251.
doi: 10.1074/jbc.275.9.6246

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