**Brefeldin A Induced Inhibition of de Novo Globo- and Neolacto-series Glycolipid Core Chain Biosynthesis in Human Cells**

**EVIDENCE FOR AN EFFECT ON β1→4GALACTOSYLTRANSFERASE ACTIVITY**

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De novo synthesis of neolacto-series glycolipids has been studied in human cell lines via metabolic labeling of ceramide with [3H]serine. Intense labeling of ceramide mono- and dihexose glycolipids occurred with increasing time. Most of the label was recovered in neutral glycolipids with about 5% of the total labeling in the ganglioside fraction. Experiments done using cell treatment with 2.5 μg/ml brefeldin A resulted in a stimulation in the total amount of labeling, accumulation of a neutral glycolipid identified as Lac due to inhibited transfer of the neolacto-series core chain terminal β-Gal residue, and a corresponding inhibition of labeling of longer chain neutral glycolipids in all cell lines. Brefeldin A also blocked synthesis of the globo-series precursor, Gb, longer chain sialylated structures such as IV³NeuAcLac, but not de novo Gm3 synthesis. Brefeldin A treatment had no effect on cellular β1→3N-acetylglucosaminyl-, β1→4galactosyl-, or α1→3fucosyltransferase specific activities, nor was it inhibitory in β1→4galactosyltransferase assays in vitro. The results describe brefeldin A-induced blocks in globo- and neolacto-series glycolipid biosynthesis, consistent with differential localization of enzymes in intracellular membranes. In particular, the results suggest that the β1→4galactosyltransferase in these cells is either not redistributed by brefeldin A or is otherwise rendered nonfunctional.

Glycosyltransferases and processing enzymes for N-linked oligosaccharides of glycoproteins have been shown to be distributed in an orderly, sequential array in the ER1 and Golgi membranes (1–4). This has also been demonstrated for enzymes associated with glycolipid biosynthesis. Recent evidence reported for ganglioside biosynthesis in rat liver indicated that many glycosyltransferases are localized in sub-Golgi fractions in the order in which they act (5, 6). Similar results were obtained in density gradient studies of enzymes involved in lacto-series type 1 and 2 glycolipid biosynthesis in Colo 205 and SW403 cells (7). These studies suggest that a considerable degree of regulation of glycosyltransferase function in the cell occurs by virtue of their membrane localization. Thus, cellular regulation of both enzyme activity and the partitioning of common precursors between enzymes that otherwise would compete randomly for these intermediates bears a great impact on the nature of cell surface carbohydrate antigens expressed. One example of this influence is the observation of almost exclusive expression of lacto-series type 1 chain-based antigens in Colo 205 cells. These cells also express high levels of enzyme activities capable of type 2 chain-based antigen expression (7, 8). Alterations in these cellular properties may have significant impact on expression of, for example, tumor-associated antigens.

Brefeldin A (BFA) has been reported to block protein transport from the ER to Golgi and cause a redistribution of the cis/medial/trans-Golgi membranes (9–12), but not the trans-Golgi network (13), to an intermediate compartment which utilizes a microtubule-dependent pathway to the ER. These studies have relied largely on ultrastructural analysis. BFA action has also been extended to biochemical effects such as synthesis of oligosaccharide moieties of ganglio-series glycolipids (14, 15) and N-linked oligosaccharides (16). Brefeldin A was found to inhibit conversion of de novo synthesized Gm3 to Gm5, indicating that Gm5 synthetase localizes to a compartment functionally distinct from the ER which is late in the intracellular trafficking pathway (14, 15). Its effect on N-linked oligosaccharides gave rise to incomplete complex-type chains with a high degree of terminal GlcNAc residues caused by diminished amounts of β-Gal substitutions. This has a further effect on other terminal modifications such as fucosylation, sialylation, or transfer of terminal α-Gal residues (16).

To gain insight into the stepwise synthesis of neolacto-series glycolipids, we have used de novo labeling of ceramide with [3H]serine on a variety of human cell lines which express these antigens. Further, BFA was applied in these studies to localize enzyme reactions of the pathway that are either closely associated or are functionally distinct after BFA.
induced redistribution. The results indicate that most enzymes responsible for core chain synthesis are redistributed to a BFA-induced compartment. Enzymes responsible for the fucosylation reactions, presumably occur in distinct compartments beyond the block. Surprisingly, evidence indicates that β-Gal transfer to Lac₄ comprising the final step in core chain synthesis, is greatly inhibited by BFA. This suggests that part of the β₁→4 galactosyltransferase activity remains functionally distinct from the ER (or from enzymes catalyzing the preceding reactions of neolacto core chain synthesis) as a result of BFA treatment.

**EXPERIMENTAL PROCEDURES**

**Materials**

The human cell lines HL-60, U937, HCT-15, and NCI-H69 were obtained from the American Type Culture Collection. Human lung carcinoma PC9 cells were obtained from M. Adachi (Immunoresearch Laboratories, Takasaki, Japan). Sodium taurodoxocholate, Triton CF-54, UDP-galactose, UDP-N-acetylglucosamine, and breafeldin A were obtained from Sigma. UDP-[14C]galactose (303 mCi/mmol) and GDP-[14C]fucose (270 mCi/mmol) were obtained from American. UDP-[14C]N-acetylglucosamine (40 mCi/mmol) and [3H]serine (20 Ci/mmol) were obtained from American Radiolabeled Chemicals, St. Louis, MO. Endoglycoceramidase was obtained from Genzyme. Lactotriaosylceramide (nLc₃) was prepared by desialylation of bovine erythrocyte glycophorin A (18). Desialylation was performed in 1% acetic acid at 100 °C for 1 h. Lactosylceramide was prepared from nLc₄ by hydrolysis with jack bean endoglycoceramidase. UDP-[14C]N-acetylglucosaminyltransferase activity was determined. In all cases under the conditions used in these experiments, no detectable label was found in oligosaccharide. Rather, only [3H] labeling of ceramide occurred.

**Enzyme Assays**

β₁→4Galactosyltransferase—β₁→4Galactosyltransferase activity was determined in reaction mixtures containing 2.5 μmol of HEPES buffer, pH 7.2, 30 μg of nLc₄, 100 μg of taurodoxocholate, 1 μmol of MnCl₂, 0.5 μmol of GDP-choline, 15 nmol of GDP-[14C]fucose (15,000 cpm/nmol), and 0.5 μmol of galactona lactone, and 0.1 mg of protein in a total volume of 0.1 ml. The reaction was conducted for 1 h at 37 °C and stopped by the addition of 6 ml of EtOH:CH₃OH (4:1). The entire reaction mixture was streaked onto a 4-cm-wide strip of Whatman No. 3MM paper and chromatographed with water overnight. The labeled product remaining at the origin was quantitated in a liquid scintillation counter. One unit of activity is defined as transfer of 1 nmol of palatose per h under the conditions of the assay.

α₁→3/4Fucosyltransferase—The fucosyltransferase activity was determined in reaction mixtures containing 2.5 μmol of HEPES buffer, pH 7.2, 4, 30 μg of nLc₄, 100 μg of taurodoxocholate, 1 μmol of MnCl₂, 0.5 μmol of GDP-choline, 15 nmol of GDP-[14C]fucose (15,000 cpm/nmol), and 0.5 μmol of galactona lactone. The reaction mixture was incubated for 2 h at 37 °C, terminated, and quantitated as described above. One unit of activity is defined as the transfer of 1 nmol of fucose per h under the conditions of the assay.

**β₁→3N-Acetylglucosaminyltransferase—**Assays of N-acetylglucosaminyltransferase activity were performed in reaction mixtures containing 2.5 μmol of HEPES buffer, pH 7.2, 40 μg of lactotriaosylceramide, 150 μg of Triton CF-54, 0.5 μmol of MnCl₂, 0.5 μmol of GDP-choline, 50 nmol of UDP-[3H]N-acetylglucosamine (5000 cpm/nmol), and 200-400 μg of protein in a total volume of 0.05 ml. The reaction mixture was incubated for 2 h at 37 °C, terminated, and quantitated as described above. One unit of activity is defined as transfer of 1 pmol of GlcNAc per h under the conditions of the assay.

**Protein Determination**

Protein concentrations of cell fractions were determined by the method of Lowry et al. (22) using bovine serum albumin as standard.

**Fractionation of Cells**

Cells, 1 to 2 ml packed volume, with and without BFA treatment and after metabolic labeling with [3H]serine as above were fractionated on a glycolipid and glycosyltransferase analysis of Golgi membranes. All steps were conducted at 0-4 °C. An aliquot of each 1-tc 2-ml cell pellet was reserved for analysis of whole cells. The cells were suspended in 40 ml of a relaxation buffer composed of 10 mM HEPES buffer, pH 7.2, 10 mM KCl, 3 mM NaCl, 1 mM ATP, 3.5 mM MgCl₂, and disopropylamine for 20 min in a nitrogen atmosphere. The disrupted cells were centrifuged at 3000 × g for 10 min, and the resulting supernatant fraction was centrifuged at 27,000 × g for 30 min. The resulting pellet was suspended in 25 ml of buffer composed of 50 mM HEPES buffer, pH 7.2, 1 mM EDTA, 0.5 mM sucrose by one stroke of a Potter-Elvehjem homogenizer and layered onto a 15-ml cushion composed of 50 mM HEPES buffer, pH 7.2, 1 mM EDTA, 1.2 mM sucrose. This was then centrifuged at 94,000 × g for 90 min. The Golgi membrane-enriched fraction was isolated at the interface of the 0.6 and 1.2 M sucrose layers. This fraction was diluted with 5 volumes of water and isolated by centrifugation at 27,000 × g for 30 min. Aliquots of each fraction were used for assay of enzyme activity and glycolipid extraction and analysis.

**Extraction of Glycolipids from Cells and Cell Subfractions**

Glycolipids were isolated from [3H]serine-labeled packed cells or cell subfractions by extraction with 10 ml of isopropl alcohol:hexane:water (55:25:20) at −20 °C.

In some experiments, labeling was also conducted in the presence of medium containing 2.5 μg/ml brefeldin A (BFA). In these experiments, the cells (2 to 4 ml packed cell volume) were incubated with the serine-free, BFA-containing medium for 3 h prior to the addition of the [3H]serine label. After labeling, the cells were harvested as before. In order to verify the distribution of the [3H] label in the glycolipid fraction, total glycolipids were hydrolyzed with endoglycoceramidase, and the recovery of [3H] in the oligosaccharide and ceramide portions was determined. In all cases under the conditions used in these experiments, no detectable label was found in oligosaccharide. Rather, only [3H] labeling of ceramide occurred.
pyridine and 1 ml of acetic anhydride. The acetylated glycolipid fraction was obtained by chromatography on a Florisil column (23) and deacetylated with NaOMe. The deacetylated glycolipid fraction, now depleted of labeled phosphatidylserine, was dissolved in a solvent containing 0.02% CaCl₂·2H₂O. After development, the plate was dried and soaked for 2 h in 5% bovine serum albumin in PBS to block nonspecific antibody binding. The plate was then incubated in culture supernatants of the derived monoclonal antibodies overnight, followed by sequential incubations with 1:1000 diluted rabbit anti-mouse Ig antibody solution and with ¹²⁵I-Protein A solution. After extensive washes with PBS between each step and after ¹²⁵I-Protein A treatment, the plate was dried, and labeled bands were detected by autoradiography.

**TLC Immunostaining of Glycolipids**

Immunostaining of glycolipids, separated on HPTLC plates, was performed using the procedure of Mann and Ledeen (24) to separate neutral glycolipids from gangliosides. The resultant total neutral glycolipid and ganglioside fractions after extensive dialysis with water were dissolved in CHCCL₃·CH₃OH (2:1). An amount of glycolipid corresponding to 2 mg of dried cell residue was spotted for TLC or used for solid-phase radioimmunoassays as indicated.

**Solid Phase Binding Assays**

Glycolipids were deposited on 96-well vinyl plates in solutions containing 3 µg of cholesterol, 5 µg of phosphatidylcholine, and total glycolipid corresponding to a known amount of dried cell residue per ml of absolute ethanol. The glycolipids were serially diluted in ethanol containing cholesterol and phosphatidylcholine alone. Aliquots of 50 µl each were dispensed into each well and allowed to air-dry. The plates were blocked with PBS containing 5% bovine serum albumin and soaked for 2 h in 5% bovine serum albumin in PBS to block nonspecific antibody binding. The plate was then incubated in culture supernatants of the derived monoclonal antibodies overnight, followed by sequential incubations with 1:1000 diluted rabbit anti-mouse Ig antibody solution and with ¹²⁵I-Protein A solution. After extensive washes with PBS between each step and after ¹²⁵I-Protein A treatment, the plate was dried, and labeled bands were detected by autoradiography.

**FIG. 1. Thin layer chromatographic analysis of time-dependent [³H]serine labeling of neutral glycolipids extracted from cultured cells.** Total neutral glycolipids were labeled and extracted as described under "Experimental Procedures." Lane a, total neutral glycolipids revealed by orcinol spray; lanes b–d, fluorography of [³H]serine-labeled glycolipids after the indicated labeling period. Lane 1, total neutral glycolipids as described in Fig. 1A; lane 2, 1 h; lane 3, 4 h; lane 4, 8 h; lane 5, 12 h; lane 6, 24 h; lane 7, 12 h followed by a 12-h chase with unlabeled medium; lane 8, 12 h followed by a 36-h chase. The results are shown for the following cell lines. Panel A, NCI-H69; panel B, PC9; panel C, HCT-15; panel D, HL-60; panel E, U937. An amount of glycolipid corresponding to 0.2 µg of dried cell residue was spotted for TLC in each case. The HPTLC plates were developed in a solvent composed of CHCCL₃·CH₃OH·H₂O, 60:40:9, containing 0.2% CaCl₂·2H₂O. Fluorography of the [³H]labeled glycolipids was conducted after spraying the plate with ENHANCE (Du Pont-New England Nuclear). The TLC mobility of the following glycolipid standards is indicated by GlcCer (a), lactosylceramide (b), and nLac (c).

**RESULTS**

Glycolipid biosynthesis proceeds via a stepwise elongation of oligosaccharide chains linked to ceramide. The nature of the products produced is a function of the glycosyltransferases expressed in a given cell which are distributed in an orderly fashion in the ER and Golgi membranes. In order to study de novo synthesis of neolacto-series glycolipid chains in expressing cells, metabolic labeling with [³H]serine was conducted taking advantage of the [³H]serine-specific labeling of the ceramide moiety.

**Time Course of [³H]Serine Metabolic Labeling of Cultured Cells—Uptake of [³H]serine into neutral glycolipids and gangliosides was studied using labeling times varying from 2 to 12 h, and the fate of the label was followed up to 36 h later as described under "Experimental Procedures." Fig. 1 shows the time-dependent [³H]serine labeling profile of neutral glycolipids isolated from a variety of transformed human cell lines, each of which shares the common property of expressing neolacto-series glycolipids. Intense labeling of glucosyl and galactosyl derivatives of ceramide is observed with each cell line, even at relatively short labeling times. In general, a progressive and stepwise labeling of slower migrating, longer chain glycolipids occurs for all cell lines tested as the [³H]serine labeling time is increased. After 12 h, the [³H] label was removed, and the fate of the labeled glycolipids was followed for an additional 36 h. In most cases, the extent of labeling of the longer chain glycolipids increased further during this time, suggesting a relatively slow turnover of the oligosaccharide chains. This was not the case for HCT-15 cells, where labeling of longer chain structures was more rapidly depleted after removal of the [³H]serine.

In contrast to the relatively efficient labeling of neutral glycolipids by [³H]serine, only weak labeling of the ganglioside fraction was observed. The extent of ganglioside labeling was approximately 6% of that found for neutral glycolipids for all
cell lines tested. Most of this label was present in GalCer, although labeling of bands co-migrating with IV3NeuAcnLc4 was observed, particularly for NCI-H69 cells (see below and Fig. 5). Despite the weak labeling by \[^3H\]serine, each cell line was found to express a significant chemical quantity of ganglioside structures, suggesting a slow turnover in the cell of these components.

**Effect of Brefeldin A on Glycolipid Synthesis**—The disruption of normal Golgi function induced by BFA provides a means to evaluate the stepwise biosynthesis of neolacto-series antigens in Golgi membranes. Fig. 2 shows the effect of BFA on the \[^3H\]serine labeling profiles of neutral glycolipids using labeling times established above to yield significant labeling of slower migrating glycolipids (generally 12 or 24 h, see Fig. 1). As previously observed, de novo synthesis of \[^3H\]labeled glycolipids corresponding to total cellular glycolipids, as revealed by orcinol staining, occurred in the absence of BFA for each cell line. Parallel cells treated with 2.5 \(\mu\)g/ml BFA, as described under “Experimental Procedures,” yielded remarkably different results. For each cell line, BFA-treated cells were characterized by relatively weak or absent \[^3H\] labeling of slower migrating glycolipids, as compared to untreated cells, along with the appearance of a strongly \[^3H\]-labeled band migrating as a ceramide-trisaccharide. This band was uniformly accumulated in all cell lines. Despite the de novo expression of this glycolipid as a consequence of short term BFA treatment (i.e., during the \[^3H\]serine labeling period prior to cell harvest), a significant chemical quantity was accumulated, as indicated by orcinol spray (see, in particular, results for NCI-H69 and HCT-15 cells). TLC immunostaining of neutral glycolipids from untreated and BFA-treated cells indicated strong staining of the BFA-induced ceramide-trisaccharide band with the anti-Lc3 TE5 antibody. These results also revealed that the Lc3 component in most cell lines was undetectable in glycolipids from untreated cells. In two cases where detectable amounts occurred (PC9 and HL-60 cells), the staining intensity was greatly stimulated after BFA treatment. Further TLC immunostaining analysis with antibodies specific for longer chain derivatives of neolacto-series core structures indicated that many of the bands labeled with \[^3H\]serine in the absence of BFA were composed of \(a1+2\)- and/or \(a1+3\)-fucosylated structures (IV3FucnLc4, IIIIFucnLc4, or IIIIVFucnLc4). Although these species were also present in whole glycolipid extracts from BFA-treated cells, their lack of \[^3H\]serine labeling indicated these structures do not represent de novo-synthesized glycolipids in the presence of BFA. In fact, the BFA biosynthetic block after synthesis of Lc3 was highly effective in all cell lines tested. Only in instances where the highest extent of \[^3H\] labeling of Lc3 occurred was there further elongation, presumably to yield nLc5, and nLc5, based upon TLC immunostaining results. A quantitative comparison of Lc3 expression in untreated and BFA-treated cells by solid phase radioimmunoassay is shown in Fig. 3. These data show the effect of glycolipid titration by
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**Figure 3. Solid phase radioimmunoassays of total neutral glycolipids derived from cells with or without 2.5 µg/ml BFA treatment for 12 h.** Glycolipids derived from 2 mg of dried extracted cell residue per ml of solution were serially diluted and assayed as described under "Experimental Procedures." Panel A, untreated cell glycolipids with TE5 antibody; panel B, BFA-treated cell glycolipids with TE5; panel C, untreated cell glycolipids with 1B2 antibody; panel D, BFA-treated cell glycolipids with 1B2. Data are given for the following cell lines, –––, NCI-H69 cells; +++, PC9 cells; +++, HCT-16 cells; +++, HL-60 cells; +++, U937 cells.

Serial dilution on binding of excess specific antibodies. Depending on the cell line, an increase of from 4- to 20-fold higher Lc3 expression occurred based upon titration curves with antibody TE5 using glycolipids derived from ±BFA-treated cells. As an internal control, little difference was observed between the treatments with 1B2 reactivity. These results are reflective of intense biosynthesis of glycolipids in the presence of BFA, leading to high accumulations of Lc3 with only weak further modification.

BFA-induced inhibition of trafficking and secretion out of the Golgi has been documented (9-13), including effects on sphingolipid distribution (14, 15). In order to confirm that the localization of neolacto-series glycolipid precursor structures after BFA treatment behaved similarly, the composition of de novo-synthesized glycolipids from isolated Golgi fractions was compared with that of whole cells after [3H]serine labeling in the presence or absence of BFA. Solid phase immunostains were shown in Fig. 4 indicate the presence of neolacto-series glycolipids from whole cells and Golgi fractions in two representative cell lines, PC9 and HCT-15. Based upon this glycolipid titration curves, the characteristic increase in TE5 reactivity (anti-Lc3) in the presence of BFA is observed both in total cellular glycolipids and in Golgi-derived glycolipids. Control assays also indicated significant expression of neolacto-series core chain and Leα antigen in all fractions. Thus, the results obtained with Golgi-derived glycolipid fractions correspond in each case to the results from whole cells. These data are consistent with the presence and enrichment of glycolipids (both short and longer chain) in Golgi-derived fractions regardless of BFA treatment. In addition, β1→3N-acetylgalcosaminyltransferase and β1→4galactosyltransferase activities were highly expressed in both crude cell homogenates and Golgi-enriched fractions from each cell line regardless of BFA exposure (results not shown). Further, fluorographs of [3H]serine-labeled, de novo-synthesized glycolipids after TLC separation indicated BFA-induced accumulations of Lc3 in both whole cells and Golgi fractions.

Moreover, very similar labeling profiles for total cellular or Golgi-derived glycolipids for both BFA-treated and untreated cell fractions were obtained (results not shown). These results indicate that neolacto-series glycolipid precursors behave similarly to other Golgi membrane components upon BFA treatment, leading presumably to accumulations of biosynthetic intermediates in redistributed Golgi membrane compartments.

**TLC immunoassaying results for total neutral glycolipids, using the anti-Gb1, 14E10 antibody, indicated intense expression of this antigen in both PC9 and U937 cells. [3H]Serine labeling of this glycolipid band was also halted in the presence of BFA in both cell lines. This indicates that BFA-induced redistribution of Golgi membranes also segregated the α1→3galactosyltransferase for biosynthesis of Gb1, such that it did not have access to the de novo-synthesized lactosylceramide precursor. Thus, enzymes responsible for globo-series glycolipids would presumably be localized in compartments not redistributed by BFA.

As indicated above, weak [3H]serine labeling of gangliosides was observed for each cell line tested. As shown in Fig. 5, stronger labeling was observed in ganglioside fractions from cells labeled in the presence of BFA. In addition, labeling of longer chain gangliosides was virtually abolished by BFA treatment. These results indicate that after BFA-induced redistribution, the α2→3sialyltransferase responsible for GM3 synthesis continued to have access to the de novo-synthesized lactosylceramide. These results are consistent with previous localization of the GM3 synthetase to the cis-Golgi (5) and the BFA-induced block in synthesis of longer chain α2→3sialyltransferase acceptors described above.

A summary of the distribution of [3H]serine label in glycolipids isolated from untreated and BFA-treated cells is shown in Table I.

**Effect of BFA Treatment on Glycosyltransferase Activities of the Neolacto-series Pathway**—The above results indicate a marked effect of BFA on neolacto-series glycolipid synthesis. To exclude the possibility of this being due to changes in levels of glycosyltransferase activities, the specific activities of β1→4galactosyltransferase, β1→3N-acetylgalcosaminyltransferase, and α1→3fucosyltransferase were determined for all cell lines under both treatment conditions. These results are shown in Table II. Although the enzyme levels varied between cell lines, there was no difference in the specific activity of any enzyme tested as a consequence of BFA treatment. Similarly, no evidence was obtained for differences in hydrolytic activities as a consequence of BFA treatment.

The [3H]galactose from *in vitro* UDP-[3H]galactose labeling of endogenous glycolipids of isolated Golgi membranes was very stable under both conditions. Similar results were observed after either N-acetylgalcosamine or fucose transfer (results not shown).

In order to account for BFA-induced accumulation of Lc3, the effect of BFA as a direct inhibitor of β1→4galactosyltransferase activity was tested using a crude cell homogenate of HCT-15 cells. These results are shown in Table III. The effect of BFA on *in vitro* enzyme activity was tested using BFA concentrations 20- and 40-fold higher that used to induce its physiological effect on the cells in culture. Further, conditions for enzyme activation were varied to include detergent, phospholipid, or no additions in case one or more of these components interfered with an inhibitory property. The results indicate that BFA had no effect on *in vitro* β1→4galactosyltransferase activity in any of the assay conditions tested.
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FIG. 4. Solid phase radioimmunoassays of total neutral glycolipids derived from whole cells or isolated Golgi membranes after treatment with or without 2.5 μg/ml BFA for 12 h. Glycolipids derived from 380 μg of dried extracted whole cell residue or 88 μg of dried extracted Golgi membrane residue per ml of solution were serially diluted and assayed as described under “Experimental Procedures.” Panels A, B, and C show results from whole cell glycolipids with antibodies TE5, 1B2, and AH6, respectively. Panels D, E, and F show results from isolated Golgi membranes with antibodies TE5, 1B2, and AH6, respectively. Data are given for the following cell lines and conditions, +--++, PC9 cells without BFA; *--*, PC9 cells with BFA; 0--0, HCT-15 cells without BFA; x--x, HCT-15 cells with BFA.

Brefeldin A, a macrocyclic lactone isolated from fungi, has been found to promote the disassembly of the Golgi and cause it to mix with the ER. The bulk of the evidence indicates this process involves redistributing the cis medial/trans-Golgi cisternae, but not the trans-Golgi network (9-13). BFA also inhibits membrane trafficking pathways (12) and association of Golgi binding proteins such as β coat protein (26) or ADP-ribosylation factors (27) to the Golgi. At present, there is no evidence to suggest that BFA is directly inhibitory to sugar nucleotide transport or of glycosyltransferase reactions. Because of this, BFA is a potent tool and has been used to analyze the sequential distribution of glycosyltransferase enzymes responsible for both glycoprotein (16) and glycolipid (14, 15, and this paper) oligosaccharide synthesis.

Biosynthesis of the oligosaccharide chains of glycolipids occurs via stepwise addition of sugar residues giving rise to, in general, ganglio-, globo-, or lacto-series type 1 and 2 chain-based structures. Each of these glycolipid classes shares lctosylceramide (Galβ1→4Glcβ1→1Cer) as a common biosynthetic precursor. The nature of the glycolipid series will be defined by the next sugar residue added. Transfer of α2→3 sialic acid or β1→4GalNAc is associated with ganglio-series synthesis, α1→4Gal for globo-series, and β1→3GlcNAc for lacto-series structures. These glycolipids are distributed in various cell types, and a given cell or cell type may express glycolipid structures of multiple series. Consequently, regu-
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The rate-limiting step in this pathway is formation of LC₃ (29), a product which is very efficiently converted to nLC₃ by β₁→4galactosyltransferase. This observation is fully expected given that β₁→4galactosyltransferase specific activity in cells may be 20- to over 100-fold higher than that of the β₁→3N-acetylglucosaminyltransferase. As a result, little of this intermediate is ordinarily present in total neutral glycolipid extracts. When it is found, evidence suggests that much of it is a hydrolytic product from turnover of longer chain structures.²

In the presence of BFA, accumulations of de novo-synthesized LC₃ occurred and resulted in diminished amounts of longer chain glycolipids for all cell lines tested. This a surprising finding because it occurred in spite of the magnitude of the excess β₁→4galactosyltransferase activity over that of the β₁→3N-acetylglucosaminyltransferase.

These observations, taken together, begin to provide a more detailed view of the organization of glycosyltransferases in the Golgi and other subcellular compartments. BFA-induced redistribution of Golgi membranes presumably yielded segregated compartments which retain the capacity for glucosyl transfer to ceramide, β₁→4galactosyl transfer to yield lactosylceramide, G₃₃₃ synthetase, and β₁→3N-acetylglucosaminyltransferase. Consequently, the major products under this condition were determined to be G₃₃₃, LC₃, and immediate precursors. Surprisingly, despite the huge excess of β₁→4galactosyltransferase over β₁→3N-acetylglucosaminyltransferase in these cells, only a small proportion of it may be active in this compartment. The slow conversion of labeled LC₃ to nLC₃, and subsequently to nLc₄ is further indication that this β₁→4galactosyltransferase enzyme pool must be very small (at least in terms of activity). These results are consistent with a previous report demonstrating increased terminal GlcNAc residues (and diminished amounts of β-Gal residues) in N-linked oligosaccharides from bovine endothelial cells after BFA treatment (16). These observations, coupled with the normally efficient conversion of LC₃ to longer derivatives, suggests the bulk of the β₁→4galactosyltransferase activity is functionally isolated from de novo-synthesized precursors in separate compartments or is otherwise rendered nonfunctional by indirect effects from the BFA-induced disruption of the normal physiological environment. The latter possibility would presumably be peculiar to β₁→4galactosyltransferase since BFA did not block LC₃ synthesis. Strous et al. (30) reported that a small amount of the total β₁→4galactosyltransferase was present and functional in the rough ER and cis-Golgi of HeLa cells. Such a distribution could account for the slow elongation of LC₃ observed.

² A. L. Sherwood and E. H. Holmes, unpublished results.

![Thin layer chromatographic analysis of [³H]serine-labeled gangliosides from cells labeled in the absence or presence of 2.5 μg/ml BFA. Odd-numbered lanes show data from cells labeled in the presence of 2.5 μg/ml BFA. Lanes 1 and 2, NCI-H69 cells; lanes 3 and 4, PC9 cells; lanes 5 and 6, HCT-15 cells; lanes 7 and 8, HL-60 cells; lanes 9 and 10, U937 cells. An amount of ganglioside corresponding to 2 mg of dried extracted cell residue was spotted for TLC in each case. The HPTLC plates were developed in a solvent composed of CHCl₃:CH₃OH:CH₃CN:H₂O:60:40:9, containing 0.2% CaCl₂·2H₂O. Fluorography was conducted after spraying the plate with EN²HANCE. The TLC mobility of ganglioside standards GM₃ and SPG (IV²NeuAcLc₄) is shown.](image)

| Table 1 | Distribution of [³H]serine label in neutral glycolipids from human cell lines with or without brefeldin A |
|-----------------|-----------------------------------------------|
| Glycolipid       | NCI-H69 -BFA +BFA | PC9 -BFA +BFA | HCT-15 -BFA +BFA | HL-60 -BFA +BFA | U937 -BFA +BFA |
| Glucosyl- or galactosylceramide | 76.4 87.2 | 75.8 77.0 | 68.4 79.1 | 29.8 52.8 | 66.4 84.0 |
| Lactosylceramide | 14.8 6.2 | 6.4 10.5 | 20.7 6.6 | 55.2 22.9 | 15.7 10.0 |
| LC₃             | 4.2 9.3 | 9.4 13.0 | 11.6 4.7 | 17.6 3.7 | 3.7 1.5 |
| nLc₄            | 5.0 1.6 | 6.4 2.4 | 7.6 1.8 | 4.3 2.7 | 3.3 0.9 |
| Longer chain and fucosylated glycolipids | 3.8 0.8 | 4.0 0.7 | 2.1 0.9 | 6.0 4.0 | 1.9 0.9 |

| Total mg dried cell residue | 22.7 21.0 | 29.8 27.1 | 166.0 152.0 | 56.0 57.1 | 80.9 58.0 |

*cpm/mg dried residue present*
Golgios and cell surface forms of the enzyme and these forms have differing intracellular fates, establishing a clearest explanation, in keeping with recognized properties of BFA, is that the bulk of the fll+4galactosyltransferase activity is not redistributed to the ER by BFA. Multiple forms of BFA after BFA-induced redistribution of the Golgi (Brefeldin A compartment) are due to inhibition of enzyme activity or sugar nucleotide amounts in the pre-vivo assay conditions. Further, accumulation of significant amounts of Lc3 would require adequate UDPGal in the pre-vivo conditions. Values shown were from duplicate experiments.

| Cell line   | Acetylglucosaminyltransferase | β1→4 Galactosyltransferase | α1→3 Fucosyltransferase |
|-------------|-------------------------------|---------------------------|-------------------------|
|             | ±BFA                          | +BFA                      | ±BFA                    |
| NCI-H69     | 94.5±0.5                      | 129.0±15                  | 2.36±0.3                |
|             |                               |                          | 3.02±0.4                |
|             |                               |                          | 1.88±0.05               |
|             |                               |                          | 5.02±0.13               |
| PC9         | 114.0±10                      | 149.0±8                   | 18.0±2                  |
|             |                               |                          | 23.0±3                  |
|             |                               |                          | 0.51±0.05               |
|             |                               |                          | 0.45±0.05               |
| HCT-15      | 97.0±9                       | 105.0±8                   | 7.31±1                  |
|             |                               |                          | 7.15±1                  |
|             |                               |                          | 0.47±0.06               |
|             |                               |                          | 0.50±0.04               |
| HL-60       | 105.0±4                      | 86.5±12                   | 7.84±1.5                |
|             |                               |                          | 5.46±1                  |
|             |                               |                          | 1.53±0.3                |
|             |                               |                          | 1.52±0.3                |
| U937        | 108.0±4                      | 104.0±5                   | 5.34±0.3                |
|             |                               |                          | 5.13±0.6                |
|             |                               |                          | 0.62±0.1                |
|             |                               |                          | 0.76±0.03               |

**Table III**

**Effect of BFA on in vitro β1→4 galactosyltransferase activity**

| Assay condition* | No BFA 50 μg/ml BFA 100 μg/ml BFA |
|------------------|-----------------------------------|
|                  | μmol/h/mg protein                 |
| None             | 0.45±0.1                          |
|                  | 0.52±0.1                          |
|                  | 0.44±0.1                          |
| Phosphatidylethanolamine | 3.51±0.4                       |
|                  | 3.30±0.3                          |
|                  | 3.64±0.3                          |
| Phosphatidylglycerol   | 2.31±0.5                       |
|                  | 6.33±0.7                          |
|                  | 6.67±0.3                          |
| Taurodeoxycholate      | 7.77±0.4                        |
|                  | 7.48±0.5                          |
|                  | 7.87±0.4                          |

*Components added to the reaction mixture to activate the enzyme reaction. Enzyme assays were otherwise conducted as described under "Experimental Procedures" in the presence of the indicated concentration of BFA. Phospholipids, when present, were at a final concentration of 250 μg/ml. The HCT-15 cell enzyme was in this analysis using crude cell homogenates as the enzyme source.

**Fig. 6. Summary of the BFA-induced effect on glycosyltransferase reactions.** Solid arrows indicate reactions which occur in a BFA redistributed compartment (Brefeldin A Compartment). Broken arrows indicate reactions which are inhibited in the presence of BFA after BFA-induced redistribution of the Golgi (Brefeldin A Inhibited Compartment). Reactions 1 and 5 are catalyzed by β1→4galactosyltransferase activities; reaction 2, α1→3N-acetylglucosaminyltransferase; reaction 3, β1→3N-acetylglucosaminyltransferase; reaction 4, α1→3galactosyltransferase.

It is unlikely that the effect observed in the presence of BFA is due to inhibition of enzyme activity or sugar nucleotide transport. No change in transferase activity occurred in cells cultured in the presence of BFA. Also, BFA did not directly inhibit β1→4galactosyltransferase activity under a variety of in vitro assay conditions. Further, accumulation of significant amounts of Lc3 would require adequate UDPGal in the preceding reaction. No other evidence consistent with either of the above possible explanations has been reported. The simplest explanation, in keeping with recognized properties of BFA, is that the bulk of the β1→4galactosyltransferase activity is not redistributed to the ER by BFA. Multiple forms of this enzyme have been described in mammalian systems due to alternative initiation at two in-frame AUG codons (31, 32), and these forms have differing intracellular fates, establishing a Golgi and cell surface form of the enzyme (33). The BFA effect would presumably be restricted to the Golgi enzyme. Perhaps much of this enzyme in the cells tested is associated with the trans-Golgi network or with elements of the trans-Golgi which are poorly redistributed by BFA and thus straddle the block. On the other hand, β1→4galactosyltransferase is reported to be a trans-Golgi marker (34, 35), and previous ultrastructural studies using antibodies specific for this enzyme showed that a BFA-induced redistribution of it occurred (36, 37). Thus, the results point out potential inconsistencies between ultrastructural and biochemical function. This leaves open the possibility that although the enzyme is redistributed, it has little activity in situ by virtue of transient effects on the enzyme. It is also possible that BFA-induced changes in Golgi membranes causes a disruption of the physiological organization of many of these enzymes, affecting the efficient movement of the reaction product from one enzyme to the next enzyme downstream. This could also result in accumulation of biosynthetic intermediates. However, in the present case, it is surprising that this would occur for the acceptor of an enzyme present in such high comparative amounts in the cell. Further study will be required to resolve these questions.

The results presented further indicate that BFA blocks elongation of lactosylceramide to yield the globo-series precursor Gbα in cells which express α1→4galactosyltransferase. Earlier reports relating to ganglio-series chain biosynthesis indicated that BFA blocked elongation of de novo-synthesized GαMα to GαM2 (14, 15). Thus, the β1→4N-acetylgalactosaminyltransferase catalyzing this reaction is also trans to the BFA block. Consequently, synthesis of the first committed step in lacto-series chain synthesis (formation of Lc3) would be expected to occur in earlier Golgi cisternae than either of these other two reactions which define differing classes of glycolipids. A previous study (7) also provided evidence for expression of β1→3N-acetylgalactosaminyltransferase in early Golgi cisternae. These observations provide a basis for understanding the relative biosynthesis of core structures for differing glycolipid classes in terms of the fate of common precursors and the distribution of the respective biosynthetic enzymes in Golgi membranes.

The relative distribution of glycosyltransferases as defined by BFA sensitivity or resistance thus provides a basis for understanding membrane regulation of enzymes which compete for common oligosaccharide precursors (and which enzymes have greater access to these precursors). This is especially significant with respect to relative synthesis of lacto-series type 1 and 2 core chains which are expressed, for example, in colorectal adenocarcinoma cells and form the basis for important tumor-associated carbohydrate antigens. In this instance, the Lc3 precursor is an acceptor for both β1→3- and β1→4galactosyltransferases. Studies focused on this topic are currently underway.

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