

Azidothymidine causes functional and structural destruction of mitochondria, glutathione deficiency and HIV-1 promoter sensitization

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Mitochondrial functional and structural impairment and generation of oxidative stress have been implicated in aging, various diseases and chemotherapies. This study analyzed azidothymidine (AZT)-caused failures in mitochondrial functions, in redox regulation and activation of the HIV-1 gene expression. We monitored intracellular concentrations of ATP and glutathione (GSH) as the indicators of energy production and redox conditions, respectively, during the time-course experiments with U937 and MOLT4 human lymphoid cells in the presence of AZT (0.05 mg·mL⁻¹) or H₂O₂ (0.01 mM) for 15–25 days. Mitochondrial DNA integrity and NF-κB-driven HIV-1 promoter activity were also assessed. ATP concentration began to decrease within several days after exposure to AZT or H₂O₂, and the decrease continued to reach 30–40% of the normal level. However, decline of GSH was detectable after a retention period for at least 5–6 days, and progressed likewise. PCR

analyses found that mitochondrial DNA destruction occurred when the ATP and GSH depletion had progressed, detecting a difference in the deletion pattern between AZT and H₂O₂-treated cells. The GSH decrease coincided with HIV-1 promoter sensitization detected by enhanced DNA binding ability of NF-κB and induction of the gene expression upon H₂O₂-rechallenge. Our results suggest that, in the process of AIDS myopathy development, AZT or oxidative agents directly impair the energy-producing system of mitochondria, causing dysfunction of cellular redox control, which eventually leads to loss of the mitochondrial DNA integrity. The mechanism of cellular redox condition-mediated NF-κB activation is discussed.

Keywords: AZT; HIV-1; mitochondria; ATP; glutathione; oxidative stress.

Azidothymidine, or AZT, is the first anti-HIV drug that is now used in effective combination therapies against AIDS. Patients with AZT administration often develop myopathy [1] and glutathione (GSH) deficiency [2]; the former represents the defect in ATP production, and the latter persistent oxidative conditions caused by insufficient redox control. The two different biological responses are attributed to the function of mitochondria, where energy production is achieved by the electron transfer system.

The thymidine analogue compound designed to block the virus reverse transcription (RT) may affect mitochondrial DNA replication [3]. Uptake of AZT to mitochondrial intermembrane space and inhibition of ADP/ATP translocator were demonstrated with rat liver mitochondria [4]. Inhibition of adenylate kinase and NADH-linked enzyme activities by AZT was also observed *in vitro* [5,6]. ROS production and poly ADP-ribose polymerase acti-

vation were detected in AZT-induced cardiomyopathy in rats [7]. Despite the significance of these problems in drug design and clinical application, the process of the functional and structural destruction of mitochondria caused by AZT administration has not been fully investigated.

HIV gene expression is activated by transcription factor NF-κB through its binding to the two NF-κB motifs in the virus promoter/enhancer region of the long-terminal repeat (LTR) [8]. Oxidative agents induce NF-κB-dependent HIV-1 gene expression, which is inhibited by *N*-acetyl-L-cysteine (NAC), an antioxidant glutathione precursor [9]. In addition to the direct activation by oxidative stress, the HIV-1 promoter undergoes 'sensitization' under low level oxidative conditions generated either by AZT or low dose H₂O₂ [10,11]. Thus, cellular redox conditions affect HIV-1 gene expression at least in two mechanisms, and are possibly involved in the onset and progression of the disease. More importantly, AZT treatment is thought to be an unexpected cause of the oxidative stress-induced HIV-1 activation [10,11].

GSH, a cysteine-containing tripeptide (γ-glutamyl-cysteinyl-glycine), is abundantly expressed in eukaryotic cells, and plays an important role in regulation of cellular redox potential by eliminating ROS including H₂O₂. Intracellular concentration of this molecule is a good indicator of the oxidative conditions [12–14].

To assess the AZT-caused alterations in mitochondrial energy production and cellular redox conditions in

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Abbreviations: GSH, glutathione; CAT, chloramphenicol acetyl transferase; AZT, azidothymidine; HIV-1, human immunodeficiency virus 1; H₂O₂, hydrogen peroxide; NAC, *N*-acetyl-L-cysteine; LTR, long-terminal repeat.

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cultured lymphoid cells, we monitored ATP and GSH levels in U937 and MOLT4 cells cultured with AZT for 15 days. Time-courses with diluted H₂O₂ were also taken, by which direct cellular responses to moderate oxidative stress were demonstrated. As a marker of structural integrity of mitochondria, mitochondrial DNA was analyzed by PCR. Also examined was the occurrence of HIV-1 promoter sensitization defined by the fact that HIV-1 gene expression was inducible by a H₂O₂ re-challenge that is ineffective on the promoter under normal conditions.

In comparison with the GSH decrease, which became detectable after a retention period for several days, suppression of ATP production more rapidly occurred in cultures with AZT, indicating that inhibition of energy production precedes the generation of oxidative conditions. Mitochondrial DNA destruction became evident after the GSH deficiency had fully developed. The GSH deficient conditions appeared to facilitate ROS-induced activation of NF- κ B. These cellular responses to AZT may be involved in the process of AIDS myopathy and in HIV activation.

MATERIALS AND METHODS

Cells and plasmids

U937 and MOLT4 cells were maintained in RPMI 1640 with 10% fetal bovine serum and antibiotics (penicillin 10 streptomycin 100 μ g·mL). Cells were transfected with pCD12 (HIV-LTR-CAT [15]); or the mutant pCD12* [8] in combination with pSV2neo that expresses the neomycin resistance gene. In pCD12*, the two NF- κ B motifs (AGGGACTTTCC and GGGGACTTTCC) are replaced with mutant NF- κ B motifs (ACTCATTTC and GCTCACTTTCC), respectively [8]. Three weeks after transfection, G418-resistant cells were obtained. U937- and MOLT4-derived cells with pCD12 were termed U937CD and MOLT4CD, respectively. MOLT-4 transfectants with p12CD* were termed MOLT4CD*. Insertion of the plasmid DNA into chromosomes was confirmed by Southern hybridization as described previously [10].

Time-course experiments with AZT and H₂O₂

AZT-treatment was carried out with 0.05 mg·mL⁻¹ of AZT for 15 days. In H₂O₂ treatment, cells were incubated with 0.01 mM of H₂O₂ for 4 h everyday for a period of 25 days. Cells were maintained with or without NAC for an additional 24 h in some experiments.

Determination of intracellular ATP concentration

ATP concentration was determined using a sensitive bioluminescence technique [16]. Cells (5×10^6) were collected, washed with NaCl/P_i, and resuspended in 50 μ L of lysis buffer (100 mM Tris, 4 mM EDTA, pH 7.75). Thereafter, 450 μ L of the same buffer was added to the cell suspension, which was then boiled for 5 min at 100 °C. Samples (500 μ L) were centrifuged at 10 000 *g* for 2 min. ATP in the supernatant was measured with ATP Bioluminescence Assay Kit CLAUS (Boehringer Mannheim, Germany) according to the manufacturer's instructions.

Intracellular GSH assay

Washed cells (5×10^6) were sonicated in ice-cold 5% metaphosphoric acid and centrifuged for 10 min at 2500 *g*. GSH in the supernatant was measured by the thioester method [14] using GSH-400 (Bioxytech S. A.).

PCR

Mitochondrial DNA integrity was analyzed by PCR. A 5.2-kb segment was amplified from 10 ng of total DNA in a 100- μ L reaction mixture containing 200 μ M of each dNTP, 1 μ M of forward and reverse primers (5'-ACGAA AATCTGTTCGCTTCA-3' and 5'-TCTTGTTTCATTGT TAAGGTT-3') [17], 5 U of *Taq* DNA polymerase (PerkinElmer Cetus), 50 mM KCl, 10 mM Tris/HCl (pH 8.3), and 1.5 mM MgCl₂. The reactions were carried out for 35 cycles using a thermal cycler (PerkinElmer Cetus). Conditions were as follows: 94 °C for 15 s (denaturation), at 45 °C for 15 s (annealing), and at 72 °C for 60 s (primer extension). Amplified fragments were analyzed by electrophoresis followed by staining with ethidium bromide.

CAT assay

The cells were re-challenged with 0.05 mM H₂O₂ for 1 h, cultured further in normal medium for 48 h, collected, and washed with NaCl/P_i. Samples of 5×10^5 cells were suspended in 0.25 M Tris/HCl (pH 8.0), and extracts prepared by five cycles of freezing (-80 °C) and thawing. Chloramphenicol acetyl transferase (CAT) activity was measured by incubating whole cell extracts with ¹⁴C-labeled chloramphenicol and 5 mM acetyl coenzyme A at 37 °C for 18 h. Acetylated chloramphenicol was separated from nonacetylated chloramphenicol by ascending thin-layer chromatography [18]. Chromatograms were examined and quantified with a Fuji image analyzer BA100.

HIV-1-LTR DNA binding assay

HIV-1-LTR DNA was digested with *Sac*I and *Pvu*II to obtain a 120-bp DNA fragment [18]. The fragment containing NF- κ B motifs was end-labeled with [γ -³²P]ATP. Nuclear extracts were prepared by the method of Dignam *et al.* [19] after treatment with AZT or H₂O₂. Three nanograms of the end-labeled DNA were incubated with 3 μ g of nuclear proteins in a solution of 20 mM Hepes buffer (pH 7.9), 100 mM KCl, 20% (v/v) glycerol, 0.2 mM EDTA, 0.5 mM dithiothreitol, 10 mM MgCl₂, 125 mM spermidine and 3 μ g poly(dI-dC) for 20 min. The reactions were subjected to electrophoresis in 4% polyacrylamide gel in a Tris/borate/EDTA buffer. For competition assays, excess amounts of the cold 120 bp fragments and a synthetic mutant sequence of the NF- κ B motif (5'-TCGACAGAA TTCACCTTCCGAGAGGCTCGA-3' [20]) were included into the binding reaction. For super-shift assays, a 10-fold dilution of the rabbit anti-(NF- κ B) Ig (p65) (Santa Cruz Biotech.) was added. NF- κ B-DNA complexes were identified by electrophoresis and quantified, as described previously [11,21].

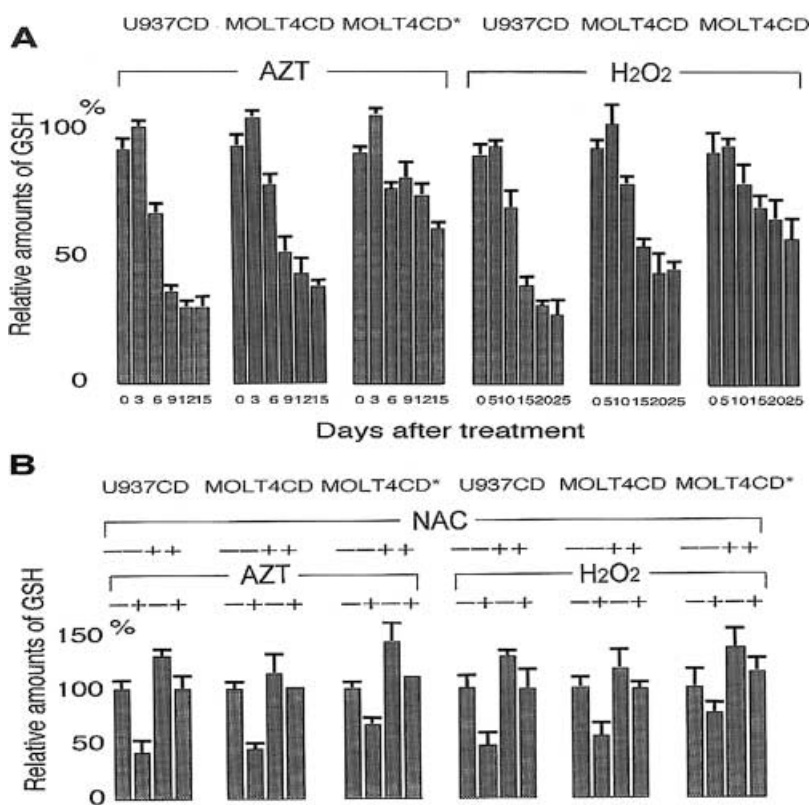


Fig. 1. GSH decrease caused by AZT- and H₂O₂-treatments. Cells were treated with 0.05 mg·mL⁻¹ of AZT for 15 days or with 0.01 mM of H₂O₂ for 25 days as described in the methods section. (A) Changes in the intracellular GSH concentration during the time-courses. AZT and H₂O₂ experiments are bracketed. Cell lines U937CD, MOLT4CD, or MOLT4CD* used for each experiment is indicated above. Time-courses are indicated in days at the bottom. GSH concentrations are expressed in relative amounts (%) to that of the control untreated cells (100%). (B) Effects of NAC treatment on the GSH level. Presence (+) or absence (-) of NAC (20 mM) in the cultures for the last 24 h before harvest is indicated on top. AZT-treated cells (after a 15-day incubation) and H₂O₂-treated cells (after 25-day incubation) are marked by +, and their control cells by -, in a bracketed section for AZT and H₂O₂ experiments. Error bands indicate standard deviation (SD) in three different experiments.

RESULTS

GSH deficiency caused by AZT-treatment

U937CD, MOLT4CD and MOLT4CD* cells were treated with 0.05 mg·mL⁻¹ of AZT for 15 days or with 0.01 mM H₂O₂ for 25 days. Cell samples (5×10^6 cells) were taken at 3-day intervals from the AZT cultures or at 5-day intervals from the H₂O₂-cultures to quantify intracellular GSH concentration. These cells and untransfected U937 cells contained ≈ 0.14 pg of GSH per cell under normal growth conditions. The GSH level was even a little augmented on day 3 after the first exposure to AZT, and began to decline on day 6. After a gradual decrease, GSH was at 32, 39 and 57% of the normal level in U937CD, MOLT4CD and MOLT4CD* cells, respectively, on day 15 (Fig. 1A). In H₂O₂-cultures, GSH alterations including the initial upregulation on day 5 and the gradual decrease in the following period were also observed. On day 25, relative amounts of GSH were approximately 29%, 39% and 59% in U937CD, MOLT4CD and MOLT4CD* cells, respectively (Fig. 1A). Both the cells in the 15-day culture with AZT and those in the 25-day culture with H₂O₂ were able to recover from the GSH deficiency by incubation with NAC (20 mM) for additional 24 h prior to cell harvest (Fig. 1B).

Impairment of ATP production in AZT-treated cells

Cellular ATP concentrations were also quantified for the cultures treated either with AZT or H₂O₂ during the time-courses. Control untreated U937CD, MOLT4CD and U937 cultures contained $\approx 4 \times 10^{-12}$ mol of ATP per cell. Figure 2A shows the changes in relative amounts of ATP in

U937CD and MOLT4CD cells treated with AZT or H₂O₂. ATP concentration decreased gradually after exposure to AZT, and reached $\approx 38\%$ of the normal level in U937CD and 35% in MOLT4CD cells on day 15. In H₂O₂-treated cells, the level of ATP declined similarly. On day 25, U937CD and MOLT4CD contained 45 and 48% of the normal concentration of ATP, respectively. Even when 20 mM of NAC, a GSH precursor, was included in the AZT- and H₂O₂-cultures for 24 h prior to cell harvest, the ATP decrease was not restored. In control cultures, however, NAC incorporation caused a 15–30% increase in ATP amount (Fig. 2B).

Thus, ATP productivity was significantly decreased in the cultures with AZT at the clinically effective concentration. Furthermore, low doses of H₂O₂ was able to mimic the drug effect. However, the impairment occurred in the early phase (day 3–5) of the experiment when the GSH level was still sufficiently retained (cf. Fig. 1). NAC rescued the GSH depletion in the late phase, but not the ATP decrease.

Destruction of mitochondrial DNA

To assess changes in the mitochondrial DNA integrity during the courses of AZT and H₂O₂ experiments, DNA from the mitochondrial fraction was subjected to PCR analysis in which a 5.2-kb region was amplified with a primer pair, 5'-ACGAAAATCTGTTTCGCTTCA-3', and 5'-TCTTGTTTCATTGTTAAGGTT-3' [17]. In control U937CD cells, the 5.2-kb segment was found intact (Fig. 3, lanes 7 and 18). However, after 12-day incubation with AZT, shorter fragments of 2.0-, 1.5- and 1.0-kb were also detectable (lane 10). On day 20 of the H₂O₂ treatment, 1.7-, 1.3- and 1.2-kb new bands appeared (lane 21).

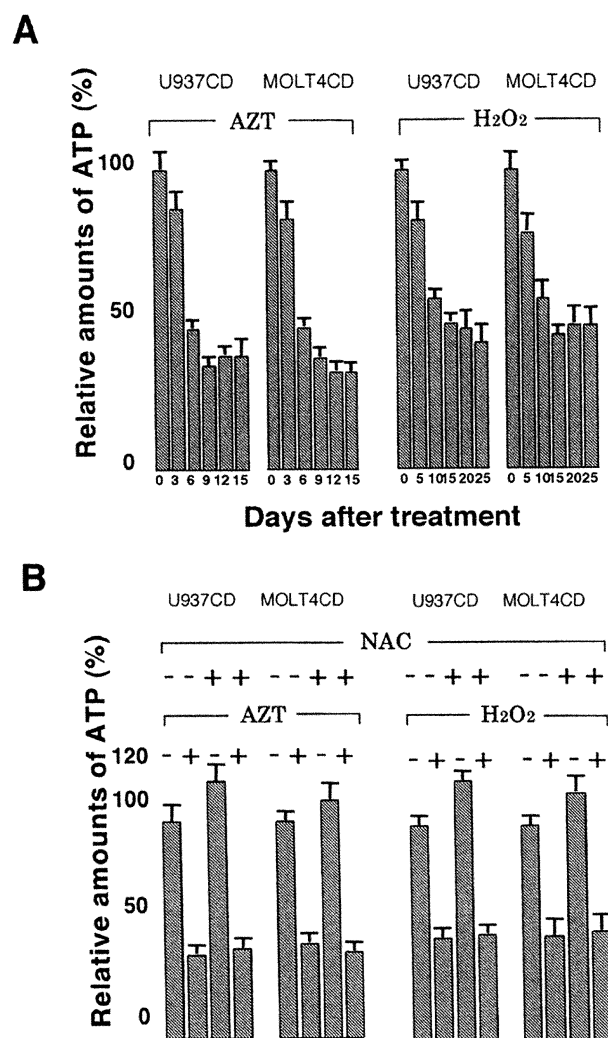


Fig. 2. Decrease of intracellular ATP concentration during the AZT and H₂O₂ experiments. (A) Rapid decrease in ATP concentration. Relative ATP quantities (%) to that in the control cells on day 0 (100%) are shown in histogram. Cell lines used are indicated. (B) Effect of NAC on the ATP decrease. Cultures incubated with NAC for the last 24 h (+) and those without NAC (–) are indicated. AZT- or H₂O₂-treated cells (+) and untreated control cells (–) are indicated. Error bands indicate SD in three independent results.

Concomitant loss in the 5.2-kb band intensity was observed with the appearance and intensification of the deleted fragments. Even when NAC was added to the AZT- and H₂O₂-cultures 24 h before each time point of cell harvest, the DNA integrity was not restored (lanes 3–5 and 14–16). However, if NAC was included in those cultures throughout the experiments, it completely protected mitochondrial DNA either in the AZT or H₂O₂ cultures (lanes 6 and 17). These results indicate that although chemical and enzymatic mechanisms acting in the mitochondrial DNA breakdown may not be exactly the same, generation of oxidative conditions are found critical for the DNA destruction caused by AZT and H₂O₂. Thus, deletion of mitochondrial DNA occurred after the significant reduction in ATP and GSH quantities, indicating that the failures in ATP production and redox control were not caused by

the DNA destruction in mitochondria. Furthermore, the deletion profile in the 5.2-kb region differed between AZT- and H₂O₂-treated mitochondria, suggesting that AZT causes damage in the DNA structure and/or replication by a molecular mechanism different from that of H₂O₂ in which oxide radical-induced DNA strand breakage is expected.

NF- κ B activation upon rechallenge by a higher dose H₂O₂

In the time-course experiments with AZT and H₂O₂, cells were re-challenged with 0.05 mM of H₂O₂ for 1 h, washed with normal medium and cultured for an additional 3 h. Nuclear extracts were obtained from the re-challenged cells and subjected to electrophoretic mobility shift assays using a 120-bp *SacI–PvuII* HIV-1 LTR fragment harboring two NF- κ B binding sites. In the AZT-cultures, the band shift efficiency was 5.3-fold elevated on day 9, and 9.5-fold on day 12. The level was retained to day 15. Similarly, the band shift efficiency in the H₂O₂-cultures increased by 6.7-fold of the control in 15 days and reached the plateau of 10.7-fold activation on day 20 (Fig. 4A). Presence of the NF- κ B p65 protein in the shifted complex was confirmed by detection of a super-shift band using a the p65 antibody either in the AZT-treated cells (day 15) or in the H₂O₂-treated cells (day 25). Furthermore, this NF- κ B activation was undetectable if cells were incubated with 20 mM NAC for additional 24 h before re-challenge (Fig. 4B). The time-course of NF- κ B activation matched the progress of GSH deficiency (Fig. 1) either in AZT- or H₂O₂-cultures. Without the re-challenge, NF- κ B activation was not detectable as described previously [10,11]. These results indicate that in the AZT-treated, GSH-deficient cells, either NF- κ B molecule itself or the signal cascade to NF- κ B activation is modified.

HIV-1 LTR-driven gene expression induced by rechallenge

Cell extracts obtained 48 h after the re-challenge were examined for CAT enzyme activity. The reporter gene expression by the HIV-1 promoter was found to be increased in a time-course reflecting the enhancement of the NF- κ B DNA binding activity in either AZT- or H₂O₂-treated cells (Fig. 5A). This induction of CAT expression was not observed when assays were performed without the re-challenge or when the promoter lacked the NF- κ B-binding sequences (Fig. 5A), as described previously [10,11]. Furthermore, NAC incorporation 24 h before the re-challenge cancelled this activation (Fig. 5B). Thus, HIV-1 LTR in GSH-deficient cells is able to exert a strong, NF- κ B-dependent transcriptional activity in response to oxidative stimuli.

DISCUSSION

In this study, we analyzed AZT-driven physiological alterations in cultured lymphoid cell lines. ATP decrease was detectable in advance to the other events. In the late phase, it could not be rescued by compensation of GSH with NAC. AZT was found to accumulate and inhibit various enzymes in mitochondria, including ADP/ATP translocator [4], adenylate kinase [6], NADH-cytochrome *c* reductase and those of NADH-linked respiration [5] in experiments

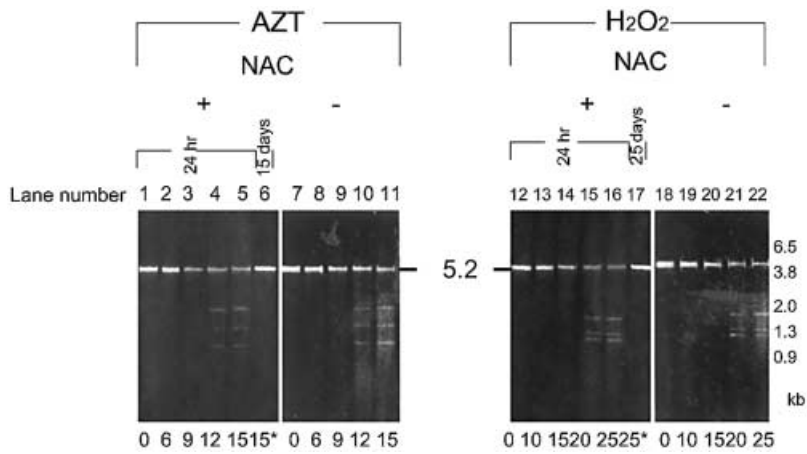


Fig. 3. Mitochondrial DNA deletion by PCR. U937CD cells incubated with AZT or H₂O₂ are examined for mitochondrial DNA integrity by PCR. Position of the amplified full-length DNA in 5.2 kb is indicated by bar. DNA size markers of 6.5, 3.8, 2.0, 1.3 and 0.9 kb are also shown with bars. Time-courses (in days) are indicated at the bottom. Deleted DNA bands found in the AZT-cell samples on days 12 and 15 are approximately 2.0, 1.5 and 1.0 kb in size. Those in the H₂O₂-cell samples on days 20 and 25 are 1.7, 1.3 and 1.2 kb in size.

with isolated mitochondria. The rapid decrease of ATP may reflect the direct effects of AZT on the mitochondrial energy producing system. The functional damage may lead to overproduction of a reactive oxygen species (ROS) [22].

In contrast to the rapid decline of ATP, GSH levels rose during the first 3–5 days of AZT or H₂O₂ administration. This might reflect the capability of the cytoplasmic redox control system. However, after the retention period, the

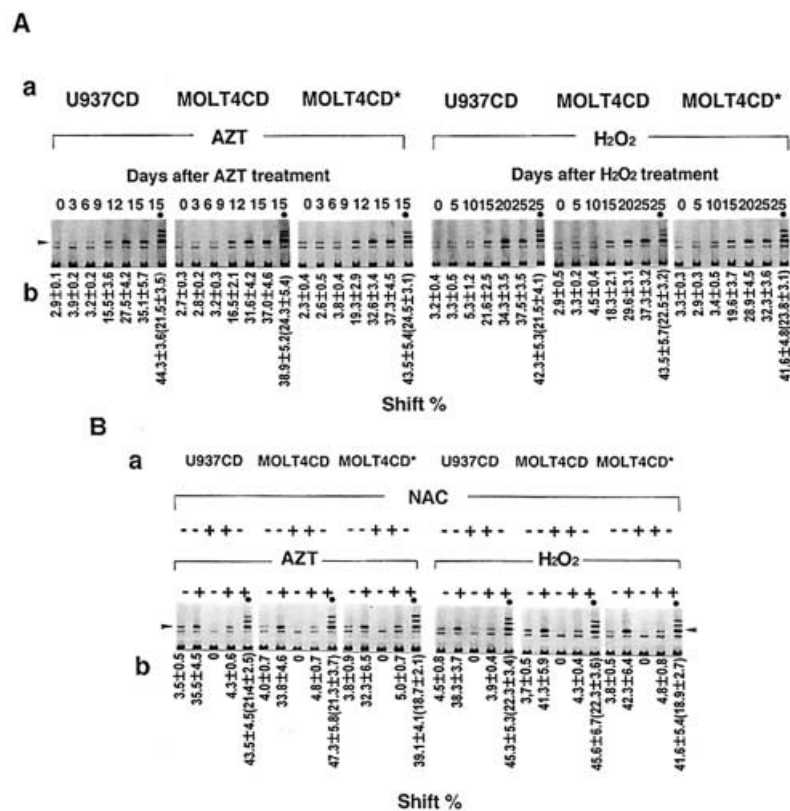
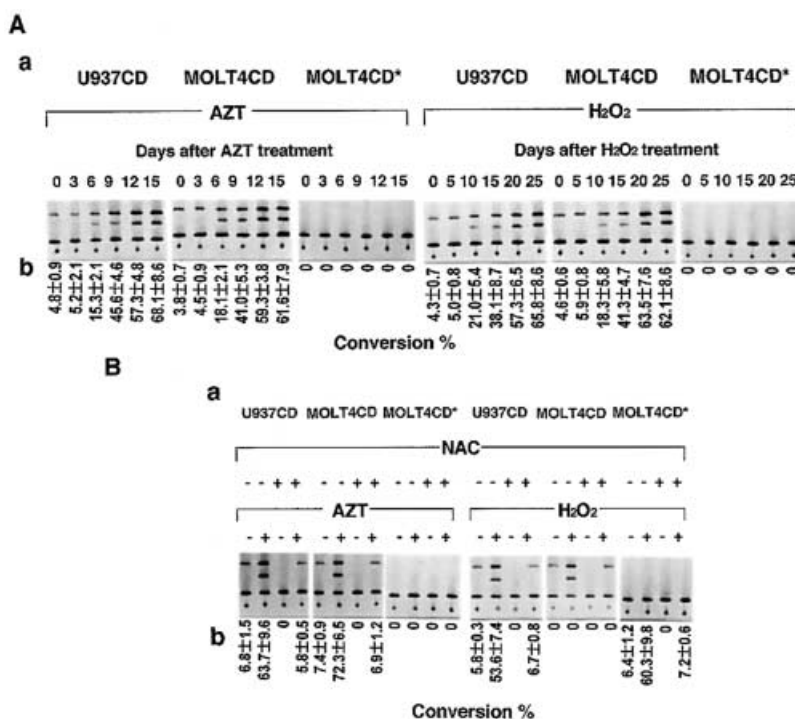


Fig. 4. Induction of the DNA binding ability of NF- κ B by rechallenge with H₂O₂. After the treatment with AZT or H₂O₂, cells were further incubated with 0.05 mM H₂O₂ for 1 h, and then in normal growth conditions for additional 3 h. Nuclear extracts were prepared for electrophoresis mobility shift assay. Typical results are shown. (A) Mobility shift assay with AZT- and H₂O₂-treated cell nuclear extracts. (a) Autoradiograms are shown. Position of the NF- κ B p65-bound DNA is indicated by arrow. Input DNA appears at the bottom in each lane. Binding experiments with an anti-(NF- κ B p65) Ig are marked with closed circles above the lanes. (b) Band shift efficiency was calculated as follows: shift (%) = (counts per min) of shifted band/(total counts per min) \times 100. Average of three experiments and SD are shown. (B) Effect of NAC on the DNA binding activity of NF- κ B. NAC-treatment was performed (+) for additional 24 h at the end of AZT- or H₂O₂-treatment before the rechallenge. (a) Autoradiograms of the experiments with NAC-treated (+) or -untreated (-) samples are shown. Cells after treatment with AZT or H₂O₂ are indicated as +, and their control cells as -. Experiments with an anti-(NF- κ B p65) Ig are indicated by closed circles. (b) The same as (b) in (A).

Fig. 5. Transcriptional activation of HIV-LTR promoter detected by CAT assay after rechallenge. (A) Activation of the CAT gene expression within 24 h after rechallenge.

(a) CAT enzyme activities determined by thin layer chromatography are shown. (b) Rate of the conversion of chloramphenicol to the acetylated form was determined as follows; Conversion (%) = (counts per min) of acetylated form of chloramphenicol/(total counts per min) × 100. Average of three experiments and SD are shown. (B) Reduction of CAT activity by NAC-incorporation after 15-day incubation with AZT or 25-day incubation with H₂O₂. Results of the CAT assay are shown in chromatograms (a) and relative enzyme activities (b).



GSH level began to decline and dropped to less than 50% of the normal level, suggesting that ROS production dominated to create chronic oxidative conditions.

Mitochondrial DNA deletion was detectable by PCR 12–15 days after AZT-treatment, or 20–25 days after H₂O₂ treatment when ATP and GSH deficiency had progressed. AZT is a thymidine analogue reverse transcription chain terminator, and has been speculated to influence the replication of mitochondrial DNA. Moreover, ROS produced by mitochondria may attack DNA as well as other molecules. In fact, mitochondrial DNA mutations derived from the modification of guanosine to 8-hydroxy-deoxyguanosine were detected in AIDS patients with myopathy [23,24]. The same modification occurs in oxygen radical reactions [25]. Cells treated with H₂O₂ also developed mitochondrial DNA deletion in the late phase of our experiment. Taken together, the DNA deletions detected in the AZT-cultures probably resulted from ROS overproduction. However, the difference in the deletion pattern between AZT- and H₂O₂-treated cells suggests involvement of a different chemical/biochemical reaction.

GSH deficiency significantly affected the activity of nuclear transcription factor NF-κB upon rechallenge with 0.05 mM H₂O₂. The antioxidant molecules play an important role in regulation of transcription factors causing nonenzymatic conformational changes [26,27]. Suppression of HIV-1 expression by GSH in chronically infected monocytic cells has been reported [28], which is consistent with our results. Conversely, thioredoxin, another reducing peptide, activates NF-κB [29]. Although the signal transduction from membrane receptor activation to nuclear translocation of NF-κB, which involves MEKK1 and IKK kinases, has been well studied [30,31], the mechanism of oxidative stress-induced NF-κB activation is not yet thoroughly understood. It is important to analyze NF-κB status

and cellular signal cascades linked to NF-κB under the GSH-deficient conditions in addition to exposure to various levels of oxidative stress.

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