Mutagenicity and loss of heterozygosity at the APRT locus in human lymphoblastoid cells exposed to 3’-azido-3’-deoxythymidine

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Previous experiments in our research group showed that 3’-azido-3’-deoxythymidine (AZT) caused increased mutant frequencies (Mf) at the X-linked hypoxanthine-guanine phosphoribosyltransferase (HPRT) and the autosomal thymidine kinase (TK) genes in human lymphoblastoid cells and that there was a significant positive correlation between AZT incorporation into cellular DNA and AZT-induced mutant Mf. In the current study, the mutagenicity of AZT was further evaluated at the autosomal adenine phosphoribosyltransferase (APRT) gene. AZH1 cells, a human lymphoblastoid cell line heterozygous at the APRT locus, were exposed to 300 µM AZT for 0, 1, 3 or 6 days or to 0, 33, 100, 300 or 900 µM AZT for 3 days (n = 5 flasks/group). A cell cloning assay was used to quantify APRT Mf. AZT-induced APRT Mf increased with extended duration and with incremental concentrations of AZT exposure. There was a positive correlation (P = 0.022, coefficient = 0.93) between AZT incorporation into DNA and AZT-induced APRT Mf. RFLP analyses indicated that AZT exclusively induced loss of heterozygosity in APRT mutants. These results, which are consistent with findings on the mutagenicity of AZT at the HPRT and TK genes, indicate the need for further investigations on the potential long-term side effects of AZT on humans, especially those who receive AZT for a prophylactic reason.

Introduction

3’-Azido-3’-deoxythymidine (AZT), a nucleoside analog widely used in the treatment of HIV-infected patients and the prevention of maternal viral transmission of HIV during pregnancy, causes vaginal epithelial cell tumors in adult mice and rats and induces liver, lung and female reproductive tumors in mice exposed during intrauterine life (Ayers et al., 1996; Olivero et al., 1997; Diwan et al., 1999). The remarkable success of AZT and other anti-retroviral treatments in providing clinical and immunological improvements in AIDS patients and in saving the lives of thousands of children born annually to HIV-infected women strongly supports the continued use of these drugs (IARC, 2000). Nonetheless, transplacental exposures to single agent or combination drug regimens may constitute a potential cancer risk for HIV-negative children born to HIV-infected women who received anti-retroviral therapy during pregnancy (IARC, 2000). Although the short-term toxicity of AZT prophylaxis in infants appears minimal, long-term effects are unknown, and very little is known about the short- or long-term effects of in utero exposure to other anti-retroviral drugs. Thus, there is a need to better define the genotoxic potential of AZT and other anti-retroviral agents, to investigate the potential long-term health hazards of perinatal anti-retroviral therapies and to determine if current therapeutic regimes can be modified in ways that decrease genetic risk without affecting efficacy.

The genotoxicity of AZT has been variably demonstrated in human cell lines, mice and HIV-infected patients using multiple end points, including micronuclei, sister chromatid exchange, chromosomal aberrations and gene mutations (Oleson and Gettman, 1990; Phillips et al., 1991; Shafik et al., 1991; Grdina et al., 1992; Gonzalez-Cid and Larripa, 1994; Ayers et al., 1996; Dertinger et al., 1996; Agarwal and Olivero, 1997). AZT incorporation into DNA, a potential mechanism underlying AZT genotoxicity, has also been detected in cultured cells from multiple species, in tissues from animal models and in blood cells from AZT-exposed adults and newborn infants (Sommadossi et al., 1989; Vazquez-Padua et al., 1990; Darnowski and Goulette, 1994; Olivero et al., 1994, 1997, 1999). Furthermore, recent studies in our research group indicate a direct correlation between DNA incorporation of AZT and AZT-induced mutagenicity at the X-linked hypoxanthine-guanine phosphoribosyltransferase (HPRT) locus in human B lymphoblastoid cells (Sussman et al., 1999). In TK6 cells, AZT accumulated in DNA as exposure time was extended and led to a significant increase in HPRT mutant frequency (Mf). Most (64%) of the AZT-induced mutations were attributable to whole gene deletions, consistent with the action of AZT as a DNA chain terminator (Sussman et al., 1999). This research was subsequently expanded to investigate the mutagenicity of AZT at the thymidine kinase (TK) and adenine phosphoribosyltransferase (APRT) loci, two autosomal genes that are more suitable targets for studying large gene deletions in cultured human cells (Liber et al., 1989).

The purpose of the present study was to evaluate the effects of duration of AZT exposure and concentration of AZT on mutant frequency and loss of heterozygosity (LOH) in the APRT gene of cultured AZH1 human B lymphoblastoid cells. AZH1 cells are heterozygous at the APRT (and TK) locus and hemizygous at the HPRT locus (Pongsaensook et al., 1997). The resulting data were compared with those obtained in parallel studies of the mutagenicity of AZT at the HPRT (Sussman et al., 1999) and TK (Meng et al., 2000a) loci of similarly exposed TK6 or AZH1 cells.

Materials and methods

Cell culture and exposure

AZH1 cells were grown in suspension in 75 cm2 tissue culture flasks containing RPMI 1640 medium supplemented with 10% fetal bovine serum, 12.5 mM HEPES buffer, 4 mM L-glutamine, 100 µM non-essential amino acids and 100 U/ml penicillin/streptomycin. Cells were counted daily and subcultured at 4×106 cells/ml. Prior to experimental exposure, cells were grown for 2 days in medium containing CAAT (cytidine, adenosine, aminopterin and thymidine)
Effects of AZT exposure on AZH1 cell survival

Relative cell survival was assessed by comparing the cloning efficiency in AZT-exposed cells versus that in unexposed cells immediately after exposure (with the control value set at 100%). Cell survival declined with extension of the exposure duration to 300 μM AZT up to 6 days (Figure 1) or with increase of the exposure concentration up to 900 μM for 3 days (Figure 2). The two highest dosing regimes of 300 μM AZT for 6 days and 900 μM for 3 days resulted in similar cell survivals of nearly 60%. These levels of cell survival indicate that there were sufficient viable cells after AZT exposure to permit meaningful measurements of APRT Mf.

Effects of AZT exposure duration and concentration on APRT mutant frequencies

To determine the effects of AZT exposure duration on APRT Mf, cultures (n = 5 flasks/group) were exposed to 300 μM AZT for 0, 1, 3 or 6 days. Immediately after exposure, cells were washed and plated at a density of 2 or 4 viable cells/well in 96-well U-bottom microtiter plates in the presence of 4x10^5 lethally irradiated feeder cells to determine relative survival of treated cells versus unexposed cells by measuring cloning efficiency. The remaining cells were subcultured daily for 6 days to allow expression of the mutant phenotype and seeded in 96-well plates as described below to measure APRT Mf.

To determine the effects of exposure concentration on APRT Mf, cultures (n = 5 flasks/group) were exposed to 0, 33, 100, 300 or 900 μM AZT for 3 days. Aliquots of cells were plated as described above for the determination of relative cell survival. The remaining cells were subcultured daily for 6 days and then plated to measure APRT Mf. To generate additional independent mutant colonies for molecular analysis, 30 extra cultures (15 control, 15 exposed to 900 μM AZT for 3 days, 10 ml/flask) were plated.

Cell cloning assay for APRT mutant frequencies

After exposure, cells were washed and subcultured in non-selective medium for 6 days to allow expression of APRT mutations. To determine the APRT Mf, a cell cloning assay was employed as previously described by Liber and Thilly (1982). Briefly, two 96-well U-bottom microtiter plates per sample were seeded with 2 viable cells/well in the presence of 4x10^5 lethally irradiated feeder cells/well to measure cloning efficiencies. To evaluate the APRT Mf for each sample, 10 96-well plates were seeded at 4x10^4 cells/well in the presence of 30 μg/ml 2,6-diaminopurine (DAP). The APRT-negative colonies were scored at 21 days after plating. Mf was calculated as the ratio of mean cloning efficiency in selective medium to that in non-selective medium (Sussman et al., 1999). One to three APRT mutants from each independent culture were collected for molecular analysis.

Restriction fragment length polymorphism (RFLP) analysis for determining LOH

A method developed by Pongsaensook et al. (1997) was used with some modifications. Genomic DNA from each mutant colony was extracted using a Tween-20/proteinase K digestion mixture. A 1410 bp fragment of APRT was PCR-amplified using primers APRT-F1429 (5'-GAGCTCTTCCTGACCTCCTC-3') and APRT-R2838 (5'-AAGGATGGTTCCTCCTG-3'). The 30 μl PCR reaction included 3 μg genomic DNA, 3 μl VM buffer (15 mM Tris-HCl, 2.75 mM MgCl2, 60 mM KCl), 2.5 mM each dNTP, 2 μM each primer and 15 U Taq polymerase. The thermal cycle consisted of a hot start (3 min at 94°C), 30 cycles of 1 min at 94°C, 1 min at 60°C and 2 min at 72°C, and a final cycle of 7 min at 72°C. The PCR reaction was electrophoresed on an 8% polyacrylamide gel. Gel bands were cut and DNA was eluted in 1xTE buffer. A second PCR using primers APRT-F1564 (5'-GACAGGC- TGCCCTGAGGAT-3') and APRT-R2838 amplified a 1274 bp fragment containing exons 3-5 of APRT. The 1410 bp APRT fragment eluted from the first round PCR was used as template and the PCR conditions were the same as described above. The PCR product was then purified using a QiAquick gel extraction kit (Qiagen) and digested with SfiI at 1 h at 37°C according to the specifications of the manufacturer. SfiI-digested DNA was then electrophoresed on an 8% polyacrylamide gel.

Statistical analyses

Statistical significance of the differences in Mf values between control and AZT-treated groups were determined using the Mann–Whitney U-statistic. The χ² test was used to measure the significance of the differences in LOH spectra between control and AZT-treated cells. A P value ≤ 0.05 was considered significant.

Results

Effects of AZT exposure on AZH1 cell survival

Relative cell survival was assessed by comparing the cloning efficiency in AZT-exposed cells versus that in unexposed cells immediately after exposure (with the control value set at 100%). Cell survival declined with extension of the exposure duration to 300 μM AZT up to 6 days (Figure 1) or with increase of the exposure concentration up to 900 μM for 3 days (Figure 2). The two highest dosing regimes of 300 μM AZT for 6 days and 900 μM for 3 days resulted in similar cell survivals of nearly 60%. These levels of cell survival indicate that there were sufficient viable cells after AZT exposure to permit meaningful measurements of APRT Mf.
LOH was not characterized. The percentages of LOH in control to 300 μM AZT for 0, 1, 3 or 6 days. A cell cloning assay was used to measure the frequency of APRT mutants. Mutant frequencies were significantly increased over background after 1 or more days of exposure (P values ranged from 0.048 to 0.004). AZT-induced mutant frequencies were obtained by subtracting the background mutant frequencies from that in AZT-exposed samples. Points, averages; bars, SE.

RFLP analysis of APRT mutant colonies

In AZH1 cells, there is a G:C→A:T transition in the non-functional APRT allele at position 1930, which results in the destruction of a StuI recognition sequence (Pongsaensook et al., 1997). This polymorphism was used to analyze LOH in APRT mutants by restriction analysis following PCR amplification. The 1274 bp fragment from the functional allele, which includes three StuI sites, including the polymorphic site, yields three fragments of 701, 366 and 207 bp. The non-functional allele, which contains two StuI sites, yields two fragments of 701 and 573 bp. Lanes 1 and 2 show four fragments of 701, 573, 366 and 207 bp, corresponding to the presence of both functional and non-functional alleles. Lanes 3 and 4 display two fragments of 701 and 573 bp, corresponding to loss of a StuI site in the functional allele of the APRT gene.

One to three mutant colonies were analyzed for LOH in each independent culture. Mutational specificity other than LOH was not characterized. The percentages of LOH in control and AZT-induced mutants are presented in Table I. The χ² test of the homogeneity of control and AZT-treated groups demonstrated that changes in LOH due to AZT treatment were significant (P = 0.014). The mutant fraction (i.e. average observed Mf/average percent of total mutants for a class of mutation) was used to estimate the induced Mf of each class of mutation (i.e. AZT-treated mutant fraction – spontaneous mutant fraction). The results indicate that the increase in the frequency of APRT mutant clones in AZT-treated cells was attributable solely to LOH.

Discussion

The proven effectiveness of durable treatment of HIV-1 infection with combination regimens that include AZT has tended to lower concerns about the potential mutagenic and carcinogenic risks of AZT and other anti-retroviral agents in AIDS patients. However, the increased use of AZT as a prophylactic agent for at-risk HIV-sero-negative individuals (Tokars et al., 1993) and pregnant HIV-positive women (Connor et al., 1994) warrants a closer look at the relationships between AZT therapy (alone or in drug combinations), DNA incorporation of AZT into host cells, the mutagenicity of AZT in mammalian cells and the potential long-term health risks in humans. Our laboratory has initiated a series of studies to assess the effects of exposure duration and concentration on DNA incorporation of AZT, induction of mutations in multiple reporter genes and the molecular nature of mutations in human lymphoblastoid cells (Sussman et al., 1999; Meng et al., 2000a; present study). These studies are part of a larger effort to investigate some of the same end points in T lymphocytes of rodents treated transplacentally with AZT and in T lymphocytes from infants of HIV-infected women who have received AZT during pregnancy.

Our previous studies demonstrated that AZT caused significant increases in TK and HPRT Mfs in TK6 cells exposed to 300 μM AZT for 3 days and the average AZT-induced TK Mfs were 2.2-fold greater than AZT-induced HPRT Mfs (Sussman et al., 1999; Meng et al., 2000a). The smaller mutagenic response at HPRT appears to be related to the hemizygous nature of this gene. Mutagenic mechanisms that involve homologous interaction, such as gene conversion and mitotic recombination, cannot occur at the X-linked HPRT
In addition, multigene deletions are likely to be lethal in HPRT mutant cells, because these gross deletions may span the adjacent genes essential for cell survival. In the current study, an average AZT-induced APRT \( M_f \) of 3.0 \( \times \) \( 10^{-6} \) was observed in cells exposed to 300 \( \mu \)M AZT for 3 days, which was similar to the average AZT-induced TK \( M_f \) value in similarly exposed cells (i.e. a significant increase of 6.5 \( \times \) \( 10^{-6} \) above background, \( P = 0.004 \)) (Meng et al., 2000a) but 3-fold greater than the average AZT-induced HPRT \( M_f \) value following identical treatment (i.e. a significant increase of 3.0 \( \times \) \( 10^{-6} \) above background, \( P = 0.004 \)) (Meng et al., 2000b).

The activation of AZT requires functional thymidine kinase, which raised concerns regarding the use of the TK gene to evaluate the mutagenicity of AZT. The current study showed that AZT-induced APRT \( M_f \)s were marginally greater than AZT-induced TK \( M_f \)s (average APRT:TK \( M_f \) ratio 1.4-fold) in human cells exposed to 33, 100, 300 or 900 \( \mu \)M AZT, but the differences were not significant (\( P > 0.05 \)) (Meng et al., 2000a). These findings indicate that the involvement of thymidine kinase in AZT anabolism had a minimal effect on the mutagenic response of AZT at the TK locus, which was in accordance with the fact that AZT triphosphate was formed before any AZT-related mutational events could occur.

Induced APRT \( M_f \)s after a 3 day exposure to 300 \( \mu \)M AZT were 3.9-fold greater than that following a 1 day AZT treatment, but no significant difference in AZT-induced \( M_f \)s between 3 and 6 days exposure was observed. These data showed that the rate of APRT mutation accumulation became slower after the initial accretion during AZT exposure. This phenomenon may be related in part to the supralinear shape of the curve describing AZT incorporation into DNA of TK6 cells exposed for 1–6 days (see Sussman et al., 1999), however, accumulation of somatic cell mutations over time should be further studied given the extended use of AZT in humans (i.e. usually months to years).

Highly significant correlations between AZT incorporation into cellular DNA and AZT-induced HPRT and TK \( M_f \)s were found in our previous investigations (Sussman et al., 1999; Meng et al., 2000a). Similarly, a positive correlation between AZT incorporation into DNA and AZT-induced APRT \( M_f \)s were observed in the current study (illustrated in Figure 6; \( P = 0.022 \), coefficient = 0.93, Pearson product moment correlation test; AZT incorporation into DNA data are from Meng et al., 2000a). These results substantiate the hypothesis that the adverse side effects of AZT are related, in part, to the incorporation of AZT into mammalian cellular DNA.

The systematic investigation of the relationships between DNA incorporation of AZT and mutagenicity in the HPRT, TK and APRT reporter genes in vitro provides a strong basis for comparing the mutagenic responses induced in cultured human lymphoblastoid cells with those produced in vivo at similar levels of AZT incorporation into DNA in T lymphocytes of mouse models exposed in utero to AZT. Such comparisons are potentially important because the levels of AZT incorporation into DNA that correlated with significant mutagenic responses in cultured human cells (Meng et al., 2000a) were similar to those found in DNA of target tissues for AZT-induced cancers in transplacentally exposed CD-1 mice (Oliverio et al., 1997). Thus, transplacental mutagenicity studies have been initiated to determine the levels of AZT incorporation into DNA and mutagenic effects at the Hprt locus of wild-type mice (CD-1 and B6C3F1 strains), at the Tk (and Hprt) locus of Tk heterozygous mice (Dobrovolsky et al., 1999) and at the Aprt (and Hprt) locus of Aprt heterozygous mice (Engle et al., 1996; Van Sloun et al., 1998; Wijnhoven et al., 1998; Liang et al., 2000).

In the current study, DAP was used as the mutant cell selection agent to facilitate future comparisons between AZT-induced mutagenic responses at the \( Aprt \) locus of AZH1 cells exposed in vitro and \( Aprt^{\pm} \) mice exposed in utero. In one of the recently established \( Aprt \) heterozygous mouse models, \( Aprt^{\pm} \) lymphocytes were selected using 50 \( \mu \)g/ml DAP and a reconstruction experiment indicated that the presence of DAP did not affect the colony-forming efficiency of \( Aprt^{\pm} \) cells (Liang et al., 2000). A comparison of DAP and 8-azaadenine

<table>
<thead>
<tr>
<th>Class of mutation</th>
<th>Spontaneous mutants*</th>
<th>AZT-treated mutants*</th>
<th>AZT-induced mutant fraction ± 10(-6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number (%)</td>
<td>Mutant fraction ± 10(-6)</td>
<td>Number (%)</td>
</tr>
<tr>
<td>LOH</td>
<td>48/63 (76.2%)</td>
<td>0.022</td>
<td>82/91 (90.1%)</td>
</tr>
<tr>
<td>Other type of mutationd</td>
<td>15/63 (23.8%)</td>
<td>3.4</td>
<td>9/91 (9.9%)</td>
</tr>
</tbody>
</table>

*Statistically significant differences between the spectra of spontaneous and AZT-treated APRT mutants (\( P = 0.014 \)) as determined by \( \chi^2 \) analysis.

**Mutant fraction = observed mutant frequency/percent of class of mutation.

Induced mutant fraction = (observed mutant frequency/percent of class of mutation in treated cells) – (percent of class of mutation in control cells).

Mutations other than LOH, including point mutations, frameshifts, small deletions/insertions, etc.
showed that selection of Aprt-deficient cells is independent of the particular selection drug in Aprt heterozygous mice (Liang et al., 2000). On the other hand, DAP, but not 8-azaadenine, has been found to induce breakage events in heterochromatin in a human cell line (Smith et al., 1998), which may elevate APRT Mfs during mutation selection. Although the use of DAP as the selection agent potentially reduces the sensitivity of the APRT mutation assay by giving higher background Mfs in vitro (Pongsansook et al., 1997), in the present study the fold increases above control Mf values were of less concern than the absolute increase in Mfs over background in APRT mutants in AZT-exposed AZH1 cells. In this case, the mutagenic potency of AZT was evaluated by determining AZT-induced Mfs (Mfs in AZT-treated cells – Mfs in control cells), therefore, measurements of the mutagenic effects of AZT should not be affected by the use of DAP considering that APRT-deficient cells in both AZT-treated and unexposed (control) cell samples were selected in the same fashion. Finally, the similarity in the frequency of LOH in DAP-selected APRT mutants in control samples in the current study (76%) was essentially the same (78%) as that reported by Pongsansook et al. (1997) in 8-azaadenine-selected mutants in control samples of the same cell line, suggesting that selection of APRT-deficient cells with 8-azaadenine would result in the same outcome as obtained with DAP in terms of the magnitude of the AZT-induced APRT Mfs and associated increases in LOH.

LOH is a common event in many types of tumor (Hansen et al., 1985; Fearson and Vogelstein, 1990) and AZT incorporation into nuclear DNA terminates chain elongation, which is a major mechanism for the induction of LOH. Therefore, the relative contribution of LOH in AZT mutation induction was determined at the HPRT (Sussman et al., 1999), TK (Meng et al., 2000a) and APRT (current study) genes in human lymphoblastoid cells. LOH accounted for 64% of AZT-induced HPRT mutants and 84% of AZT-induced TK mutants. The current study further indicated that AZT exclusively induced LOH in APRT mutants. Although there were minor differences in the percentage of LOH in AZT-induced HPRT, TK and APRT mutations, which were assumed to be related to the intrinsic nature of the individual genes (the simultaneously determined occurrences of LOH in spontaneous HPRT, TK and APRT mutations were 15.8, 63.3 and 76.2%, respectively), one consistent finding was that large deletion or LOH was the major mechanism of AZT mutation induction.

In summary, the genotoxicity of AZT has been demonstrated by several laboratories in multiple systems (Oleson and Gettann, 1990; Phillips et al., 1991; Shafik et al., 1991; Grdin et al., 1992; Gonzales-Cid and Larripa, 1994; Ayers et al., 1996; Dertinger et al., 1996; Agarwal and Olivero, 1997). Our studies have shown that AZT caused increased Mfs at the HPRT, TK and APRT loci in human lymphoblastoid cells and there were positive correlations between AZT incorporation into mammalian cellular DNA and AZT-induced TK or APRT Mfs. LOH, a cancer-related event, was responsible for the majority of AZT-induced mutations. Taken together, these data indicate the need for further cell culture, animal model and population studies on the potential long-term side effects of AZT and other anti-retroviral agents on humans, especially those who receive these drugs for a prophylactic purpose.

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References


Hansen,M.F., Koufos,A., Gallie,B.L., Phillips,R.A., Fodstad,O., Brogger,A., Darnowski,J.W. and Goulette,F.A. (1994) 3'/H11032 and Of Mutagen. Res.,7, 117–158. Differences are due to an additional class of mutations at the autosomal tk locus of human lymphoblastoid cells. In this case, the relative contribution of LOH in AZT mutation induction was determined by several laboratories in multiple systems (Oleson and Gettann, 1990; Phillips et al., 1991; Shafik et al., 1991; Grdina et al., 1992; Gonzales-Cid and Larripa, 1994; Ayers et al., 1996; Dertinger et al., 1996; Agarwal and Olivero, 1997). Our studies have shown that AZT caused increased Mfs at the HPRT, TK and APRT loci in human lymphoblastoid cells and there were positive correlations between AZT incorporation into mammalian cellular DNA and AZT-induced TK or APRT Mfs. LOH, a cancer-related event, was responsible for the majority of AZT-induced mutations. Taken together, these data indicate the need for further cell culture, animal model and population studies on the potential long-term side effects of AZT and other anti-retroviral agents on humans, especially those who receive these drugs for a prophylactic purpose.
Q. Meng et al.


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