Antiretroviral nucleoside reverse transcriptase inhibitors (NRTIs), given to human immunodeficiency virus-1–infected pregnant women to prevent vertical viral transmission, have caused mitochondrial dysfunction in some human infants. Here, we examined mitochondrial integrity in skeletal muscle from offspring of pregnant retroviral-free Erythrocebus patas dams administered human-equivalent NRTI doses for the last 10 weeks of gestation or for 10 weeks of gestation and 6 weeks after birth. Exposures included no drug, Zidovudine (AZT), Lamivudine (3TC), AZT/3TC, AZT/Didanosine (ddI), and Stavudine (d4T)/3TC. Offspring were examined at birth (n = 3 per group) and 1 year (n = 4 per group, not including 3TC alone). Circulating levels of creatine kinase were elevated at 1 year in the d4T/3TC-exposed group. Measurement of oxidative phosphorylation enzyme activities (complexes I, II, and IV) revealed minimal NRTI-induced changes at birth and at 1 year. Histochemistry for complex IV activity showed abnormal staining with activity depletion at birth and 1 year in groups exposed to AZT alone and to the 2-NRTI combinations. Electron microscopy of skeletal muscle at birth and 1 year of age showed mild to severe mitochondrial damage in all the NRTI-exposed groups, with 3TC inducing mild damage and the 2-NRTI combinations inducing extensive damage. At birth, mitochondrial DNA (mtDNA) was depleted by ~50% in groups exposed to AZT alone and the 2-NRTI combinations. At 1 year, the mtDNA levels had increased but remained significantly below normal. Therefore, skeletal muscle mitochondrial compromise occurs at birth and persists at 1 year of age (46 weeks after the last NRTI exposure) in perinatally exposed young monkeys, suggesting that similar events may occur in NRTI-exposed human infants.

Key Words: zidovudine; lamivudine; stavudine; didanosine; electron microscopy; mitochondrial DNA quantity; oxidative phosphorylation.
HIV-1 uninfected, may be at risk of developing potential long-term sequelae from perinatal NRTI exposures.

In adult HIV-1–infected patients, long-term NRTI use has been associated with mitochondrial toxicity that includes skeletal muscle and cardiac wasting (Beach, 1998; Dalakas et al., 1990; Lewis and Dalakas, 1995), elevated serum lactic acid (Brinkman, 2001; Gerard et al., 2000), abnormal oxidative phosphorylation (OXPHOS) enzyme activity (Mhiri et al., 1991; Tomelleri et al., 1992), and depletion in the quantity of mitochondrial DNA (mtDNA) (Dagan et al., 2002; Lewis and Dalakas, 1995). Few abnormal clinical findings have been reported at birth or during the early years of life in HIV-1–uninfected NRTI-exposed infants (Brogly et al., 2007; Caselli et al., 2000; Culinare et al., 1999; Hanson et al., 1999; Lipshultz et al., 2000; Mofenson and Munderi, 2002; Newschaffer et al., 2000; Tuomal et al., 2002); however, two children born to mothers receiving Combivir during pregnancy (Blanche et al., 1999) died at about 1 year of age of severe mitochondrial toxicity. In addition, in a cohort of approximately 2600 children exposed in utero to NRTIs, mitochondrial dysfunction was found in about 28 children under the age of 10 years, many of whom had no clinical symptoms (Barret et al., 2003). Morphological (electron microscopy [EM]) and molecular (mtDNA) evidence of mitochondrial compromise have been reported in umbilical cord, as well as cord and peripheral blood, from HIV-1–uninfected infants born to HIV-1–infected mothers (Divi et al., 2004; Poirier et al., 2003; Shiramizu et al., 2003). In one study, depletion of leukocyte mtDNA was found in a large fraction of AZT-exposed infants at birth and persisted in children at 2 years of age (Poirier et al., 2003), even though all of these children were clinically asymptomatic.

In order to explore the long-term effects of morphological and molecular compromise in primate infants exposed in utero to NRTIs, in the absence of virus, we are studying a breeding colony of Erythrocebus patas monkeys, where pregnant dams are given human-equivalent protocols of the NRTI combinations typically used in clinical practice. In this study, some of the monkey offspring were exposed to NRTIs in utero and taken by cesarean section at term. Others were exposed in utero, born naturally, exposed to NRTIs for the first 6 weeks of life, and followed up to 1 year of age. Skeletal muscle mitochondrial integrity was examined at birth and 1 year of age using several approaches that included morphological evaluation by EM, OXPHOS enzyme assays, OXPHOS tissue histochemistry for complex IV, and mtDNA quantification by Hybrid Capture-Chemiluminescence Immunoassay (HC-CIA).

### MATERIALS AND METHODS

**Monkey maintenance, NRTI sources, and exposure protocols.** Monkeys were maintained and exposed to NRTIs at Bioqual Inc. (Rockville, MD) under conditions approved by the American Association for Accreditation of Laboratory Animal Care, and exposures were performed in accordance with humane principles for laboratory animal care. Protocols were reviewed by the Institutional Animal Care and Use Committee of Bioqual, Inc. Female patas monkeys were kept with the males until the female was assessed to be pregnant. Pregnancies were ascertained as previously described (Lu et al., 1993). Pregnant dams were randomized by weight and assigned into different exposure groups. They were individually housed in stainless steel wire mesh metabolic cages under controlled conditions of temperature (20 ± 2°C) and light (12 h)-dark (12 h) circadian rhythm, with access to water and diet ad lib throughout the study. Pregnant dams were given NRTIs during the final 10 weeks or 4 weeks (depending on the exposure protocol) of the 20-week gestation.

The drugs and exposure protocols for pregnant monkeys and offspring are summarized in Table 1. For evaluation at birth, three to four monkeys were included in the study: no drug (n = 4), AZT (n = 3), 3TC (n = 3), AZT/3TC (n = 3), AZT/ddI (n = 3), and d4T/3TC (n = 3). For the 1-year studies, which did not include 3TC alone, the unexposed and NRTI-exposed groups each contained four monkeys.

AZT was obtained from Sigma Chemical Co. (St Louis, MO), and 3TC (Epivir) was obtained as a pediatric liquid clinical formulation from GlaxoWellcome (Raleigh, NC). All other NRTIs were purchased as clinical formulations from the National Institutes of Health Veterinary Pharmacy (Bethesda, MD). Each drug was dissolved in a simple syrup, Oral Plus (oral suspending vehicle), and banana cream flavoring to make it palatable. The banana cream–flavored drug mixture was placed in a groove made inside of a 1/4 to 1/3 piece of a banana, and the entire piece of banana was administered orally. AZT was given b.i.d. in 20 mg doses for a total of 40 mg AZT/day, 5 days/week for the last 10 weeks of gestation. 3TC was given in two 12 mg doses for a total of 24 mg/day, 5 days/week for the last 4 weeks of gestation. d4T (Zerit) was given as two 4.5 mg doses, for a total daily dose of 9 mg, 5 days/week for the last 10 weeks of gestation. DdI (Videx) purchased in powder form, and weighed into 20 mg aliquots, was subsequently mixed into a banana-flavored drink for dosing twice daily. DdI was given 30 min before a meal, along with an antacid (Zantac) for stomach protection. Monkeys were given a total of 40 mg ddI/day, 5 days/week for the last 10 weeks of gestation. Unexposed monkeys received the banana-flavored drink twice daily.

Monkeys offspring were taken either near term by cesarean section (Gerschenson et al., 2000–2004) or born naturally, hand raised for the first

### TABLE 1

| NRTI Treatment Protocols for Patas Dams during Gestation and for Offspring after Birth |
|-------------------------------|------------------|------------------|------------------|
| NRTI exposure | Mother | Infant | | |
| | Dose (mg/day) | Durationa (weeks) | Dose (mg/day) | Exposure (weeks after birth) |
| | | | | |
| None (n = 4) | — | — | — | — |
| AZT | 40 | 10 | 4 | 1–4 |
| | | | 6 | 5 and 6 |
| 3TC | 24 | 4 | — | — |
| AZT and 3TC | 40 | 10 | 4 | 1–4 |
| | | | 6 | 5 and 6 |
| | 24 | 4 | 2 | 1–4 |
| | | | 3 | 5 and 6 |
| AZT and ddI | 40 | 10 | 4 | 1–4 |
| | | | 6 | 5 and 6 |
| | 40 | 10 | 4 | 1–4 |
| d4T and 3TC | 9 | 10 | 0.6 | 1–4 |
| | | | 1.0 | 5 and 6 |
| | 24 | 4 | 2 | 1–4 |
| | | | 3 | 5 and 6 |

*aRefers to the last 4 or 10 weeks of gestation.
6 weeks of life, and grown to 1 year of age. Offspring were hand raised in order to decrease experimental variability and facilitate NRTI dosing of the infants for the first 6 weeks of life. For the 1-year study, newborn monkeys were dosed twice daily per os by syringe for 6 weeks of age, according to the protocols and doses shown in Table 1. NRTI doses were adjusted at 4 weeks of age in order to offset the weight gain due to growth. Offspring were taken 46 weeks after the last dose of NRTI was given and a complete necropsy performed to obtain tissue samples for evaluation of mitochondrial toxicity.

Isolation of skeletal muscle mitochondria. All steps for the isolation of mitochondria were performed at 4°C. Fat and connective tissue were first removed from skeletal muscle, 3–4 g of which was minced using a scalpel blade. The minced sample was homogenized in three steps of 30 s each in a 15–20 ml of homogenization buffer containing 210 mM mannitol, 70 mM sucrose, 1 mM EDTA, 20 mM HEPES (pH 7.4), 2 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride using a Polytron tissue processor (Sorvall, Asheville, NC). The tissue homogenate was centrifuged twice at 1000 × g for 5 min to remove cellular debris and nuclei. The mitochondria were collected by centrifugation at 20,000 × g for 20 min, gently resuspended in 50–100 μl aliquots of homogenization buffer, frozen in liquid nitrogen, and stored at −70°C. Mitochondrial protein was quantified by the Coomassie Brilliant Blue method using bovine serum albumin as a standard (Bradford, 1976).

OXPHOS enzyme-specific activity and protein assays. Specific activities of the OXPHOS enzyme complexes I, II, and IV were quantified using a Hewlett Packard diode array spectrophotometer as previously described (Trounce et al., 1996). Complex I: Nicotinamide Adenine Dinucleotide-reduced ubiquinone oxidoreductase, sensitive activity was measured by the oxidation of NADH. Complex II: succinate-ubiquinone oxidoreductase activity was measured by the reduction of 2,6-dichlorophenol-indophenol when coupled to complex II–catalyzed reduction of decylubiquinone. Complex IV: ferrocytochrome-c:oxygen oxidoreductase (COX) activity was measured by following the oxidation of reduced cytochrome c. Mitochondrial protein concentrations were measured by the Coomassie Brilliant Blue method using bovine serum albumin as a standard (Bradford, 1976).

Histochimical staining for OXPHOS enzymes. Staining for complex IV (cytochrome c oxidase, COX) activity was performed as detailed by Seligman et al. (1968). Sectioning of frozen blocks was followed by specific staining, dehydration, a xylene wash, and mounting with permount. The staining method involves oxidation of exogenous cytochrome c and 3,3′-diaminobenzidine by COX resulting in formation of insoluble brown color pigmentation at the site of enzyme activity.

Transmission EM of skeletal muscle and procurement and scoring of EM photomicrographs. Skeletal muscle from quadriceps was taken from one set of monkeys at birth and from additional monkeys at 1, 3, 6, 9, and 12 months of age. Muscle tissue was cut into 1–2 mm slices and fixed in 4% paraformaldehyde, 2% glutaraldehyde in 100 mM phosphate buffer, pH 7.4, at 4°C overnight. After 24 h, the fixative was replaced with sodium cacodylate buffer (0.1M). In preparation for sectioning, tissues were postfixed in 1% osmium tetroxide in the same buffer for 1 h at room temperature, rinsed in sodium cacodylate once, followed by two rinses with 0.1M sodium acetate buffer (pH 4.5), and en bloc staining with 0.5% (wt/vol) uranyl acetate in 0.1M sodium acetate buffer (pH 4.5) for 1 h. The tissues were dehydrated in a series of graded alcohol washes (e.g., 35, 50, 75, 95, and 100%) followed by 100% propylene oxide. The tissues were then infiltrated in equal volumes of propylene oxide and epoxy resin (LX-112, Ladd Research, Williston, VT), embedded in pure epoxy resin, and allowed to cure for 48 h at 55°C. Thin sections were made with an ultramicrotome (Ultracut E, Leica, Northvale, NJ) and mounted on a 200-mesh grid. The mounted thin sections were stained with uranyl acetate and lead citrate (EM Stain, Leica), stabilized by carbon and by electron evaporation (Denton Vacuum, Moores?ton, NJ), observed, and photographed with a ×10,000 magnification with an electron microscope (H7000, Hitachi, Tokyo, Japan) operated at 75 kV. Images were captured randomly while manually moving in a Z-line pattern on the grid. For each monkey, 10 EM photomicrographs, enlarged to ×30,000, were evaluated for mitochondrial morphology. The piece of tissue (2 × 4 mm) collected for EM represents a portion of the whole organ, and EM photomicrographs were captured randomly from different areas within that sample. The degree of mitochondrial and cellular pathology was scored by four different investigators and one outside pathologist, all of whom were given coded EM photographs to eliminate bias.

DNA preparation. DNA was extracted from tissues using the QiAamp DNA mini kit according to the manufacturer’s protocol (Qiagen, Valencia, CA). Briefly, tissues were equilibrated to room temperature, sliced into small pieces, washed with 100 mM phosphate-buffered saline (PBS) without calcium and magnesium (pH 7.4), homogenized in three volumes of PBS, lysed with Qiagen ATL buffer, and digested with proteinase K and RNase A. The DNA was separated using spin columns provided with the kit and was subsequently extracted into 10 mM Tris pH 7.4 containing 1 mM EDTA and stored at −70°C until analysis. The quality and quantity of DNA were determined spectrophotometrically by absorbance at 260 nm.

MtDNA quantification by HC-CIA. The protocol for HC-CIA is described in detail elsewhere (Divi et al., 2005) and was used with some modifications to measure the mtDNA in the patas skeletal muscle samples. Sample DNA (1.0 μg diluted in 10 μl of 10 mM TRIS, pH 8.0, 0.3 mM EDTA buffer) containing both nuclear and mtDNA was mixed with 0.1 μM patas monkey complex I (NADH dehydrogenase) ND4/ND5 (5′-amino-TTCCTAATTCGCCCACGGA) (GenBank ID: D85291) oligonucleotide (Invitrogen, Carlsbad, CA) in 10 μl of

| TABLE 2 | OXPHOS Complex I, II, and IV Enzyme Activities (nmol/min/mg protein ± SD) in Skeletal Muscle Mitochondria from Patas Infants Taken at Birth |
|----------------------|----------------------|----------------------|----------------------|
| NRTI exposure | Number of monkeys | Complex I | Complex II | Complex IV |
| None | 4 | 62.6 ± 2.3 | 137.5 ± 44.1 | 460.0 ± 68.5 |
| AZT | 3 | 66.2 ± 1.9 | 225.2 ± 79.1 | 440.5 ± 45.3 |
| AZT/3TC | 3 | 51.2 ± 35.0 | 153.7 ± 79.9 | 333.0 ± 184.9 |
| AZT/ddI | 3 | 67.1 ± 8.6 | 230.6 ± 13.8 | 299.4 ± 42.9 |
| d4T/3TC | 3 | 60.6 ± 3.6 | 133.9 ± 25.0 | 124.1 ± 52.2 |

*Value is significantly different (p < 0.05) from that found in unexposed monkeys by t-test.

| TABLE 3 | OXPHOS Complex I, II, and IV Enzyme Activities (nmol/min/mg protein ± SD) in Skeletal Muscle Mitochondria from 1-Year-Old Patas Monkeys |
|----------------------|----------------------|----------------------|----------------------|
| NRTI exposure | Number of monkeys | Complex I | Complex II | Complex IV |
| None | 4 | 82.6 ± 5.4 | 292.9 ± 15.2 | 404.5 ± 104.0 |
| AZT | 4 | 94.1 ± 11.9 | 291.7 ± 22.2 | 426.5 ± 38.3 |
| AZT/3TC | 4 | 51.86 ± 9.24b | 270.3 ± 18.1 | 481.3 ± 55.3 |
| AZT/ddI | 4 | 66.7 ± 10.5 | 241.8 ± 10.4ab | 503.2 ± 26.6 |
| d4T/3TC | 4 | 47.1 ± 9.8ab | 223.0 ± 16.0a | 336.9 ± 22.5 |

*Values are significantly different (p < 0.05) from those found in unexposed monkeys by t-test.

Values are not significantly different from those found in unexposed monkeys by Holm-Sidak test.
2X Expand High Fidelity PCR buffer without MgCl2 (Roche Diagnostics, Indianapolis, IN) and denatured together at 95°C for 5 min followed by hybridization at 60°C for 30 min. The hybrid was labeled with 1.0 µl of Digoxigenin (DIG)-Chem-Link (Roche) at 55°C for 30 min. Excess unreacted Chem-Link was quenched using 5.0 µl of stop solution provided by the manufacturer. The DIG-DNA was separated from unhybridized oligo and unreacted Chem-Link using YM-10 microcon spin columns (Millipore, Billerica, MA). An incubation mixture containing the ND4/ND5 oligonucleotide and a 500-bp ampiclon of the human cystic fibrosis (CFTR-Human) gene was prepared by PCR amplification, using primers specific for the CFTR gene (Ensembl gene ENSG00000001626; forward: 5'-AGAATCTAGATCAAATGGAAAGG; reverse: 5'-GACTTGCACTTGCTTGAGTTCCG) and the human genomic DNA as template, was processed through the above procedure to provide a negative control.

The purified hybrid was diluted to 1 ml in binding buffer (10mM phosphate buffer [pH 7.4] containing 1mM EDTA) and an aliquot (100 µl) in four replicates was transferred to a DNA-Bind (N-oxysucinimamide) 96-well plate (Corning, Acton, MA) and incubated at 37°C for 1 h to capture the hybrid. The plates were washed three times with 2X saline sodium citrate solution containing 0.1% sodium dodecyl sulfate and rinsed three times with the binding buffer. The void spaces in the wells were blocked with 0.25% I-block (Casein, PE Applied Biosystems, Foster City, CA) in PBS for 1 h at 37°C and washed three times with PBST (PBS with 0.05% Tween 20). Anti-DIG-alkaline phosphatase (Roche), diluted 1:2000 in I-block buffer, was added to all the wells and incubated for 1 h at room temperature before wells were washed three times with PBST and rinsed with 10mM Tris buffer (pH 8.0). Following incubation with alkaline phosphatase substrate (CDP-Star with Emerald II, PE Applied Biosystems) for 30 min at room temperature, luminescence was measured using a TR 717 Luminometer (PE Applied Biosystems). The luminescence signal from non-specific wells was always 6- to 10-fold lower than the luminescence signal from the specific wells. The values for mtDNA quantity are expressed as luminescence units (LU) per ng of total DNA.

Statistical methodology. For comparing the NRTI-exposed groups to the unexposed group for EM photos and mtDNA quantity, the data were first adjusted at 1 month of age to accommodate the patas monkey’s increase in body weight (Table 1).

Exposed and unexposed patas offspring did not show differences in physical activity, behavior, or birth weight. Clinical chemistry profiles were obtained at birth, 2 weeks of age, and at 1, 2, 3, 6, 9, and 12 months of age. For most clinical chemistry parameters, there were no differences between exposed and unexposed monkeys (data not shown). However, the level of circulating creatine kinase (CK), an indicator of muscle wasting, averaged 471 ± 129 (mean ± SE) U/l in unexposed offspring and ranged between 932 and 1482 U/l in NRTI-exposed offspring at birth. In monkeys at 1 year of age, the CK values in unexposed offspring averaged 657 ± 96 U/l (mean ± SE) and most NRTI-exposed groups were similar to the controls; however, the d4T/3TC group averaged 1740 ± 734 (mean ± SE) U/l. The large variability in the values among monkeys, when the number of monkeys per group is small, makes statistical evaluation difficult, but it is clear that some NRTI-exposed patas offspring have CK levels that are much higher than those found in any unexposed patas offspring. Therefore, NRTI exposure appears to result in sustained and persistent muscle damage in some individuals.

RESULTS

Overall Toxicity and Response to NRTI Exposures

The Centers for Disease Control and Prevention (CDC) have recommended that NRTIs be given for the last 6 months (67%) of human pregnancy. In the monkey model, however, in the interest of avoiding undue toxicity and possible teratogenic effects, we chose to administer the NRTIs to the pregnant monkey dams for the last either 10 weeks (approximately 50%) or 4 weeks (approximately 20%) of gestation (Table 1). In a clinical setting, NRTI therapy may be given throughout the pregnancy or only at the very end. Though the NRTI doses and treatment protocols given to HIV-1–infected women vary to some extent due to differences in body weights, for the purposes of designing the patas experiments, it was assumed that a pregnant woman weighs 70 kg and a near-term pregnant patas monkey weighs 7 kg. Given these assumptions, the patas monkey daily doses (Table 1) calculated on a gm/kg body weight basis for AZT, 3TC, ddI, and d4T were approximately 80, 83, 100, and 96% of the daily doses given to pregnant women, respectively.

The CDC also recommends that infants born to HIV-1–infected women be given NRTI prophylactic therapy for the first 6 weeks of life. In order to model this, the same NRTI combination that the dam had received was given orally to the infant monkeys for the first 6 weeks of life. The drug doses were adjusted at 1 month of age to accommodate the patas infant’s increase in body weight (Table 1).

The OXPHOS enzyme specific activities in skeletal muscle mitochrondia were determined by enzymatic assay. At birth, OXPHOS changes were not remarkable (Table 2) except that complex IV was decreased by 73% in the d4T/3TC group. Interestingly, at 1 year of age, which is 46 weeks after the last NRTI exposure, the OXPHOS changes were more pronounced (Table 3). For complex I, the AZT/3TC and d4T/3TC groups had decreases of 37 and 43%, respectively, compared to the unexposed group. For complex II, the AZT/ddI and d4T/3TC groups had decreases of 17 and 24%, respectively, compared to the unexposed group. For complex IV, the d4T/3TC group had a reduction of 17% (Table 3).

Histochemistry of OXPHOS Complex IV (COX) at Birth and 1 Year of Age

Skeletal muscle samples taken at birth and at 1 year of age were stained for COX activity using cytochrome c and 3,3’-diaminobenzidine to reveal localization of active enzyme
through formation of an insoluble brown color. Representative stained sections are shown in Figure 1. Tissue from unexposed monkeys showed uniform COX staining within each sarcomere fiber, with a typical darker staining in type I fibers and lighter staining in type II fibers (Fig. 1, control). In contrast, there were clear staining abnormalities in both types of skeletal muscle fibers, at birth and at 1 year of age, in monkeys exposed in utero or perinatally to NRTIs (Fig. 1). Clear definition of perimysium, the connective tissue which groups individual muscle fibers, and endomysium, the layer of connective tissue surrounding a muscle fiber, was apparent in sections from unexposed monkeys but absent in sections from NRTI-exposed monkeys. For the groups exposed to AZT alone and AZT/3TC, at 1 year of age, the interfiber spaces (endomysium) were abnormally wide, compared to the unexposed controls, suggesting peripheral loss of COX activity in muscle fiber. The groups exposed to pairs of NRTIs showed depletion of enzyme activity in most muscle fibers, and it was difficult to distinguish between type I and type II fibers. In addition to COX enzyme loss, all of the groups exposed to two NRTIs showed breaks/separation and rounded holes in the muscle fibers. There was clear evidence of widespread abnormality in COX activity, even after 46 drug-free weeks, demonstrating that focal and peripheral loss of complex IV, occurring as a result of perinatal NRTI exposure, is not reversible by 1 year of age.

EM of Skeletal Muscle and Scoring of Photomicrographs

Substantial damage to mitochondria and sarcomeres was generally apparent by EM in skeletal muscle of offspring at birth and 1 year of age. Figure 2 shows representative photomicrographs for skeletal muscle taken at birth from infant monkeys exposed in utero to no drug (A), 3TC (B), AZT (C), AZT/3TC (D), AZT/ddI (E), and d4T/3TC (F). Figure 3 shows representative photomicrographs for skeletal muscle taken at 1 year of age from infant monkeys exposed in utero and for the first 6 weeks of life to no drug (A), AZT (B), AZT/3TC (C), AZT/ddI (D), and d4T/3TC (E). Because there was little 3TC-induced mitochondrial damage at birth (Fig. 2B), we did not follow 3TC-exposed animals to 1 year of age. However, for the other NRTI-exposed groups, there was substantial damage to mitochondria and sarcomeres in offspring skeletal muscle, both at birth (Figs. 2C–F) and at 1 year of age (Figs. 3B–E). Manifestations of NRTI-induced mitochondrial damage found

FIG. 1. Representative histochemical complex IV (COX) activity staining of skeletal muscle from patas offspring exposed in utero and taken at birth or exposed in utero and for the first 6 weeks of life and taken at 1 year of age. Exposures included no drug (control), AZT, AZT/3TC, AZT/ddI, and d4T/3TC. Samples are visualized at ×60 and ×300 magnifications using bright field microscopy. Blue triangles indicate endomysium, red triangles indicate perimysium, and black arrows point to focal loss of COX activity in muscle fiber.
in skeletal muscle from these animals (see legends to Figs. 2 and 3) included mitochondrial proliferation, mitochondrial swelling with loss of cristae, sarcomere disruption (Z-line and M-line irregularities), clusters of similar-appearing abnormal mitochondria (reminiscent of clonal expansions), and occasional formation of giant mitochondria. The most abnormal photographs were from the AZT/ddI-exposed offspring, where some regions contained clusters of cristae suggesting that the mitochondria had become damaged, proliferated, and then experienced widespread membrane decomposition (Figs. 2E and 2F).

For each photomicrograph, the ultrastructural damage in skeletal muscle was scored on a scale of 0–5 by a team of investigators that included a pathologist. An average score for 10 photomicrographs captured randomly for skeletal muscle from each monkey was derived based on the scoring scheme in the footnote to Table 4. The Table shows values for monkeys taken at birth and at 1 year of age, and the values suggest that, in the AZT/3TC and d4T/3TC groups, there may be an improvement in the level of mitochondrial morphological damage between birth and 1 year of age; however, the values were not statistically significant. In the AZT/ddI-exposed group,
group, where damage at birth was severe, there was no improvement during the first year of life. A photomicrographic comparison, at increased magnification, between skeletal muscle taken from monkeys at birth and at 1 year of age is shown in Figure 4, where, 46 weeks after the last dose of NRTI, the persistence of mitochondrial abnormalities in the NRTI-exposed groups is clearly evident.

Quantification of Skeletal Muscle mtDNA by HC-CIA

In these experiments, relative mtDNA levels were quantified using the HC-CIA procedure described previously for hearts from these same animals (Divi et al., 2005). Figure 5 shows mtDNA values (expressed as LU/μg DNA) for unexposed monkeys and NRTI-exposed monkeys at birth and 1 year of age. Compared to the unexposed controls, significant depletion of mtDNA was observed in each of the NRTI-exposed groups at birth and at 1 year of age \( (p < 0.05) \). At birth, AZT/3TC-, AZT/ddI-, and d4T/3TC-exposed animals had 54, 58, and 51% depletion of skeletal muscle mtDNA, respectively; and at 1 year of age, the depletions in the same exposure groups were 26, 47, and 19%, respectively. The group exposed to AZT alone showed little change during the first year of life, as the mtDNA depletion was 45% at birth and 47% at 1 year of age.
TABLE 4
Scoringa of EM photos for Mitochondrial Morphological Damage (scale 0 to +5) in Skeletal Muscle from Patas Monkeys Taken at Birth and 1 Year of Age

<table>
<thead>
<tr>
<th>NRTI exposure</th>
<th>Number of photomicrographs/monkey</th>
<th>Damage score (Mean ± SE)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>At birth (n = 3)b</td>
<td>At 1 year (n = 4)</td>
</tr>
<tr>
<td>None</td>
<td>40</td>
<td>1.4 ± 0.3</td>
<td>0.5 ± 0.3</td>
</tr>
<tr>
<td>AZT</td>
<td>40</td>
<td>2.8 ± 0.4c</td>
<td>4.0 ± 0.4c</td>
</tr>
<tr>
<td>3TC</td>
<td>40</td>
<td>1.5 ± 0.6</td>
<td>NSd</td>
</tr>
<tr>
<td>AZT/3TC</td>
<td>40</td>
<td>3.8 ± 0.7c</td>
<td>2.8 ± 0.9c</td>
</tr>
<tr>
<td>AZT/ddI</td>
<td>40</td>
<td>4.0 ± 0.6c</td>
<td>4.0 ± 0.6c</td>
</tr>
<tr>
<td>d4T/3TC</td>
<td>40</td>
<td>4.0 ± 0.6ce</td>
<td>2.3 ± 0.8c</td>
</tr>
</tbody>
</table>

aScoring was based on a range of values between (0) and (+5), according to the following scheme:
(0) Virtually all mitochondria had the typical oblong shape, with compact, intensely dark, intact cristae, while an infrequent mitochondrion showed minimal swelling that was considered to be within normal limits, and the sarcomeres were uniform with clearly visible tight Z-lines and M-lines.
(+1) Most mitochondria had the typical oblong shape, and although a few were rounded with discontinuous membranes, no loss of cristae and matrix material or alterations in sarcomere structure was evident.
(+2) The number of mitochondria per cell was nearly doubled compared with samples graded (0) and (+1), and many mitochondria were swollen with membrane disruptions but minimal loss of cristae. Most of the remaining mitochondria looked essentially normal, but there was some sarcomere disruption and Z-line misalignment.
(+3) Mitochondrial proliferation was evident and most mitochondria were swollen with multiple membrane breaks, partial loss of cristae and matrix material, and widespread sarcomere damage with Z-line misalignment and M-line depletion.
(+4) Mitochondrial proliferation was evident and virtually all mitochondria were swollen with fragmented membranes and effacement of central architecture, while sarcomere disruptions, dispersed Z-lines and Z-line misalignments were widespread. Clusters of highly damaged, similar-appearing, mitochondria could be identified.
(+5) In some areas the mitochondrial membrane was completely dissolved, and the cristae were fragmented and disorganized, in other areas giant swollen mitochondria containing no cristae or central architecture were apparent, and all other areas looked like those in (+4).

bThere were four unexposed monkeys at birth.

cBy Holm-Sidak pairwise multiple comparison test, values for any NRTI-exposed group were significantly different from the corresponding unexposed control group for monkeys taken at birth (p < 0.05) and at 1 year of age (p < 0.05).

dNS = no sample.

DISCUSSION

In this study, we have shown that skeletal muscle taken from infant monkeys exposed in utero to human-equivalent NRTI protocols sustain complex IV activity abnormalities, mitochondrial morphological damage, and mtDNA depletion. The NRTI-exposed patas offspring were examined both at birth, and at 1 year of age or 46 weeks after last NRTI dose. The monkeys taken at birth showed mitochondrial compromise similar to that previously reported at birth for infants born to pregnant patas dams exposed to AZT/3TC during gestation (Gerschenson et al., 2004). This study extends the previous observations by examining 3TC alone, by adding additional NRTI combinations, AZT/ddI and d4T/3TC, and by following the infant monkeys for the first year of life. In addition, we have used a newly developed method for quantification of mtDNA and have applied a paradigm for scoring mitochondrial integrity in the EM photomicrographs.

Perhaps the most important contribution of this study is the demonstration that mitochondrial morphological damage (by EM), COX activity (by histochemistry), and depletion of mtDNA (by HC-CIA) in NRTI-exposed patas fetuses do not return to the normal levels found in unexposed patas offspring, even after 46 weeks with no drug exposure. The persistent mitochondrial compromise observed in skeletal muscle parallels the cardiac mitochondrial damage observed in these same animals and reported previously (Divi et al., 2005).

In this study, OXPHOS abnormalities were minor and observed only in the monkeys exposed to pairs of NRTIs. The value for complex I in unexposed patas at 1 year of age was 82.6 ± 5.4 nmol/min/mg protein, similar to the value of 82 ± 26 nmol/min/mg protein reported for human muscle complex I using ubiquinone2 as electron acceptor (Birch-Machin and Turnbull, 2001). In unexposed monkeys, complex II was 292.1 ± 15.2 nmol/min/mg protein at 1 year of age, comparable to the reported value for human muscle of 293 ± 55 nmol/min/mg protein (Birch-Machin and Turnbull, 2001). The value for complex IV in unexposed monkeys at 1 year of age was 473.8 ± 109.7 nmol/min/mg protein, similar to the reported human values of 430.8 nmol/min/mg protein (Birch-Machin et al., 1994). Exposure to NRTIs did not alter complex I at birth, although at 1 year of age there were depletions of 37 and 43% in the AZT/3TC- and d4T/3TC-exposed monkeys, respectively. Decreases were found for complex II at 1 year of age in the AZT/ddl and d4T/3TC groups and complex IV in the d4T/3TC group at birth. In a previous publication (Gerschenson et al., 2004), using a different group of patas fetuses, with OXPHOS assays performed on different days by multiple staff members, we reported a substantial depletion of complex I at birth in skeletal muscle from patas monkeys exposed in utero to AZT/3TC. The current data were obtained in a more consistent fashion, with all the assays for a single OXPHOS complex performed on the same day. We believe the values presented here to be more accurate because of improved quality control and the fact that the numbers obtained from 1-year-old monkeys closely match the published human data. The fetuses exposed to AZT and AZT/3TC reported here were not the same animals as those reported in the previous study (Gerschenson et al., 2004), but both sets of monkeys showed no clinically evident toxicity.

Perhaps more informative than the actual OXPHOS values, were histochemical staining patterns that indicate the localization of OXPHOS activity. Staining for complex IV (COX) allowed visualization of activity for this enzyme, and in
skeletal muscle from NRTI-exposed patas, we found an
abnormal, nonuniform, sometimes punctuate, loss of COX
staining in the type I and type II muscle fibers. More
importantly, aberrant patterns of COX enzyme loss were
persistent in skeletal muscle from all NRTI-exposed groups at
1 year of age indicating that, during 46 weeks without drug, the
skeletal muscle was unable to reestablish a completely normal
pattern of COX activity.

The persistent COX staining aberrations, observed here in
NRTI-exposed fetuses, were consistent with observations of
skeletal muscle mitochondrial compromise by EM. Unexposed
monkey skeletal muscle showed relatively small mitochondria
that were dense with cristae, had visible double membranes,
and were uniformly layered between sarcomeres. In addition,
there was the characteristic ordered alignment of Z-lines and
M-lines. In the 3TC-exposed monkey at birth, there were only
occasional areas showing mitochondria with loss of cristae or
Z-line disruption. However, mitochondrial membrane damage
with resulting rounding, enlargement, and loss of cristae in the
other NRTI-exposed groups was accompanied by Z-line mis-
alignment, M-line disappearance, and what appear to be clus-
ters of damaged mitochondria lacking double membranes. The
overall integrity of the NRTI-exposed skeletal muscle mito-
chondria appeared to be as compromised at 1 year of age as at

FIG. 4. Representative digitally magnified (∗1.5) comparisons of ∗30,000 magnified EM photomicrographs showing patas offspring skeletal muscle at birth
(A–E) after in utero exposure to NRTIs and at 1 year of age (F–J) after perinatal exposure to no drug (A and F), AZT (B and G), AZT/3TC (C and H), AZT/ddI (D
and I), and d4T/3TC (E and J). White arrows indicate mitochondria, white triangles indicate Z-lines, and black arrows point to damaged sarcomeres. The photos
show sarcomere disruption in all groups except the controls, mitochondrial proliferation (E and G), dissolution and high matrix luminosity in matrix (D, E, G, H, I,
and J), and, Z-line dispersions and misalignments (D, E, H, and I).

FIG. 5. Skeletal muscle mtDNA (LU/ngDNA) determined by HC-CIA at
birth (filled bars) and at 1 year of age (open bars) in patas offspring after
in utero and/or perinatal exposure to: no drug (control), AZT, AZT/3TC, AZT/
ddI, and d4T/3TC. Bars show mean ± SE for four monkeys in each control
group, three NRTI-exposed monkeys per group at birth, and four NRTI-
exposed monkeys per group at 1 year of age.
birth. Numerical scoring of EM photomicrographs confirmed the persistence of significant mitochondrial morphological damage at the end of the first year of life, again demonstrating that during 46 weeks without drug exposure skeletal muscle did not recover from damage occurring as a result of perinatal NRTI exposure. Heart muscle from these same animals responded similarly with respect to mitochondrial morphological damage at birth and 1 year of age (Divi et al., 2005), with a high degree of mitochondrial damage visible by EM at both times. Many of the more peculiar aberrations, including giant mitochondria, clusters (possible clonal expansions) of mitochondria with similar abnormal appearances, and rounded mitochondria missing cristae and double membranes, were visible in both heart and skeletal muscle at birth and were persistent at 1 year of age.

Depletion of mtDNA is a classic consequence of NRTI exposure, occurring both because the drug is a DNA chain terminator and because it is an effective inhibitor of the mtDNA polymerase $\gamma$. In hearts from these same patas infants (Divi et al., 2005), there was also a depletion of mtDNA at birth in the NRTI-exposed groups, but, by 12 months of age, the hearts had become abnormally large, producing an excess of mtDNA. We know from the EM studies that there was widespread aberrant morphology in the heart mitochondria at 1 year of age, and, therefore, we assume that the cardiac enlargement was a compensation for the stress of the NRTI exposure. Skeletal muscle, which is presumably less essential to the organism than heart, appeared to lack the compensatory response observed in cardiac muscle. Both organs sustained depletion of mtDNA as an initial response, and whereas the skeletal muscle did improve somewhat during the first year of life, the mtDNA levels were still significantly depleted at 1 year of age.

The patas mitochondrial morphological damage and mtDNA depletion in heart and skeletal muscle parallel investigations in humans using available tissues such as leukocyte DNA and cord blood DNA (Divi et al., 2004; Poirier et al., 2003; Shiramizu et al., 2003). Umbilical cord mitochondrial morphological damage and mtDNA depletion were observed (Divi et al., 2004, 2007; Poirier et al., 2003; Shiramizu et al., 2003) in infants exposed to AZT/3TC and AZT/ddI. In addition, mtDNA depletion was observed in HIV-1-uninfected 1- and 2-year-old human infants born to HIV-1-infected mothers who received AZT therapy during pregnancy (Poirier et al., 2003). In that study, the human infants were exposed to AZT in utero and during the first 6 weeks after birth, and mtDNA values were determined in DNA extracted from cord blood and peripheral blood leukocytes. The study showed that even after 46 and 98 drug-free weeks, the AZT-exposed infant mtDNA levels were significantly lower than those found in infants born to uninfected women and those in infants born to HIV-1-infected mothers who did not receive AZT therapy during pregnancy. In another study, the extent of mtDNA depletion found in both human and monkey umbilical cord DNA and leukocyte DNA at birth was similar in both species after perinatal exposure to AZT/3TC or AZT/ddI (Divi et al., 2004, 2007; Poirier et al., 2003; Shiramizu et al., 2003), although the monkey dams were not retrovirus infected. The similarity in mitochondrial compromise in umbilical cords and leukocytes from monkeys and humans suggests that human infants may also sustain some cardiac and skeletal muscle mitochondrial compromise and should be followed long term for signs of mitochondrial dysfunction.

**FUNDING**

Intramural Research Program of the NIH, National Cancer Institute, and Center for Cancer Research.

**ACKNOWLEDGMENTS**

We wish to thank our pathologist colleague Dr U. Thorgeirsson for scoring the coded EM photos, Dr C. Thamine for statistical assistance, and Dr M. Gerschenson for help with the early monkey studies.

**REFERENCES**


Connor, E. M., Sperling, R. S., Gelber, R., Kiselev, P., Scott, G., O’Sullivan, M. J., VanDyke, R., Bey, M., Shearer, W., Jacobson, R. L.,
et al. (1994). Reduction of maternal-infant transmission of human immuno-


