Comparison of Rates of Intracellular Metabolism of Zidovudine in Human and Primate Peripheral Blood Mononuclear Cells

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3'-Azido-3'-deoxythymidine (AZT) is a drug of choice for the treatment of AIDS. On the basis of pharmacokinetic data, the nonhuman primate Macaca nemestrina has been shown to be a suitable animal model for use in the study of the disposition of AZT. However, since AZT is activated to its metabolite, the AZT triphosphate (AZTTP), intracellularly, we investigated the intracellular activation of AZT in peripheral blood mononuclear cells (PBMCs) of healthy and simian immunodeficiency virus-infected macaques and compared it with that in PBMCs obtained from human volunteers. At 5 μM extracellular AZT, both human and macaque PBMCs rapidly convert AZT to AZT monophosphate (AZTMP) (84% of total phosphorylated products) in 4 h. Increases in AZTMP levels of 7.7- and 12-fold were observed in human and macaque PBMCs, respectively, when the extracellular AZT concentration increased from 0.45 to 14.4 μM. Similar ratios of AZT metabolites, AZT diphosphate (AZTDP)/AZTTP (0.7 to 1.4), AZTMP/AZTDP (3 to 14), and AZTMP/AZTTP (3 to 19), over the same AZT concentration range were observed in both human and macaque PBMCs, suggesting that these cells have similar capacities to phosphorylate AZT. Simian immunodeficiency virus-infected macaque PBMCs showed a fivefold increase in intracellular AZT and AZTMP levels and a twofold increase in AZTDP and AZTTP levels (picomoles per 10^7 cells) when compared with those in the uninfected cells (at 4 h with 0.9 μM extracellular concentration). This increase in AZT metabolite levels has also been reported for human immunodeficiency virus-infected PBMCs. Collectively, given the similarities in phosphorylation profiles between healthy and infected human and macaque PBMCs, we conclude that the macaque is a suitable animal model for use in the study of factors that can affect the in vivo phosphorylation of AZT.

The human immunodeficiency virus (HIV), a lymphotropic retrovirus, is known to be the etiologic agent of AIDS and AIDS-related complex. In order to expedite research on and the development of new anti-HIV drugs, there is an urgent need to develop and validate a representative animal model that can be used to test the efficacy and safety of new anti-HIV drugs. The simian immunodeficiency virus (SIV)-infected nonhuman primate is believed to be one such representative animal model (for reviews, see references 20 and 33). SIV is a retrovirus-like group of viruses that produce an AIDS-like syndrome similar to that produced by HIV in a variety of nonhuman primates. SIV has genetic, antigenic, and biological similarities to HIV types 1 and 2, the viruses that cause AIDS (19, 21).

The deoxynucleoside analog 3'-azido-3'-deoxythymidine (AZT) is the first clinically approved drug for the treatment of AIDS (for a review, see reference 34). Intracellularly, this produg is metabolized through a series of cellular kinases to its active moiety, the AZT triphosphate (AZTTP). AZTTP inhibits the HIV enzyme reverse transcriptase by a premature termination in the synthesis of proviral DNA (5, 13, 34). AZTTP is also capable of inhibiting DNA polymerases alpha and beta (6, 10, 13, 27) and gamma (13). Therefore, the intracellular concentration of AZTTP is an important determinant of the efficacy and toxicity of AZT. Interestingly, the extracellular concentration of AZT is not linearly correlated with the intracellular concentration of AZTTP (10, 12, 28).

Data from our laboratory and other laboratories have indicated that the nonhuman primate, the macaque, is a suitable animal model for use in the study of the disposition of AZT and dideoxynosine in adults (18, 25) and the disposition of AZT in neonates (16, 17, 22) and pregnant women (16, 18, 32). As part of this series of studies to verify the suitability of Macaca nemestrina as an animal model for the study of the disposition of deoxynucleosides, we investigated the intracellular phosphorylation of AZT in healthy and SIV-infected macaque peripheral blood mononuclear cells (PBMCs). Since the efficacy and toxicity of AZT in SIV-infected macaques are analogous to those observed in the human population with AIDS (11, 31), the studies described here may aid in elucidating the mechanism responsible for the toxicity and loss of efficacy of AZT against HIV replication observed in the clinic.

MATERIALS AND METHODS

Chemicals. 3'-Azido[meH]3'-deoxythymidine ([3H]AZT; radiodegradable purity >99.7%; 14 Ci/mmol) was purchased from Moravek Biochemical (Brea, Calif.). AZT monophosphate (AZTMP), AZT diphosphate (AZTDP), and AZTTP were obtained from Burroughs Wellcome Co. (Research Triangle Park, N.C.). Lymphocyte separation medium (LSM) was purchased from Organon Teknika, Durham, N.C. Fetal calf serum (FCS) was purchased from Hyclone (Logan, Utah). RPMI 1640 and antibiotics were purchased from Gibco BRL (Grand Island, N.Y.). All other chemical and reagents were of analytical or high-pressure liquid chromatography (HPLC) grade.

Subjects. All healthy human volunteers (three Caucasians and two Asians) were HIV negative. All adult Macaca nemestrina used in our experiments were from the Regional Primate

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Research Center of the University of Washington. Five of these macaques were infected with SIV and four were SIV negative. The SIV-infected macaques were exposed to 10^4 to 10^5 50% tissue culture infective doses of SIV intrarectally. Two to 4 months prior to conducting the experiments, the infected macaques were determined to be SIV positive by viral antigen test (p27 core antigen of SIV), antibody detection, and PCR of PBMCs.

Isolation of PBMCs. Fresh venous blood (maximum of 20 and 200 ml per macaque and human subject, respectively) was collected in heparinized Vacutainer tubes. Following dilution with an equal volume of cold phosphate-buffered saline (PBS), 20-ml aliquots of the diluted blood were loaded onto 20 ml of LSM in a 50-ml conical centrifuge tube, and the tubes were centrifuged at 400 × g for 20 min to isolate the PBMCs. For isolation of macaque PBMCs, 95% LSM (diluted with PBS) was used. The isolated PBMCs were washed two times in RPMI 1640 medium and were finally resuspended in the culture medium for cell counting and incubation. Differential cytology examination was performed to measure the purity of the isolated cells. PBMCs constituted 94% ± 3% and 93% ± 5% of the isolated cells from human and macaque blood, respectively.

Incubation of PBMCs with AZT, extraction, and analysis of the phosphorylated metabolites. The isolated PBMCs were incubated in RPMI 1640 medium (Gibco) containing 10% FCS supplemented with 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 0.25 μg of amphotericin B (Fungizone; Gibco) per ml. Various (0.45, 0.9, 1.8, 3.6, 7.2, and 14.4 μM) or fixed (5 μM) [3H]AZT concentrations were added to the PBMC medium, and the cells were maintained at 37°C in 5% CO_2 for constant (4 h) or various (1, 2, 4, 8, 16, and 24 h) times prior to removal for extraction. The PBMCs were scraped from the tissue culture plates at the indicated time points and were extracted and analyzed for AZT metabolites by gradient HPLC and scintillation counting by a method previously described by our laboratory (24).

RESULTS

Kinetics of intracellular AZT phosphorylation in macaque and human PBMCs. The time course of AZT phosphorylation in macaque PBMCs was determined by incubating the cells for 1 to 24 h with 5 μM [3H]AZT. As shown in Fig. 1A, AZT was rapidly converted to AZTMP in the macaque cells (maximal accumulation in 4 h). During this period, AZTMP constituted 73 to 84% of the total phosphorylated metabolites. The intracellular AZTMP level rose from 0.77 to 0.93 pmol/10^7 cells within the 4-h period, while AZTDP and AZTTP levels (0.11 and 0.15 pmol/10^7 cells, respectively) peaked at 8 h. Over the time course of the study, the levels of AZTDP and AZTTP constituted less than 12 and 15%, respectively, of the total phosphorylated metabolites. After achieving the peak, the intracellular level of AZTMP declined approximately 50% over the next 20 h of incubation, while AZTDP and AZTTP levels decreased only slightly during the 8- to 24-h incubation period.

The time course of AZT phosphorylation in human PBMCs was similar to that of macaque PBMCs (Fig. 1B). Under the same experimental conditions, AZTMP, AZTDP, and AZTTP levels peaked at 4 h after incubation with 5 μM [3H]AZT. AZTMP constituted approximately 83% of total phosphorylated metabolites, whereas AZTDP and AZTTP constituted approximately 7 and 10% of the total phosphorylated metabolites, respectively. The intracellular peak level of AZTMP did not change during 4 to 24 h of incubation. AZTDP and AZTTP profiles in human PBMCs were similar to those in macaque PBMCs over the same time period.

Concentration-dependent intracellular phosphorylation of AZT in PBMCs. To determine the concentration effect of AZT on its intracellular metabolism over a clinically relevant concentration range, PBMCs were incubated with 0.45 to 14.4 μM (6.3 to 203 μCi) AZT for 4 h. The distributions of phosphorylated AZT products were assayed in both macaque and human cells. In macaque PBMCs, a 32-fold increase in the AZT concentration (from 0.45 to 14.4 μM) resulted in a 12-fold increase in the intracellular AZTMP level (from 0.2 to 2.4 pmol/10^7 cells). The rise in either AZTDP or AZTTP level was less than threefold (Fig. 2A). Similarly, in human PBMCs, a 7.7-fold increase in intracellular AZTMP levels was detected over the same AZT concentration range. The increases in AZTDP and AZTTP levels in human PBMCs were also quite similar (two- to fourfold increase) to those in macaque PBMCs (Fig. 2B). Regardless of the source of the PBMCs or the extracellular concentration of AZT, AZTMP was found to be the predominant metabolite (>61%). AZTDP and AZTTP constituted the remaining phosphorylated products.

To characterize any differences in the efficiency of AZT phosphorylation between macaque and human PBMCs, we compared the ratio of AZT metabolites as AZTDP/AZTTP, AZTMP/AZTDP, and AZTTP/AZTMP. The AZTDP/AZTTP, AZTMP/AZTDP, and AZTTP/AZTMP ratios for macaque PBMCs over extracellular AZT concentrations of 0.45 to 14.5 μM were 0.7 to 1.4, 3 to 14, and 3 to 19, respectively (Fig. 3A). Similar intracellular ratios of AZT metabolites were observed over the same AZT concentration range observed for human PBMCs (Fig. 3B).

Intra- and interindividual variations in AZT metabolites in
human PBMCs. To determine the variation among human subjects in the ability of their PBMCs to phosphorylate AZT, PBMCs isolated from a number of individuals were incubated with 0.9 μM [3H]AZT for 4 h, and the phosphorylated metabolites were assayed by HPLC. As shown in Fig. 4A, the intracellular metabolism of AZT to AZTMP, AZTDP, and AZTTP was reproducible when measured in PBMCs obtained from a single individual (coefficient of variation [CV], <7.5% for all metabolites). In contrast, the intersubject variability (n = 5) in the levels of intracellular AZT metabolites was much greater (Fig. 4B), being prominent for AZTMP (CV, <60%) and least prominent for AZT (CV, <10%).

AZT phosphorylation in healthy and SIV-infected macaque PBMCs. PBMCs isolated from healthy (n = 4) and SIV-infected (n = 5) macaques were incubated with 0.9 μM [3H]AZT for a 4-h period, and intracellular metabolite levels were determined. The average levels of intracellular AZT, AZTMP, AZTDP, and AZTTP in healthy macaque PBMCs were 0.30 ± 0.09, 0.04 ± 0.06, 0.05 ± 0.01, and 0.04 ± 0.01 pmol/10^7 cells, respectively (Fig. 5). Under the same experimental conditions, the average levels (in picomoles per 10^7 cells) of intracellular AZT (1.58 ± 0.24), AZTMP (2.17 ± 1.12), AZTDP (0.13 ± 0.02), and AZTTP (0.13 ± 0.02) in SIV-infected PBMCs were 5.3-, 5.5-, 2.5-, and 2.9-fold greater than those in uninfected PBMCs, a difference which was highly significant (Fig. 5).

**DISCUSSION**

The kinetics of intracellular AZT phosphorylation in human PBMCs showed that AZT is rapidly converted to AZTMP (Fig. 1B). Similar observations for human PBMCs (1, 29) and human placental trophoblast and Hofbauer cells (24) have been reported previously. Beyond the 4-h period, an additional increase in intracellular metabolite levels was observed. The kinetics of intracellular AZT phosphorylation to AZTMP in macaque PBMCs were also found to be rapid (Fig. 1A). In contrast to human PBMCs, a decrease in AZTMP levels was observed after 4 h in macaque PBMCs. A similar phenomenon was also observed in human placental trophoblast cells (24); human lymphocytic lines HL60, H9, and K-562 (8), ATH8, Molt-4/F, and MT4 (3, 4), CEM (2), and CCRE-CEM (9); and activated human PBMCs (1). The mechanism for this decline in AZTMP levels is not clear.

A large increase (7.7-fold) in intracellular AZTMP levels was detected in both human and macaque PBMCs over the extracellular AZT concentration range of 0.45 to 14.4 μM (Fig. 2). In contrast, the time-dependent accumulation of intracellular AZTDP and AZTTP levels was smaller for these cells. These observations are in agreement with those reported previously over a similar AZT concentration range in both activated and resting PBMCs (29). These data support the notion that the rate-limiting step of AZT phosphorylation is the conversion of AZTMP to AZTDP by thymidylate kinase, as evidenced by the significantly lower AZTDP/AZTTP ratio when compared with the AZTMP/AZTTP ratio (Fig. 3). Both human and macaque PBMC studies showed that 0 to 8 μM extracellular AZT produced an increase in intracellular AZT levels and in the levels of its phosphorylated metabolites. However, higher extracellular AZT concentrations (8 to 16 μM) failed to produce further increases in the levels of intracellular phosphorylated products (Fig. 2). Hence, an
increase in the extracellular AZT concentration produces diminishing returns in terms of the amount of the active moiety (AZTTP) that is formed. These data therefore probably explain the clinical findings that a 100-mg dose is as effective as a 250-mg dose of AZT (7).

Although the intraindividual variation (CV, <7.5% for all metabolites) in AZT phosphorylation in healthy human PBMCs is low (Fig. 4A), indicating the excellent reproducibility of the assay, the interindividual variability in phosphorylation of AZT is significant (CVs, 60% for AZTMP, 41% for AZTDP, and 33% for AZTTP; Fig. 4B). A similar interindividual variation in AZTMP levels was observed in the PBMCs of SIV-infected macaques (CV, 54%) but not in the PBMCs of healthy macaques (Fig. 5). Although this variability has previously been reported for human PBMCs by Kuster et al. (15) and Törnevik et al. (29), the underlying basis for the variation is not clear.

Comparison of AZT phosphorylation between healthy and SIV-infected macaque PBMCs showed that the levels of AZT metabolites were significantly (2.5- to 5-fold) higher in SIV-infected macaques (Fig. 5). These data are similar to those reported by Slusher et al. (26), who observed that the intracellular levels of AZT, AZTMP, AZTDP, and AZTTP in HIV-infected patient PBMCs were nearly 10-fold higher than those in the PBMCs of healthy volunteers. Toyoshima et al. (30) also reported a similar increase in intracellular AZT metabolite levels in the PBMCs of two HIV-infected patients. The higher intracellular levels of AZT (picomoles per 10⁷ cells) in SIV-infected PBMCs suggest an increased cellular volume (although alternate explanations such as modulation of the nucleotide pools in the infected cells are possible) of the infected cells. Assuming that the extra- and intracellular concentrations of AZT should be at equilibrium, we computed the intracellular volume (cellular volume = mean intracellular amount of AZT/extracellular concentration of AZT) and
therefore the intracellular concentration of the AZT metabolites. On the basis of that computation, the mean AZT TMP concentrations were found to be similar (1.2 μM) in both SIV-infected and uninfected cells. However, the mean concentrations of AZTDP and AZTTP were found to be twofold lower in SIV-infected PBMCs (Table 1), suggesting the possible use of these intracellular AZT metabolites in the presence of productive SIV infection. We recognize that because of the efflux of AZT during the washing procedure, the calculated volumes may be underestimate. However, since the inter- and intra-individual variabilities in the AZT levels were small, the relative magnitudes of the difference in these levels in SIV-infected and uninfected should be an accurate reflection of the differences in AZT levels prior to washing.

The mechanism for the increase in AZT phosphorylation in HIV- or SIV-infected PBMCs is not known. Arnér et al. (1) recently reported a fivefold increase in AZT phosphorylation in HIV-infected PBMCs. They hypothesized that a mitogen-like stimulatory factor(s) in the HIV-bearing supernatant may be responsible for an induction in thymidine kinase activity. However, in their study the increase in thymidine kinase activity was found to be only twofold. Alternatively, a change in cellular volume may explain the increase in AZT TMP levels (on a per-cell basis) because of lymphocyte activation in HIV- and SIV-infected macaque PBMCs. Because of the well-documented increase in the cellular volume of lymphocyte activation by phytohemagglutinin or HIV infection (1, 14, 23), the cellular concentration (micromolar) rather than amount (picomoles per 10^7 cells) may better reflect cellular metabolite profiles. When the cellular AZTMP contents expressed as amounts per cell are converted to intracellular concentration (1.2 μM), there was no difference in the AZT TMP concentration in infected versus uninfected macaque PBMCs. Interestingly, the intracellular concentrations of AZTDP and AZTTP were lower in the SIV-infected PBMCs. This may be a reflection of the use of AZTTP by the productively infected cells.

In conclusion, healthy and SIV-infected macaque PBMCs showed an AZT phosphorylation profile comparable to that of healthy and HIV-infected human PBMCs. Thus, these data provide further evidence that the macaque, M. nemestrina, is a suitable animal model for use in the study of factors affecting in vivo intracellular AZT metabolism during infection with an immunodeficiency virus.

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