Original article

Antiretroviral nucleoside analogues suppress antibody synthesis in human B-lymphocytes

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Background: Some antiretroviral nucleoside reverse transcriptase inhibitors (NRTI) impair mitochondrial polymerase-γ and T-cell proliferation, possibly by pyrimidine depletion. We aimed to analyse NRTI effects on the content of mitochondrial DNA (mtDNA) and B-cells, and on their proliferation and antibody synthesis.

Methods: Peripheral blood B-lymphocytes from six healthy individuals were stimulated in vitro with interleukin-4 and Staphylococcus aureus superantigen in the presence or absence of NRTI in concentrations equivalent to, or fivefold exceeding, human peak plasma levels. We also tested the effects of uridine, a pyrimidine precursor, which has antagonized NRTI toxicities in other models.

Results: During 9 days of culture, B-lymphocyte proliferation and vitality were not affected by NRTI. Didanosine and stavudine, but not zidovudine, dose-dependently induced mtDNA depletion. All three NRTI significantly and dose-dependently impaired the synthesis of all immunoglobulin classes. The lymphocytopoietic effects of the thymidine analogues zidovudine and stavudine on B-lymphocytes were antagonized by the addition of uridine.

Conclusions: Didanosine, stavudine and zidovudine induce mitochondrial toxicity in human B-lymphocytes and impair the immunoglobulin synthesis in vitro, warranting further studies on their in vivo effects.

Introduction

HAART induces a significant immune reconstitution in HIV-infected patients, resulting in a decrease of HIV-associated morbidity and mortality [1]. Yet, even in the HAART era, HIV-positive patients have an increased morbidity from bacterial and viral infections compared to HIV-negative individuals, and the restoration of immune competence and response to cognate antigens remains incomplete. Cohort studies in HIV-infected individuals, for example, demonstrate that despite HAART and antimicrobial prophylaxis, the incidence of pneumococcal infections remains considerably increased compared with the general population [2,3].

In a screening of HIV antiretrovirals, it has been shown that some nucleoside reverse transcriptase inhibitors (NRTI), particularly didanosine (ddI), stavudine (d4T) and zidovudine (AZT), interfere with mitochondria [4,5]. It has also been demonstrated that NRTI decrease the levels of mitochondrial DNA (mtDNA) in human T-lymphocytes, and impairs their proliferation [6,7].

The mechanism by which mitochondrial toxicity induces this immunosuppressive effect involves decreased synthesis of mtDNA-encoded respiratory chain subunits and defective oxidative phosphorylation. The impairment of mitochondrial respiration also inhibits dihydroorotate dehydrogenase (DHODH), an enzyme that requires an intact electron flux through the respiratory chain and is indispensable for the de novo synthesis of all pyrimidines in the body [8]. Respiratory chain dysfunction and consecutive DHODH impairment therefore could decrease the intracellular availability of pyrimidine building blocks required for mtDNA synthesis, for the maintenance of an accurate mtDNA multiplication process (replication fidelity), and also for other metabolic processes [9–11]. The observed effects of mitochondrial toxicity and subsequent DHODH blockade on T-lymphocytes therefore closely resemble those of leflunomide, a direct DHODH inhibitor and immunosuppressive drug, which is licensed for the treatment of rheumatoid arthritis [8,12].

The observed NRTI-related mitochondrial toxicity in T-lymphocytes also raises the possibility that similar mechanisms may operate in B-lymphocytes. Such B-lymphocyte toxicity could explain some of the sustained effects of NRTI on B-lymphocytes.
susceptibility towards influenza and pneumococcal pathogens in HIV-infected patients, as these infections are normally mitigated or prevented by protective antibodies.

Up to now, there are only limited data about the isolated effects of NRTI on B-cell mitochondria and function [13,14]. The few studies that have examined the effects of HAART on antibody responses to pneumococcal vaccines indicate that HAART components may indeed impair T-cell-independent immunoglobulin synthesis [14,15]. In vivo mtDNA measurements in B-lymphocytes are difficult to conduct because of the heterogeneity and relative paucity of B-cells in the peripheral blood compartment, as well as the fact that mtDNA levels may also be altered during the natural course of HIV infection [16–19]. Difficulties also arise from contaminating platelets [20]. We therefore established an in vitro model in which we aimed to investigate if mtDNA depletion can indeed be detected in primary human B-lymphocytes under NRTI exposure and to investigate the functional effects of NRTI on B-cell proliferation and antibody production. In our model we selected ddI, d4T and AZT as known in vivo inhibitors of mtDNA replication [21,22]. NRTI were used at concentrations equivalent to steady-state peak plasma concentrations ($C_{\text{max}}$) of humans during antiretroviral therapy (NRTI product data sheets), for example, 11.8 μM of ddI, 3.6 μM of d4T and 7.1 μM of AZT [6]. In addition, concentrations corresponding to 5×$C_{\text{max}}$ were tested (for example, 59 μM of ddI, 18 μM of d4T and 35.5 μM of AZT). Cultures were tested in parallel without or with uridine at a concentration of 200 μM; controls were incubated in medium without NRTI.

**Nucleoside reverse transcriptase inhibitors**

ddI, d4T and AZT were purchased from Sigma (Taufkirchen, Germany) and freshly dissolved in medium. Final NRTI concentrations corresponded to $C_{\text{max}}$ of humans during antiretroviral therapy (NRTI product data sheets), for example, 11.8 μM of ddI, 3.6 μM of d4T and 7.1 μM of AZT [6]. In addition, concentrations corresponding to 5×$C_{\text{max}}$ were tested (for example, 59 μM of ddI, 18 μM of d4T and 35.5 μM of AZT). Cultures were tested in parallel without or with uridine at a concentration of 200 μM; controls were incubated in medium without NRTI.

**Mitochondrial DNA quantification**

Total DNA was extracted with the QIAamp DNA isolation kit (Qiagen, Hilden, Germany). mtDNA and native DNA (nDNA) copy numbers were determined by quantitative PCR using the ABI 7700 sequence detection system (Applied Biosystems, Foster City, CA, USA) [7]. We amplified the mtDNA ATP6 gene between nucleotide positions 8981 and 9061 with the forward primer, 5′-ACCAATAGCCCTGGCCGTAC-3′ and the backward primer 5′-CGGGGATCCGCTACTTTAGA-3′. mtDNA was quantified with a FAM fluorophore labelled probe (5′-FAM-CCTTCCAGAACATCATTACATACTGCAGGCCACC-TAMRA-3′). For the detection of nDNA, we selected exon number 8 of the GAPDH gene between nucleotide positions 4,280–4,342, using the forward primer 5′-GGTGCGCTTCCAATTAGGT-3′ and the backward primer 5′-GGTGCGCTTCCAATTAGGT-3′. mtDNA was quantified with a VIC fluorophore labelled probe (5′-VIC-CCCTGCCCTACTGGCCTGCC-TAMRA-3′). For the detection of nDNA, we selected exon number 8 of the GAPDH gene between nucleotide positions 4,280–4,342, using the forward primer 5′-CCCTGCCCTACTGGCCTGCC-TAMRA-3′. Each 25 μl reaction contained 25 ng of genomic DNA, 100 nM probe, 200 nM primers and TaqMan Universal Master Mix (Applied Biosystems). Amplifications of mitochondrial and nuclear products were separately performed in optical 96-well plates (Applied Biosystems). An initial incubation at 50°C for 2 min was followed by 10 min at 95°C and 40 denaturing steps at 95°C (15 s), alternating with combined annealing/extension at 60°C (1 min). All samples were run in 730
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triplicate. Absolute mtDNA and nDNA copy numbers were calculated using serial dilutions of plasmids with known copy numbers.

Immunoglobulin synthesis
A quantity of 10 μl of supernatants were centrifuged at 9,000 g for 10 min to remove cells and debris. Immunoglobulin M (IgM), IgG, and immunoglobulin A (IgA) concentrations were measured with immunoglobulin class-specific ELISA kits from Dianova (Hamburg, Germany) according to the manufacturer’s protocol. Measurements for each supernatant were performed in triplicates on an ELISA microplate reader (Dynatech Laboratories, Chantilly, VA, USA) at 405/490 nm.

Statistics
Group means were compared by paired Student’s t-test or Wilcoxon tests, as appropriate. All calculations were performed using the SigmaStat statistical package version 1.0 (SPSS, Inc., Chicago, IL, USA).

Results

Effects of nucleoside reverse transcriptase inhibitors on proliferation and mortality
In the absence of NRTI, B-lymphocytes divided rapidly after stimulation with SAC to a mean density of 1.5×10⁶ cells/ml until day 4. After day 5 the proliferation almost ceased, leading to a mean final concentration of 1.87×10⁶ cells/ml (±0.44×10⁶) at day 9. In control B-lymphocytes, there was no mortality until day 4; afterwards, the mortality increased to approximately 20% at day 9.

The addition of NRTI did not significantly impair the proliferation of B-lymphocytes (Figure 1A). NRTI supplementation did not affect mortality (data not shown).

Effects of nucleoside reverse transcriptase inhibitors on mitochondrial DNA
ddI and d4T induced a decline of mtDNA in B-lymphocytes (Figure 1B). After 9 days of exposure with 11.8 μM of ddI, residual mtDNA levels were only 70.0% of control levels, and 11.2% of control levels with 59 μM of ddI. d4T also induced mtDNA depletion in B-lymphocytes after SAC stimulation, although residual mtDNA amounts were higher than with ddI (88.5% of control levels for 3.6 μM of d4T and 68.0% for 18 μM of d4T). The mtDNA depletion induced by ddI and d4T was dose-dependent (P=0.009 and P=0.043, respectively). AZT, in contrast to ddI and d4T, did not deplete mtDNA in B-lymphocytes. Day 4 mtDNA levels were similar to those at day 9 for ddI, d4T and AZT (data not shown).

Effects of nucleoside reverse transcriptase inhibitors on antibody production
At 4 days after SAC stimulation, the production of all immunoglobulin classes was negligible. After day 4, there was a time-dependent increase of immunoglobulin synthesis with the synthesis of class-switched
immunoglobulin molecules (IgG and IgA) being quantitatively less pronounced than that of unswitched IgM (Figure 2). ddI, d4T and AZT were all potent inhibitors of immunoglobulin production and reduced the synthesis of all immunoglobulin classes in a dose-dependent manner. Table 1 provides the rates of immunoglobulin synthesis at day 9 in percentage of control values.

Effects of uridine

Uridine has antagonized the mitochondrial toxicity of thymidine analogue NRTI (d4T and AZT) in a number of models [24–27], probably by replenishing the pyrimidine building blocks required for mtDNA synthesis [10,28]. We therefore investigated the effects of uridine supplementation on the mitochondrial toxicity of NRTI on B-cells.
In the absence of NRTI, there was no unspecific effect of uridine on B-lymphocyte proliferation (Figure 1A). Although in the absence of uridine none of the NRTI significantly impaired B-cell proliferation, uridine slightly increased cell numbers of B-cells exposed to d4T.

With regard to the mtDNA depletion (Figure 1B), we did not detect an effect of uridine in counteracting the toxicity of ddl, a pyrimidine analogue consistent with previous reports in other models [29,30]. Uridine, however, appeared to antagonize the mtDNA depletion of d4T, a pyrimidine analogue.

With respect to antibody production, uridine significantly counteracted the impaired synthesis of all three immunoglobulin classes induced by d4T and AZT (Table 1). The effects of uridine on ddl-associated B-cell dysfunction were less clear, and a consistent improvement of immunoglobulin synthesis was not observed.

### Discussion

We investigated the in vitro effects of ddl, d4T and AZT on the proliferation, vitality, mtDNA copy number and antibody production of primary human B-lymphocytes of healthy volunteers. Whereas lymphoproliferation and B-cell vitality were unaffected, both ddl and d4T induced a dose-dependent mtDNA depletion, extending previous findings in which lasting mtDNA depletion was induced by NRTI in a human immortalized myelogenous leukaemia cell line [31] and in primary human T-cells [6,7]. Even more importantly our findings demonstrate that all tested NRTI have the capacity to dose-dependently impair the synthesis of immunoglobulins. Lymphocytes must react quickly to proinflammatory stimuli. To cope with the demands of rapid division upon stimulation, lymphocytes are equipped with nucleoside transporters [19,32,33]. The finding of a time- and dose-dependent mtDNA depletion makes it likely that NRTI or their phosphorylation products are transported to the intramitochondrial compartment, allowing for an inhibition of polymerase-γ [9]. AZT inhibited the antibody synthesis unrelated to mtDNA depletion, similar to its effects in T-cells [6]. AZT is a known inhibitor of ATP/ADP translocation in the mitochondrial membrane, and thus may also impair the respiratory

<table>
<thead>
<tr>
<th>Antibody class and type of NRTI</th>
<th>Immunglobulin synthesis, μg/10⁶ B-cells [μg]</th>
<th>Percentage of control</th>
<th>P-value versus control</th>
<th>Immunglobulin synthesis, μg/10⁶ B-cells [μg]</th>
<th>Percentage of control</th>
<th>P-value versus control</th>
<th>P-value uridine versus no uridine</th>
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<td>IgM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
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<td>29.02 (4.29)</td>
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<td>0.0002</td>
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<td>ddI5</td>
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<td>22</td>
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</tr>
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<td>6.45 (1.11)</td>
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<td>0.0004</td>
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<td>93</td>
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<tr>
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<td>97</td>
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<td>30.05 (2.82)</td>
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<td>23.13 (2.24)</td>
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<tr>
<td>Control</td>
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<td>10.91 (1.61)</td>
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<td>0.043</td>
<td>11.07 (0.97)</td>
<td>101</td>
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<td>9.28 (0.48)</td>
<td>85</td>
<td>0.041</td>
<td>&lt;0.0001</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
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<td>100</td>
<td>NA</td>
<td>7.02 (0.68)</td>
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<td>NA</td>
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<td>0.0002</td>
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<td>48</td>
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<td>0.0048</td>
<td>7.83 (0.51)</td>
<td>111</td>
<td>0.0009</td>
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<td>3.20 (0.14)</td>
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<td>60</td>
<td>&lt;0.0001</td>
<td>6.63 (0.82)</td>
<td>94</td>
<td>0.015</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

*Controls represent B-lymphocytes without nucleoside reverse transcriptase inhibitor (NRTI) exposure from the same subjects (n=6). The amount of immunoglobulin produced is given as group means (μg) per 10⁶ B-lymphocytes at day 9; P-values represent pairwise comparisons. AZT, zidovudine; AZT5, 35.5 μM of zidovudine; ddl, didanosine; ddl5, 59 μM of didanosine; d4T, stavudine; d4T5, 18 μM of stavudine; IgA, immunoglobulin A; IgG, immunoglobulin G; IgM, immunoglobulin M; NA, not applicable.*
chain in coupled mitochondria, among other potential mechanisms [34].

In the T-cell compartment, late-onset decreases of T-lymphocyte numbers were observed in patients with slightly increased plasma concentrations of ddI [35], but there are only few data about the effects of NRTI on antibody production. Murine immunotoxicological studies have demonstrated that the active metabolite of ddI impairs IgM synthesis in response to T-cell independent antigens [13]. Clinical studies have demonstrated that HIV-infected patients have a significantly lower frequency and magnitude of antibody responses to the T-cell independent 23-valent vaccine against Streptococcus pneumoniae, than those without HIV infection [15,36]. One recent study examined the influence of antiretroviral therapy on the immunogenicity of vaccinations and found that the intake of NRTI reduced the odds of response by 70%, compared to patients taking antiretrovirals without NRTI or HAART [14]. Another study examined the effects of HAART on antibody synthesis after vaccination and found a significant positive correlation between the induced immunoglobulin levels and the time on HAART; the components of the antiretroviral combination were however not separately analysed [37].

mtDNA depletion induces a defect in the respiratory-chain-dependent DHODH function and de novo pyrimidine synthesis, an effect that contributes to the mitochondrial toxicity of thymidine nucleoside analogues [38] and can be overcome by the supplementation of uridine, a pyrimidine precursor that is eagerly taken up by B-lymphocytes [39]. It was generally believed that lymphocytes are dependent on nucleoside de novo synthesis to cover their demand during proliferation and differentiation [40]. Recent data, however, indicate that at least the deoxycytidine kinase, a rate-limiting enzyme in deoxynylribonucleoside salvage is also necessary both for T- and B-lymphocytes [41]. Thus, recycling of metabolic products of DNA degradation seems to be part of the normal lymphocyte physiology. The fact that uridine was previously demonstrated to antagonize the immunosuppressive effects of the direct DHODH inhibitor leflunomide is a further indication of a functional nucleoside salvage pathway in lymphocytes [23,42].

In line with these data, we have demonstrated that the inhibitory effects of thymidine NRTI on mtDNA replication and immunoglobulin synthesis in B-cells can, in similarity to the immunosuppressive effects of leflunomide, be antagonized with uridine as a pyrimidine precursor [23]. The lack of a clear-cut effect of uridine on ddI induced B-cell toxicity is similar to our observations in other tissues [29,30]. This indicates that uridine or its intracellular metabolites competes with either pyrimidine NRTI uptake, phosphorylation, or directly with pyrimidine NRTI triphosphates at polymerase-γ [10,38]. In the work of Soler et al. [39], however, uridine uptake was not suppressed by thymidine uptake, suggesting different transporters at least into the cellular compartment.

In summary, our findings indicate that some antiretroviral polymerase-γ inhibitors induce a concentration- and time-dependent mtDNA depletion in primary human B-lymphocytes and a functional impairment in terms of reduced immunoglobulin synthesis, a finding that could contribute to the incomplete recovery of B-cell responses under HAART [13–15]. The immunosuppressive effects of thymidine NRTI were partially or fully antagonized by the addition of uridine, warranting clinical studies.

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Disclosure statement

The authors declare no competing interests.

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