3′-Azidothymidine (Zidovudine) Inhibits Glycosylation and Dramatically Alters Glycosphingolipid Synthesis in Whole Cells at Clinically Relevant Concentrations*

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Recent in vitro work with Golgi-enriched membranes showed that 3′-azidothymidine-5′-monophosphate (AZTMP), the primary intracellular metabolite of 3′-azidothymidine (AZT), is a potent inhibitor of glycosylation reactions (Hall et al. (1994) J. Biol. Chem. 269, 14355–14358) and predicted that AZT treatment of whole cells should cause similar inhibition. In this report, we verify this prediction by showing that treatment of K562 cells with AZT inhibits lipid and protein glycosylation. AZT treatment dramatically alters the pattern of glycosphingolipid biosynthesis, nearly abolishing ganglioside synthesis at clinically relevant concentrations (1–5 μM), and suppresses the incorporation of both sialic acid and galactose into proteins. Control experiments demonstrate that these changes do not result from nonspecific effects on either the secretory apparatus or protein synthesis. On the other hand, studies using isolated nuclei as a model system for chromosomal DNA replication show that AZTTP is a very weak inhibitor of DNA synthesis. These observations strongly suggest that the myelosuppressive effects of AZT in vivo are due to inhibition of protein and/or lipid glycosylation and not to effects on chromosomal DNA replication.

3′-Azidothymidine (AZT) is one of the primary chemotherapeutic agents used in the treatment of HIV infection (1). This drug is effective because the triphosphate form of AZT, AZTTP, is a potent and somewhat selective inhibitor of HIV reverse transcriptase (2). Unfortunately, AZT therapy is often accompanied by side effects such as severe anemia and neutropenia due to inhibition of the maturation of blood stem cells, especially in the late stages of the disease (3).

The current paradigm to explain AZT’s hematologic toxicity focuses on DNA replication. AZT is proposed to impede growth or development of stem cells through incorporation of the analog into chromosomal DNA (3). This hypothesis is consistent with the rapid proliferation of blood stem cells and their general sensitivity toward inhibitors of DNA replication (for example cancer chemotherapeutics). However, AZTTP is a remarkably weak inhibitor of the three nuclear replicative DNA polymerases, α, δ, and ε (4, 5). Under physiological nucleotide concentrations, the amount of AZTTP needed to inhibit these enzymes is much higher than the concentrations that accumulate in treated cells (6) and raises the possibility that the myelosuppressive effects of AZT are not related to inhibition of chromosomal DNA replication.

We recently demonstrated that the primary intracellular metabolite of AZT, AZTMP, is a potent competitive inhibitor of pyrimidine nucleotide sugar import into Golgi-enriched membrane fractions (7). Consequently, the glycosylation reactions that occur within the Golgi lumen were almost completely inhibited. Since AZTMP is known to accumulate to millimolar levels in several cell types (8), these observations suggested a novel mechanism for AZT toxicity, namely selective inhibition of lipid and protein glycosylation.

Several lines of evidence indicate that inhibition of glycosylation could indeed lead to cytotoxicity. Small changes in glycosphingolipid synthesis can profoundly affect signal transduction, differentiation, and cell-cell interactions. Ganglioside synthesis varies in a characteristic manner during growth and differentiation (9, 10), and subtle changes in glycolipid composition dramatically alter the properties of many receptors and enzymes (10). For example, variations in ganglioside composition as small as 15% can block activation of some growth factor receptors (11, 12). Alterations in glycosylation pattern of proteins could also contribute to cytotoxicity since previous studies have established that inhibition of N-linked protein glycosylation is toxic and can block development (13, 14).

We therefore examined the effects of AZT on lipid and protein glycosylation in whole cells and found that treatment with AZT did inhibit these reactions at clinically relevant concentrations (0.5–5 μM). In particular, AZT treatment dramatically altered the pattern of glycosphingolipid biosynthesis in the human blood cell line K562. In contrast, the AZT metabolites AZTMP and AZTTP were extremely weak inhibitors of DNA synthesis by isolated K562 nuclei. The significance of these results with respect to the side effects associated with AZT therapy are discussed.

EXPERIMENTAL PROCEDURES

Materials—Cell lines were obtained from the American Type Culture Collection and were maintained in RPMI 1640 supplemented with 10% fetal calf serum, 100 μg/ml penicillin G, and 100 μg/ml streptomycin. Cell culture media and bovine fetal serum were obtained from Life Technologies, Inc. or Sigma. AZTMP and AZTTP were generously provided by Wayne Miller (Wellcome Research Laboratories, Research Triangle Park, NC). [6-3H]Acetylmannosamine, [1-14C]galactose, and [α-32P]dATP were purchased from DuPont NEN. [α-3H]Galactose was obtained from American Radiochemical and trans-[15N]metabolic labeling was from ICN. AZT, ddI, ddC, and glycolipids...
used as carriers for extraction were from Sigma. SPG and PG glycolipid standards were generously provided by Dr. Bruce Macher (San Francisco State University), while the other TLC lipid standards were obtained from Matreya Inc. The goat anti-human transferrin receptor serum was a generous gift of Dr. Caroline Enns (Oregon Health Sciences University).

DNA Synthesis in Isolated Nuclei—Isolation of nuclei and measurements of DNA replication were as described previously (15, 16). Control experiments established that DNA synthesis was linear over the time points used. IC50 values for nucleotide analogs were determined using Dixon plots.

Labeling and Quantitative Analysis of Glycosphingolipids—Unless otherwise indicated, sphingolipids were labeled by growing 1–1.5 × 106 cells in the presence of (1-14C)galactose (0.6 μCi/ml) or [U-14C]serine (2 μCi/ml) for 24 h in RPMI 1640. Lipids were analyzed using a slight modification of the method described by Sandhoff et al. (17). Briefly, metabolically labeled cells were transferred to glass tubes, washed with PBS, and extracted consecutively with 5 ml each of 1:2, 1:1, and 2:1 CHCl3:MeOH (v/v) (18). Purified ceramide, galactocerebroside, gangliosides, and gangliosides (2.5 μg of each) were added before the first extraction to improve and verify recovery. The pooled extracts were treated with methanolic NaOH (50 ml) for 2 h at 37°C to hydrolyze phospholipids. Following neutralization, lipids were desalted by chromatography over a Waters C-18 SepPak column (19). Sphingolipids were separated by silica gel TLC (Merck) or HPTLC (Whatman) developed using CHCl3:MeOH:25% aqueous CaCl2 (60:35:5, v/v/v) indicated, separation of neutral and acidic glycosphingolipids was accomplished prior to treatment with methanolic NaOH by chromatography on DEAE-Sepharose A25 (20). [14C]-Labeled compounds were visualized and quantified using a PhosphorImager. Lipid standards were detected with orcinol/H2SO4 reagent.

Measurement of Lipid Degradation Rates—K562 cells were labeled with [14C]galactose for 24 h as described above. Following two washes to remove free [14C]galactose, cells were resuspended in fresh media (RPMI 1640 plus 7.5% fetal calf serum) containing 0 or 20 μM AZT. After 0, 7, or 24 h, cells were harvested, and the glycosphingolipids were analyzed as described above.

Metabolic Labeling and Immunoprecipitation of the Transferrin Receptor—K562 cells were grown in the presence of 0 or 20 μM AZT for 3.5 h and then transferred to the identical medium lacking methionine. After a 15-min pretreatment, [35S]Trans-label (100 μCi/ml) was added, and incubation continued for 20 min. The labeled cells were then chilled, washed once in ice-cold Hank's buffered saline, and chased at 37°C in complete medium containing 500 μg/ml methionine in the continued presence of 0 or 20 μM AZT. At various chase times, 5 × 105 cell aliquots were transferred to ice to arrest protein transport. At the end of the chase, all samples were washed once with Hank's buffer and incubated for 1 h with 1 μl of a previously characterized goat anti-human transferrin (TF) receptor serum, an amount sufficient to bind all receptors on the cell surface (21, 22). Cells were recovered by centrifugation and resuspended in 1 ml of Hank's buffered saline containing a 5-fold excess of unlabeled K562 cells to quench any remaining free antibody. Receptor-antibody complexes were recovered as described previously (21), except that protein A-Sepharose Fast Flow (Pharmacia Biotech Inc.) was substituted for the fixed Staphylococcus aureus suspension. Immunoprecipitates were resolved on 7.5% SDS-polyacrylamide gels, and the amount of receptor was quantitated by PhosphorImager analysis. Background observed at 0 chase time was less than 10% of maximum signal and was subtracted from all values.

Metabolic Labeling with [3H]N-Acetyllactosaminosamine and [3H]Galactose—Cells (1 × 106) were washed in labeling medium, plated in a final volume of 300 μl/well of a 24-well plate (Falcon), and incubated in the presence of various concentrations of AZT and [3H]N-acetyllactosaminosamine (9 μCi/ml) or [3H]Galactose (1.8 μCi/ml) or Trans-[35S]Metabolic label (6 μCi). Unless otherwise indicated, labeling was performed for 24 h in complete RPMI 1640 (2 g/liter glucose) supplemented with 7.5% fetal calf serum. At the end of the incubation, cells and several rinses with phosphate-buffered saline from each well were transferred to individual glass tubes kept on ice.

The extent of [3H] incorporation into proteins and lipids was determined by collecting cells onto glass fiber filters (G4) quenched in 1 ml 1N HCl for 4 h at 100°C to release carbohydrates. The hydrolysates were then lyophilized and resuspended in H2O for analysis by descending paper chromatography in EtOAc/pyridine/H2OAc/H2O (5:5:1:3).

RESULTS

AZT Metabolites Have Little Effect on Nuclear DNA Synthesis—Previous work has established that AZTTP is a very poor inhibitor of the purified eukaryotic DNA polymerases α, δ, and ε (4, 5). However, since chromosomal replication is a highly complex and coordinated process, it nonetheless remained possible that AZTTP affected nuclear DNA synthesis in vivo. Furthermore, since AZTMP accumulates to millimolar levels in AZT-treated cells, inhibition of DNA synthesis by AZTTP could be potentiated by the known ability of AZTMP to inhibit the 3′–5′ exonuclease activity of DNA polymerase δ (24). We tested these possibilities by measuring DNA synthesis in isolated nuclei, a model system that is thought to accurately mimic cellular DNA replication (16).

In assays containing 10 μM dNTPs, AZTTP poorly inhibited DNA synthesis in nuclei obtained from K562 and CEM cells, with IC50 values greater than 500 μM (Table I). The presence of 1 mM AZTMP slightly enhanced inhibition in K562 nuclei, but the AZTTP concentration needed for significant inhibition remained several hundred-fold higher than the AZTTP concentration reported in AZT-treated cells (about 1 μM (6)). In contrast, two other antiviral nucleotide analogs, ddCTP and ganciclovir triphosphate, inhibited DNA synthesis in isolated nuclei much more potently than AZTTP, in agreement with the fact that these are more potent inhibitors of purified nuclear DNA polymerases (15, 25). These results suggest that inhibition of nuclear DNA replication is unlikely to account for the side effects associated with AZT therapy.

AZT Treatment Dramatically Alters Glycosphingolipid Synthesis—To investigate inhibition of glycosylation by AZT, we turned to the erythroleukemia cell line K562 as a model system. This cell line is ideal for these studies because anemias are a major side effect of AZT treatment, and K562 cells can be induced to differentiate into hemoglobin-producing cells (26). Cells were incubated with [3H]Galactose to label newly synthesized glycosphingolipids, and the composition of these lipids was then analyzed by HPTLC (Fig. 1); sphingolipids were identified by comigration with standards under three solvent conditions as described in Table II. The major acidic glycosphingolipids were GD1a, SPG, GM2, and GM3, while the primary neutral glycosphingolipids were lactosylceramide and glucosylceramide, a composition in agreement with that previously reported for K562 cells (18, 27).

Fig. 1 shows that AZT treatment dramatically altered the pattern of glycosphingolipid biosynthesis in a dose-dependent manner. Quantitative analysis of the data demonstrated that AZT treatment nearly abolished synthesis of GD1a, GM2, and
SPG and had the opposite effect on lactosylceramide and material that comigrated with GM3 and PG standards (Fig. 2, left panel). Since AZT treatment typically results in 2–4 μM AZT in the serum of patients (28), these results demonstrate that AZT can cause a dramatic remodeling of the glycolipid composition of cells at clinically relevant concentrations. Identical results were obtained in several separate experiments.

The analysis of total sphingolipids presented in Fig. 1A suggests that the synthesis of GM3, unlike that of the other acidic lipids, is stimulated by AZT. However, this analysis is complicated by the comigration of GM3 and PG, the immediate precursor to SPG, under standard chromatography conditions. To investigate the effects of AZT on PG and GM3 production, neutral and acidic lipid fractions were isolated by ion exchange chromatography and separately analyzed by HPTLC (Fig. 1, B and C). Analysis of the acidic glycolipids revealed that the synthesis of GM3, in contrast to that of GD1a, SPG, and GM2, increases in the presence of AZT (Fig. 1B). Note that HPTLC under basic conditions resolves GM3 from PG. Quantitation of several independent experiments shows that 20 μM AZT stimulates GM3 synthesis by 50 ± 10%.

The observed decrease in SPG synthesis could have resulted either from inhibition of the synthesis of its precursor, PG, or from a block in the conversion of PG to SPG. Analysis of the neutral glycosphingolipids from AZT-treated cells shows that relatively little PG normally accumulates in K562 cells but that treatment with 20 μM AZT resulted in a 2.2-fold increase in PG levels (Fig. 1C). The species identified as PG cochromatographed with authentic PG under several solvent conditions, thereby confirming its identification. The observed increase in PG accounts for nearly 60% of the decrease in SPG production, which suggests that the decrease in SPG synthesis results in great part from inhibition of the sialylation of PG.

**FIG. 2.** Quantitative analysis of the effects of AZT on glycosphingolipid synthesis. Left panel, K562 cells were labeled with [3H]galactose for 24 h in the presence of various AZT concentrations. The amount of radioactivity present in each of the selected glycolipid was expressed as a fraction of the total present at each AZT concentration and then normalized to the fraction observed in the absence of AZT. The average of two to four independent experiments is shown (standard deviation < 6%). The lipids analyzed and their corresponding plot symbols are as follows: ○, cerebroside; ●, lactosylceramide; ■, PG/GM3; ▲, GM2; ▼, SPG; △, GD1a. Right panel, K562 cells were labeled with [3H]serine for 24 h in the presence of various AZT concentrations. Glycolipids were analyzed and quantified as described for the left panel.

Because the neutral and acid glycolipids were separated prior to analysis, it was not possible to directly determine the stimulation of PG production relative to total glycolipids. Therefore, effects of AZT on PG accumulation were quantified by normalizing the amount of PG to that of lactosylceramide and cerebrosides.

**TABLE II**

Structure of glycosphingolipids synthesized by K562 cells

The primary acidic and neutral glycosphingolipids synthesized by K562 cells are listed (18, 27, 29). The identification of these lipids (Figs. 1 and 2) was based on their Rf value and comparison with known standards (27). This identification was confirmed by comigration of the radiolabeled species with the appropriate standards after silica TLC or HPTLC developed with three solvents of very different pH (CHCl3, MeOH, 0.22% aqueous CaCl2, HOAc, NH4OH (60:35:8:0:0, 60:35:7:1:0, or 60:35:7:0:1)). The band identified as GM2 migrates slightly faster than standard brain GM2, as previously reported (27).

| Glycosphingolipid            | Structure                                      |
|------------------------------|------------------------------------------------|
| Glucosylceramide             | Glc(1→1)Gal                                 |
| Lactosylceramide             | Gal(1→4)Glc                                  |
| Globotriosylceramide         | Gal(1→4)Gal(1→4)Glc                         |
| Paragloboside                | NeuAc(1→3)Gal(1→4)Glc                      |
| Sialosylparagloboside        | NeuAc(1→3)Gal(1→4)Glc                      |
| GM3                          | NeuAc(1→3)Gal(1→4)Glc                      |
| GM2                          | NeuAc(1→3)Gal(1→4)Glc                      |
| GD1a                         | NeuAc(1→3)Gal(1→4)Glc                      |

* Cer, ceramide.
radiation rates. To test the possibility that AZT treatment alters glycosphingolipid breakdown, K562 cells were metabolically labeled with [14C]galactose for 24 h and then chased in the presence of 0 or 20 μM AZT for an additional 7 or 24 h (Fig. 3). Nearly identical amounts of glycosphingolipids were recovered from all incubations (data not shown), and treatment with AZT had no significant effect on the pattern of labeled glycosphingolipids present after either a 7- or 24-h chase (Fig. 3). These results argue strongly that the AZT-induced changes in newly synthesized glycosphingolipids result from altered synthesis rates rather than from changes in degradation rates.

The Effects of AZT Do Not Result from Changes in Nucleotide Sugar Pools or from a General Block of the Secretory Apparatus—Our previous in vitro work with AZTMP suggests that the dramatic effects of AZT on glycosphingolipid synthesis most likely result from inhibition of nucleotide sugar import into the lumen of the Golgi complex. However, similar results could be observed if AZT treatment affected incorporation of galactose into nucleotide sugars or blocked other aspects of the secretory apparatus.

To verify that the effects of AZT did not result from changes in the incorporation of [14C]galactose into the precursor pool, we repeated our analysis of glycosphingolipid synthesis using a non-carbohydrate metabolic label, [14C]serine, that is readily incorporated into the sphingosine backbone of all sphingolipids. As shown in Fig. 2, AZT treatment reduced ganglioside synthesis and caused accumulation of neutral species to levels very similar to those observed when using [14C]galactose as a label. In contrast, the synthesis of sphingomyelin, a non-glycosylated sphingolipid, was completely unaffected by AZT. These results strongly suggest that the effects of AZT are not mediated by changes in sugar uptake or nucleotide sugar precursor pools.

The small quantitative differences probably arise because only one [14C]serine is incorporated per sphingolipid, whereas different numbers of [14C]galactose can be incorporated as either glucose or galactose into each lipid species. In addition, direct measurements of nucleotide sugar pools established that treatment with 5 μM AZT did not alter intracellular levels of either hexosyl or N-acetylhexosyl-nucleotides.

The possibility that AZT treatment interferes with the secretory pathway was excluded by measuring the synthesis and transport of a well-characterized glycoprotein of K562 cell membranes, the Tf receptor. Cells were labeled with [35S]methionine for 20 min, and movement of the Tf receptor to the cell surface in the absence or presence of 20 μM AZT was monitored. Fig. 4 shows that maximal accumulation of the Tf receptor at the cell surface occurred in about 60 min, as previously observed.4 AZT affected neither the amount nor the rate at which the Tf receptor was incorporated into the plasma membrane, demonstrating that AZT does not act as a general inhibitor of the secretory pathway. The lack of effect of AZT on transport despite clear effects on protein glycosylation (see below), is in agreement with previous work showing that inhibition of complex carbohydrate synthesis does not affect transport of the Tf receptor (21). Incorporation of [35S]methionine into bulk proteins was also used to measure the effects of AZT on protein synthesis. AZT concentrations as high as 50 μM had no effect on [35S]methionine incorporation in trichloroacetic acid-precipitable material. This lack of effect on the synthesis and transport of proteins is consistent with previous work showing that these low concentrations of AZT do not reduce the growth rate of K562 cells (30).

In the lipid-labeling experiments presented in Figs. 1 and 2, cells were treated and labeled for 24 h prior to analysis. To test the possibility that effects of AZT were due to alterations in the biosynthesis of the glycosylation machinery (sugar transferases, etc.), short treatment and labeling times were also examined. After treating cells with AZT for 60 min to allow accumulation of AZTMP, [14C]galactose was added, and newly synthesized glycolipids were analyzed following an additional 2-h incubation. In this short term labeling, 20 μM AZT inhibited the synthesis of GM2, SPG, and GD1a by 82, 54, and 65%, respectively, and stimulated the synthesis of lactosylceramide and GM3/PG by 92 and 43%, respectively, values similar to those obtained in the 24-h labeling experiments shown in Fig. 2. These results indicate that changes in glycolipid synthesis are rapid and unlikely to result from AZT-induced alterations in the levels of proteins involved in glycosylation.

Inhibition of Glycosylation by AZT

Fig. 3. AZT treatment dramatically alters the synthesis but not the breakdown of glycosphingolipids in K562 cells. Cells were metabolically labeled with [14C]galactose in the absence of AZT for 24 h. Labeled cells were then washed and chased in media lacking [14C]galactose for 0, 7, or 24 h in the presence or absence of 20 μM AZT. Remaining labeled glycosphingolipids were analyzed by TLC. Cerebr, cerebroside; LacCer, lactosylceramide.

Fig. 4. AZT treatment does not inhibit the secretory pathway. K562 cells were labeled with [35S]methionine and chased for various lengths of times in the presence of either 0 or 20 μM AZT, as indicated. The synthesis and transport of the Tf receptor was monitored as described under "Experimental Procedures." The mobility of the receptor was the same at all chase times, both in the presence and absence of AZT. The amount of Tf receptor present at the cell surface after various chase times is shown. Similar results were obtained in two separate experiments.

3 T. Kline, P. Melançon, and R. D. Kuchta, unpublished results.

4 Enns, C., personal communication.
The effects of ddC and ddI, two other anti-HIV nucleosides, on lipid and protein glycosylation were examined. Treatment of K562 cells with 2 μM ddC or 100 μM ddI, concentrations 10-fold above values observed in patients' serum (28), had no significant effects on either total glycosylation as measured by [3H]sialic acid incorporation into total biomolecules or [14C]serine incorporation into glycosphingolipids (data not shown). Thus, inhibition of protein and lipid glycosylation is not a general property of anti-HIV nucleosides.

**DISCUSSION**

We previously demonstrated that AZTMP potently inhibits the import of pyrimidine nucleoside sugars into the lumen of Golgi-enriched membrane fractions (7). Since K562 cells treated with only 10 μM AZT accumulate greater than 1 mM AZTMP (8), these data suggested that AZT should selectively inhibit glycosylation reactions. Here, we have verified this prediction and established that AZT treatment dramatically alters glycosphingolipid synthesis and suppresses protein glycosylation in K562 cells at clinically relevant concentrations. AZT may be a general modulator of glycosphingolipid synthesis, since we have found that AZT alters glycolipid synthesis in HEL and A431 cells.5

Several observations support competitive inhibition of the import and accumulation of nucleotide sugars in the Golgi apparatus as the most likely molecular mechanism for these effects of AZT: (i) AZT treatment does not alter lipid degradation, protein biosynthesis, or protein secretion; (ii) AZT affects both lipid and protein glycosylation; and (iii) the onset of AZT’s effects are rapid. Additionally, ddC and ddl, two nucleosides that do not accumulate as monophosphates (6), did not affect glycosylation.

AZT treatment of K562 cells inhibited the synthesis of the SPG, GD1a, and GM2, and caused accumulation of GM3, PG, and lactosylceramide. While AZT treatment generally inhibited the synthesis of complex, acidic glycosphingolipids, this result cannot be explained by a simple model in which the import of a single nucleotide sugar is affected (see Table II). Whereas the decrease in SPG and compensatory increase in PG demonstrate that import of CMP-sialic acid was inhibited, the increase in GM3 and decrease in GD1a and GM2 indicate that AZT also blocked the import of UDP-GaINAc.

The selective effects of AZT treatment on different glycosylation reactions could result from several causes. Since AZTMP inhibits the import reaction competitively with respect to the nucleotide sugar (7), AZTMP will have greater impact on the import of those nucleotide sugars whose concentrations are lowest and whose transporters are most sensitive to inhibition. In addition, AZTMP could affect the various subcompartments of several glycosphingolipids. If, as our data suggest, these effects are due to a block of nucleotide sugar import into the Golgi complex, then AZT should also inhibit protein glycosylation since nucleotide sugar import is required for most protein glycosylation reactions. This possibility was tested using established metabolic labeling procedures to measure incorporation of sialic acid and galactose into glycoproteins (31).

We first assayed sialic acid incorporation into total lipids and proteins by incubating cells for 24 h with [6-3H]N-acetylmannosamine, a specific precursor of sialic acid (32), and measuring the amount of [3H]sialic acid present in acid-insoluble material. Fig. 5 shows that AZT reduced sialic acid incorporation at concentrations as low as 0.5 μM. The effects of AZT were examined over a wide range of labeling times (3–24 h) with identical results.

Galactosylation of lipids and proteins was similarly examined by incubating K562 cells with [3H]galactose. As shown in Fig. 6, AZT reduced incorporation of galactose into total lipids and proteins at concentrations as low as 1 μM. Since [3H]galactose can be catabolized to non-carbohydrate precursors upon conversion to glucose and transfer of the [3H] to NADP⁺, we tested the effect of altering the amount of glucose in the labeling medium. Increasing the glucose concentration from 0.075 g/liter to 2 g/liter decreased the rate of [3H] incorporation by 70% but did not alter the extent of inhibition by AZT (Fig. 6).

We then verified that AZT suppressed protein glycosylation by extracting lipids from the trichloroacetic acid precipitates with CHCl₃:MeOH prior to scintillation counting. Less than 15% of the radiolabeled material was extracted by the organic solvent, indicating that most of the label incorporated into biomolecules was present in proteins and that the results in Figs. 5 and 6 reflect effects on protein glycosylation. Analysis of the CHCl₃:MeOH insoluble material produced results identical to those presented in Figs. 5 and 6, thereby confirming that AZT inhibited protein glycosylation. Further control experiments established that >95% of the radioactivity incorporated into protein during labeling with [3H]galactose co-migrates with galactose by paper chromatography and that this fraction is unchanged by AZT (data not shown). No [3H]glucose was detected in this analysis because glucose is not permanently incorporated into proteins during N- and O-linked glycosylation.

Inhibition of Glycosylation Is Not a General Effect of Anti-HIV Nucleosides—The effects of ddC and ddl, two other anti-HIV nucleosides, on lipid and protein glycosylation were examined over a wide range of labeling times (3–24 h) with identical results. The selective effects of AZT treatment on different glycosylation reactions could result from several causes. Since AZTMP inhibits the import reaction competitively with respect to the nucleotide sugar (7), AZTMP will have greater impact on the import of those nucleotide sugars whose concentrations are lowest and whose transporters are most sensitive to inhibition. In addition, AZTMP could affect the various subcompartments

5 R. Steet, R. D. Kuchta, and P. Melançon, unpublished data.
eral observations, however, suggest that inhibition of DNA synthesis is largely based on the observation that treatment of blood toxicity is due to effects on nuclear DNA replication. This model the killing of blood progenitor cells by AZT maintains that the Cytotoxic Effects of AZT

Tests to test this possibility are in progress. Inhibition of Glycosylation Reactions May Be Responsible for the Cytotoxic Effects of AZT—The current paradigm to explain the killing of blood progenitor cells by AZT maintains that toxicity is due to effects on nuclear DNA replication. This model is largely based on the observation that treatment of blood progenitor cells with AZT results in incorporation of AZT into DNA, and this incorporation correlates with toxicity (3). Several observations, however, suggest that inhibition of DNA replication by AZT is not the cause of toxicity. AZTTP is an extremely poor inhibitor of DNA polymerases α, δ, and ε, the three enzymes likely involved in nuclear DNA replication (4, 5), and as shown in this report, AZTTP is likewise a very weak inhibitor of chromosomal replication in isolated nuclei. Furthermore, Sommadossi and co-workers have recently reported that addition of uridine prevents toxicity without causing any reduction in the extent of AZT incorporation into chromosomal DNA (34). Together, these results suggest that effects of AZT on DNA metabolism are not responsible for the growth suppression of blood progenitor cells.

In contrast, the effects of AZT on protein and lipid glycosylation would readily account for the cytotoxicity of AZT. As described in the Introduction, changes in ganglioside content as small as 15% can compromise the function of membrane receptors (11, 12). It is therefore quite likely that the dramatic changes in ganglioside synthesis reported here will interfere with the function of proteins such as the erythropoietin receptor. Whereas such changes may have minimal impact on the growth of most cultured cell lines, they will most likely be toxic to those cell types that are dependent on extracellular signals for growth or differentiation. For example, rapidly growing erythrocyte precursor cells may show such sensitivity, since erythropoietin-dependent cells undergo apoptosis when deprived of erythropoietin (35). Direct evidence that inhibition of glycosylation can lead to anemia is provided by the observation that congenital dyserythropoietic anemia type II is due to defective poly-N-acetyllactosamine addition to proteins in erythrocyte precursors (36).

Our demonstration that AZT inhibits glycosylation may not only facilitate development of methods to better control AZT toxicity but will also open up new avenues to study the role of gangliosides in regulating receptor function and cell-cell interactions. The lack of methods to depress ganglioside biosynthesis has limited previous attempts to determine the effect of adding exogenous lipids. In the future, suppression of ganglioside synthesis by AZT treatment followed by selective incorporation of different glycosphingolipids should allow for more control over membrane composition to better define the role of this important class of molecules.

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