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Effect of two different combinations of antiretrovirals (AZT + ddI and AZT + 3TC) on cytokine production and apoptosis in asymptomatic HIV infection

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Abstract

Nineteen HIV-seropositive antiretroviral therapy-naive and asymptomatic individuals (200–500 CD4/μl) were enrolled in a prospective study aimed at analyzing the immunologic and virologic effects of two different combinations of nucleoside reverse transcriptase inhibitors (AZT + ddI and AZT + 3TC), and randomly assigned to one of the treatment group. Immunologic (CD4 and CD8 counts, mitogen-stimulated cytokine production, unstimulated and mitogen-stimulated apoptosis) and virologic (HIV viral load) determinations were performed pre-therapy and 15, 30, 90, 200 and 360 days after initiation of therapy. Results showed that the two combinations had comparable effects on increasing CD4 counts and the CD4/CD8 ratio and in reducing HIV viral load. In contrast, AZT + 3TC was more efficient in improving interleukin-2 (IL-2) and interferon gamma (IFNγ) production as well as the type 1/type 2 cytokine ratio and in down modulating the susceptibility of peripheral blood mononuclear cells to in vitro mitogen-stimulated apoptotic cell death. These data suggest that the combination of AZT + 3TC has a stronger effect on potentially beneficial immune parameters (IL-2 production; reduction of apoptosis) than the one between AZT + ddI. The combination of AZT + 3TC could be more advantageous in the therapy of HIV infection even when used in association with a protease inhibitor.

Keywords: HIV infection; Antiviral therapy; Apoptosis; Cytokine; Immunology

1. Introduction

A number of pharmacological compounds is currently available for the therapy of human immunodeficiency virus (HIV) infection; these compounds can be roughly divided into two different

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families. Thus, the first class includes nucleoside reverse transcriptase inhibitors (RTI) which are compounds aimed at inhibiting HIV replication by interfering with reverse transcriptase, the enzyme that retrotranscribes viral RNA into DNA (Richman, 1996; Saag et al., 1996; Flexner, 1998). The second class is composed by protease inhibitors (PI), which act on viral proteases and have been introduced into HIV therapy more recently (Flexner, 1998). One of the great challenges facing clinicians is to properly define the most effective combinations capable of controlling the progression of HIV infection in each patient. Currently used protocols are mostly based on the association of two RTI with one PI; it has been repeatedly shown that these combinations can effectively suppress HIV replication for an extended period of time (Richman, 1996; Saag et al., 1996; Flexner, 1998).

Whereas it is clear that triple therapy is effective at controlling viral replication, other fundamental questions remain unanswered. Thus, there is not a general agreement on which RTI should be used in association with PIs and on which RTIs should be used first. Both RTI and PI are antiretroviral drugs effective in suppressing HIV replication (Richman, 1996; Saag et al., 1996; Flexner, 1998). HIV infection and its progression to AIDS are nevertheless the product of a complex interplay between two different factors: HIV itself and the immune response to the virus (Levy, 1993; Cohen et al., 1997). Despite an increasing number of reports, the effects of antiretroviral therapy (ART) on the immune system are not clear. An in-depth analysis of the action of these compounds on the immune response could facilitate clinical choices and the decision on which drugs association to privilege. To address this question we designed a prospective study in which the effect of AZT used either in association with 3TC or with ddI on different immune parameters (CD4 and CD8 counts; type 1 and type 2 cytokine production; susceptibility to apoptotic cell death) was analyzed in antiretroviral naive, asymptomatic HIV-seropositive individuals. The results showed that despite a potent and comparable suppression of HIV replication, these two regimens have different effects on the immune system.

2. Materials and methods

2.1. Patients

Nineteen HIV-infected, antiretroviral naive asymptomatic patients (200–500 CD4/μl) were selected for this study. Patients were randomized into two different groups and were treated for 12 months with AZT (500 mg/day) (ten patients) in association with either ddI (400 mg/day) or 3TC (300 mg/day) (nine patients). Immunologic and virologic parameters were evaluated before therapy and 15, 30, 90, 200 and 360 days after initiation of therapy. AZT + ddI was interrupted (after 1 month of therapy) in two patients reporting appearance of nausea and diarrhea. In the AZT + 3TC-treated group two patients were lost to follow-up (after 3 months of therapy) because of lack of compliance; a protease inhibitor was added to the therapeutic regimen of a third patient (after 9 months of therapy) in whom HIV plasma viremia was not properly controlled by the association of two drugs. Therapy was well tolerated in all the patients who completed the study protocol (eight patients in the AZT + ddI protocol; six patients in the AZT + 3TC-treated group).

2.2. Processing of samples

Whole blood was drawn in vacutainer tubes containing preservative free heparin (Becton Dickinson & Co., Rutherford, NJ). PBMC were separated on lymphocyte separation medium (Organon Teknika Corp., Durham, NC) washed in PBS, and the number of viable leukocytes was determined by trypan blue exclusion and a hemocytometer. PBMC were tested as fresh samples. EDTA-plasma samples, kept at 4°C, were processed within two hours. After two-step centrifugation the plasma samples were aliquoted, frozen and kept at −80°C until use.

2.3. In vitro cytokine production

IFNγ, IL-2, IL-4 and IL-10 production by PBMC was determined by culturing 3×10^6/well PBMC in 24 well plates (COSTAR, Cambridge, MA) at 37°C in a moist, 7% CO_2 atmosphere.

PBMC were either unstimulated or were stimulated with PHA (Grand Island, NY) diluted 1:100. Cultures were supplemented with 5% pooled AB^+ human serum (SIGMA Diagnostic, Saint Louis, MO). Supernatants were harvested and assayed after 48 h of culture. Cytokine production was evaluated with the commercially available ELISA (Genzyme, Cambridge, MA). Values for all the cytokines were calculated from a standard curve of the corresponding recombinant human cytokine.

2.4. Apoptotic cell death

Apoptotic cell death was measured using a DNA fragmentation ELISA assay (Cellular DNA Fragmentation Elisa KIT-Boeringer Mannheim GmbH, Kohln, Germany) detecting BrdU-labelled DNA fragments in the cytoplasm of PBL, 5×10^5 PBL/ml were incubated (18 h at 37°C) in RPMI 1640 + BrdU (1% final concentration) in T-75 flasks (COSTAR). PBMC were subsequently washed, plated (5×10^5/ml) (96-well microtiter plates), and either unstimulated or stimulated (3 h) with pokeweed mitogens + staphylococcal enterotoxin B (SIGMA Diagnostics). This combination of mitogens was chosen because previous studies indicated that apoptotic cell death is optimally stimulated when PBMC are triggered in this experimental condition (Clerici et al., 1994). Lysis was induced with a specific solution; the BrdU-labeled DNA fragments released in the culture supernatants were quantified with an ELISA assay (1st step: double helix DNA fragment; 2nd step: BrdU). Data are indicated in relative O.D. (O.D. × 100).

2.5. Quantitation of plasma HIV-1 RNA

The determination of HIV-1 copy numbers was carried out using a quantitative PCR assay (AMPLICOR HIV MONITOR™, Roche Diagnostic Systems, Basel, Switzerland) which is based on a single, combined reverse transcription and amplification of a conserved region of the gag gene as well as an internal standard (quantitation standard). The former corresponds to a 142 bp sequence defined by primers SK 462 and SK 431 while the latter is a synthetic RNA molecule identical to the target except for a 20 bp long probe binding region. HIV-1 RNA, obtained from 200 μl of plasma, was co-extracted with a known amount of quantitation standard (QS) and processed with a single round of reverse transcription and amplification carried out by the Tag DNA polymerase as follows: 30 min at 60°C; 10 s at 95°C; 10 s at 55°C; 10 s at 72°C for four cycles; 10 s at 90°C, 10 s at 60°C, 10 s at 72°C for 26 cycles. To avoid false positive results due to carry-over contamination, all reactions were preceded by a short incubation (2 min at 50°C) with uracyl-*N*-glycosylase (Amperase™, Roche Diagnostic Systems) which inactivates dUTP containing double-stranded DNA. Amplification products, generated using biotinylated primers, were then serially diluted and hybridized to a microwell plate coated with HIV and QS specific probes. After detecting the bound amplicons using HRP-labelled avidin and the colorimetric substrate TMB, the absorbance of each well was measured at 450 nm. The highest dilution for both HIV and QS which corresponds to an optical density included in the range 0.2–2.0 was selected and used to calculate the HIV RNA copies/ml.

2.6. Statistical analyses

Because of the small sample size only descriptive statistic was employed.

3. Results

3.1. AZT + ddI and AZT + 3TC have a similar effect on plasma viremia

Modifications in plasma HIV-1 viremia induced by the two associations studied are shown in Fig. 1, panel A. Mean HIV plasma viremia was comparable in the two groups of patients at baseline (AZT + ddI = 43, 411 copies/μl; AZT + 3TC = 64, 99 copies/μl). Both combinations of antiretroviral were capable of significantly reducing plasma HIV-1 genome copies; the effect was evident as early as 15 days after the initiation of therapy. Reduction in HIV plasma viremia was more prompt in AZT + ddI-treated patients

(AZT + ddI = 837 copies/μl; AZT + 3TC = 1485 copies/μl) and was maintained throughout the study period (day 360: AZT + ddI = 4271 copies/μl; AZT + 3TC = 1910 copies/μl). The temporary increase in HIV viral load observed in the AZT + 3TC-treated group after 90 days of therapy (Fig. 1, panel A) was caused by results obtained in two of the six patients analyzed. Thus, in patient # 2 viremia increased from < 500 to 2063 copies/ml; infection with influenza virus was detected in this patients in correspondence with the increase in HIV viral load. In patient # 6 viremia increased from < 500 to 14 720 copies/ml; therapy was suspended for 10 days in this individuals, again in correspondence with the increase in viremia, because of a minor surgical intervention.

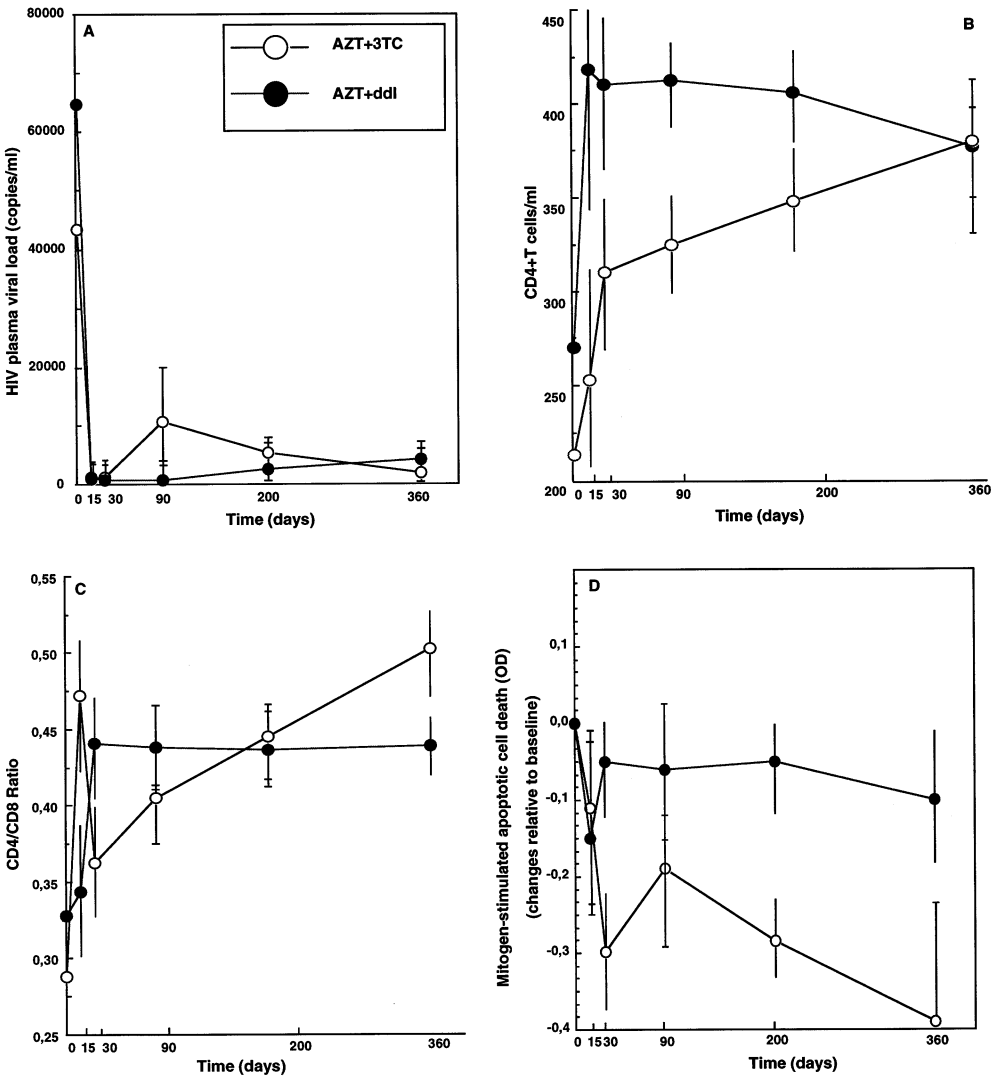

Fig. 1. Effect of AZT + 3TC (O) and AZT + ddI (●) on a series of parameters. Means are shown. Means are derived from the following number of patients. AZT + 3TC, time 0, 15, 30 days = nine patients; time 200 days = seven patients; time 360 days = six patients. AZT + ddI: time 0, 15, 30 days = ten patients; all other times = eight patients. Panel A = HIV viral load (copies/ml), panel B = CD4 + T cells (absolute changes), panel C = CD4/CD8 ratio, panel D = susceptibility to apoptotic cell death (changes relative to baseline).

3.2. AZT + ddI and AZT + 3TC improve CD4 cell counts and the CD4/CD8 ratio

CD4 and CD8 counts were evaluated in all patients. Both regimens resulted in a significant increase in CD4 counts and in an improvement of the CD4/CD8 ratio. Mean CD4 counts at the end of the study period were increased in both groups of patients compared to the pre-therapy data (AZT + ddI: baseline = 270 CD4/μl, day 360 = 377 CD4/μl; AZT + 3TC: baseline = 213 CD4/μl, day 360 = 378 CD4/μl) (Fig. 1, panel B). Whereas the greatest positive change in CD4 counts was comparable in both groups (AZT + ddI = + 166 CD4/μl; AZT + 3TC = + 165 CD4/μl) it is interesting to observe that the dynamics of CD4 changes were different. Thus, the effect of AZT + ddI on CD4 counts was precocious and peaked 15 days after initiation of therapy whereas the use of AZT + 3TC was associated with a constant improvement in this parameter such that the highest positive increase in CD4 counts was observed after 360 days of therapy (Fig. 1, panel B). A similar trend was noticed in the CD4/CD8 ratio which was maximally increased within 30 days after initiation of AZT + ddI therapy but showed a double peak in AZT + 3TC-treated patients in whom the maximum positive modification was nevertheless observed after 360 days of therapy (Fig. 1, panel C).

3.3. Susceptibility to apoptotic cell death is reduced by AZT + 3TC more than by AZT + ddI

Mitogen-stimulated apoptotic cell death (ACD) was evaluated in both groups of patients. The assay employed detects DNA fragments in the cytoplasm of PBL and the results are expressed in optical density (OD); this assay has been validated by a number of studies in different biological systems (Ito et al., 1995; Hines and Allen-Hoffman, 1996; Su et al., 1997). Unstimulated ACD was comparable in AZT + ddI- and AZT + 3TC-treated patients at baseline (0.04 and 0. 05, respectively) and was only marginally modified during therapy (AZT + ddI = 0.08; AZT + 3TC = 0.07). Susceptibility of PBMC to mitogen-stimulated ACD was similarly comparable in both groups of patients at baseline (AZT + ddI = 0.424; AZT + 3TC = 0.471) and was reduced early in the course of therapy in both groups of individuals (AZT + ddI = 0. 235 at day 15; AZT + 3TC = 0.191 at day 30). Interestingly, whereas susceptibility to ACD rebounded to values similar to those observed at baseline in the AZT + ddI-treated patients, this parameter was steadily reduced in the group of individuals receiving AZT + 3TC. The changes in mitogen-stimulated programmed cell death relative to the values detected at baseline are shown in Fig. 1, panel D.

3.4. AZT + 3TC improves type 1 cytokine production more than AZT + ddI

Mean PHA-stimulated type 1 cytokine production was reduced at baseline in the patients subsequently treated with AZT + 3TC (IL-2: AZT + ddI = 603 pg/ml; AZT + 3TC = 279 pg/ml; IFNγ: AZT + ddI = 1228 pg/ml; AZT + 3TC = 936 pg/ml) whereas IL-4 and IL-10 production was comparable in the two groups. Mean changes in PHA-stimulated IL-2, IFNγ, IL-4, and IL-10 production by PBMC relative to the values detected at baseline are shown in Fig. 2. We measured all cytokines after PHA stimulation to equalize the experimental conditions. Cytokine production was modified by both therapies but important differences were observed between the two groups. Thus, IL-2 production peaked early after initiation of therapy both in AZT + ddI − (+ 432 pg/ml) and in AZT + 3TC − (+ 778 pg/ml) treated patients. Nevertheless, whereas IL-2 production constantly declined after the initial peak in the AZT + ddI group (− 265 pg/ml at day 360), the generation of this cytokine was significantly increased at the end of the study period in the AZT + 3TC group (+ 547 pg/ml) (Fig. 2, panel A). A similar trend was observed in IFNγ production which was increased early in both groups of patients (AZT + ddI = + 161 pg/ml at day 30; AZT + 3TC = + 582 pg/ml at day 30) and then declined in AZT + ddI treated patients (− 346 pg/ml at day 360) whereas it increased in the group receiving AZT + 3TC (+ 230 pg/ml at day 360) (Fig. 2, panel B). PHA-stimulated production of type 2 cytokines was

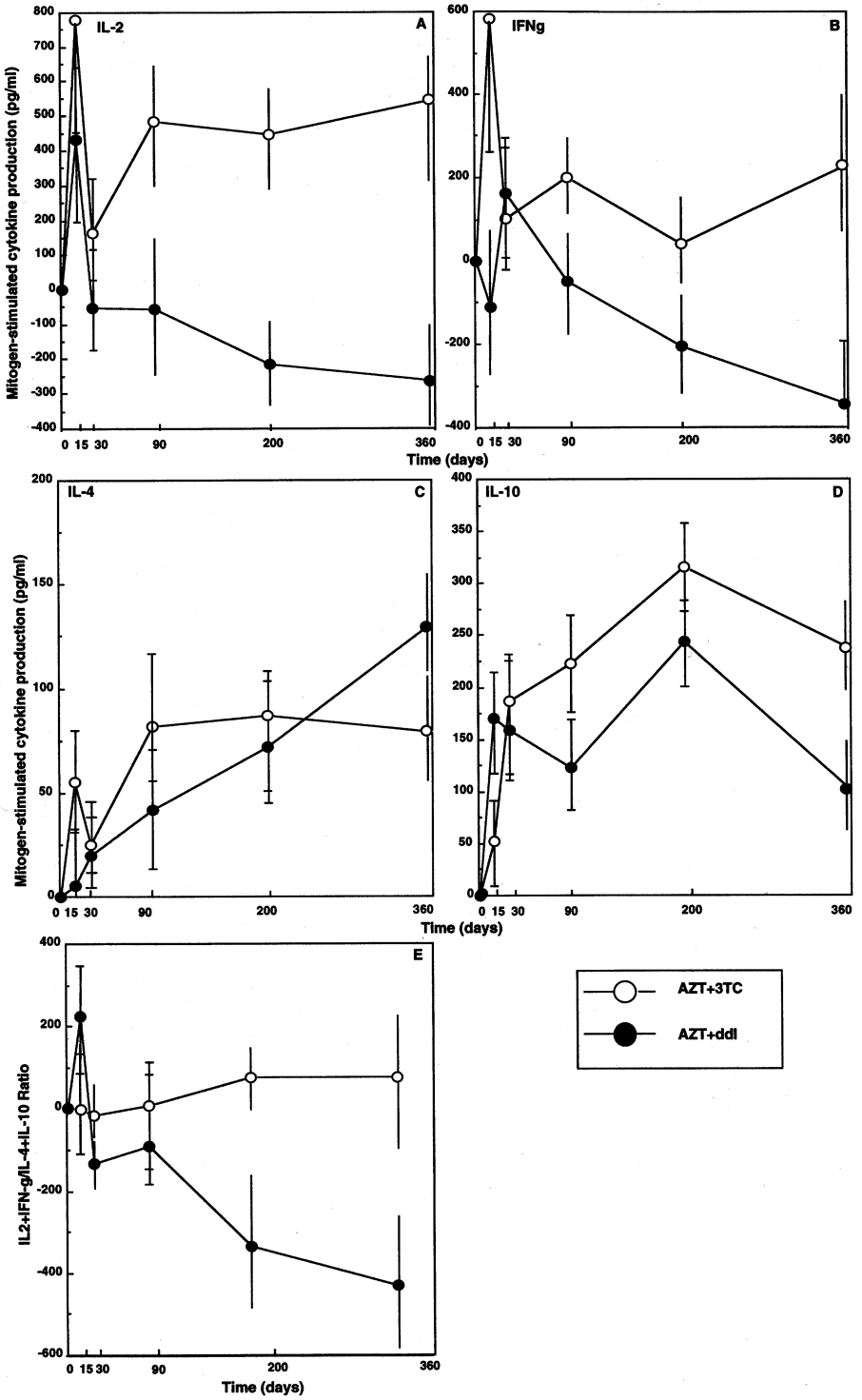

Fig. 2. Effect of AZT + 3TC (O) and AZT + ddI (●) on a series of parameters. Means of changes relative to baseline are shown. Means are derived from the following number of patients. AZT + 3TC: time 0, 15, 30 days = nine patients; time 200 days = seven patients; time 360 days = six patients. AZT + ddI: time 0, 15, 30 days = ten patients; all other times = eight patients. Panel A = IL-2, panel B = IFNγ, panel C = IL-4, panel D = IL-10, panel E = type-1/type-2 (IL2 + IFNγ/IL-4 + IL-10) ratio.

also increased, albeit less strongly, in both groups of patients. Thus, compared to the baseline values IL-4 was steadily increased at the end of the study period both in AZT + ddI- (+131 pg/ml at day 360) and in AZT + 3TC- (+79 pg/ml) treated patients (Fig. 2, panel C), and a similar trend was observed when the production of IL-10 at the end of the study period was compared to the baseline period (AZT + ddI = +101 pg/ml; AZT + 3TC = +236 pg/ml).

The next parameter analyzed was the IL-2 + IFNγ/IL-4 + IL-10 ratio in which the ability of AZT in association with either 3TC or ddI to modulate functionally similar cytokines was studied. Changes in the IL-2 + IFNγ/IL-4 + IL-10 ratio relative to baseline are shown in Fig. 2, panel E, and can be summarized as follows: (1) a peak is observed 15 days after therapy in AZT + ddI-treated patients; and (2) compared to baseline, AZT + 3TC-treated patients show a positive IL-2 + IFNg/IL-4 + IL-10 ratio at the end of the study period whereas this ratio is negative in AZT + ddI treated individuals.

4. Discussion

The progression of HIV infection is associated with increases in plasma HIV viral load and a complex pattern of immune alterations (Levy, 1993; Cohen et al., 1997); thus it is generally accepted that low plasma viremia and the presence of strong cell mediated immunity correlate with lack of such progression. Drugs designed as a therapeutic tool against HIV infection should consequently be aimed at reproducing these correlates: reduction of HIV viral load and stimulation of a quantitatively and qualitatively adequate immune response. The efficacy of anti-HIV drugs currently available is nevertheless almost exclusively evaluated based on their ability to suppress HIV replication, and very limited data on the immunomodulatory properties (if any) of these compounds are available. Because we wanted to perform an ex vivo study on immunologic parameters in HIV-infected and antiviral-treated patients, we decided to analyze immune function by whole PBMC rather than subdividing peripheral blood cells into different subpopulations. This approach did not allow us to evaluate parameters such as differential antiviral activity and intracellular metabolism between AZT and ddI in lymphocytes versus monocytes (Mukherji et al., 1994) or the rate of drugs phosphorilation in resting or activated cells (Gao et al., 1993). On the other hand, the study design we employed has the advantage to more closely reflect the effects that AZT, ddI, and 3TC have in vivo on immune parameters.

A panel of antiretrovirals is employed in the therapy of HIV infection; these antiretrovirals are used in association and the problem of which of the different associations to utilize is heatedly discussed (Richman, 1996; Saag et al., 1996; Flexner, 1998). Viral parameters are the only ones used to determine which drugs combination should be utilized, even if reduction of viral load below the threshold of detection is achieved with most therapeutic associations (Richman, 1996; Saag et al., 1996; Flexner, 1998). Current reports on immunomodulatory activity of antivirals do not include a prospective assessment and rarely have been carried out on a longitudinal basis (Kelleher et al., 1996; Autran et al., 1997; Pakker et al., 1997; Kelleher et al., 1997; Angel et al., 1998; de Jong et al., 1998; Marschner et al., 1998; Wilkinson et al., 1999; Pontesilli et al., 1999). Because the knowledge of possible immune modulatory abilities may be useful in the process of therapeutic decision, we analyzed two of the most commonly used antiretroviral combinations in the treatment of HIV infection from an immunologic standpoint. The results showed that, the powerful effect of both AZT + ddI and AZT + 3TC on reducing HIV plasma viremia notwithstanding, interesting differences are noticed when these different associations are compared. Therefore, whereas the effect of AZT + ddI and AZT + 3TC was comparable on increasing CD4 counts and the CD4/CD8 ratio (quantitative immune parameters), AZT + 3TC was more efficient in improving interleukin-2 (IL-2) and interferon gamma (IFNγ) production and the type 1/type 2 cytokine ratio and in down modulating susceptibility of PBMC to in vitro mitogen-stimulated cell death (qualitative immune parameters).

When compared to the baseline, CD4 counts were similarly augmented after 1 year of therapy in either AZT + ddI- or AZT + 3TC-treated patients, nevertheless qualitative immune parameters (cytokine production) were different between the two groups. These results underline the concept that CD4 count and immune function are independent variables. T helper lymphocytes functionality is often impaired in HIV-seropositive asymptomatic individuals even before a critical decline in CD4 count (Clerici et al., 1989); in analogy cytokine production can be stimulated independently of differences in CD4 counts in patients undergoing therapy (Clerici et al., 1992). We previously measured in vitro stimulated T lymphocyte proliferation and IL-2 production in adult and pediatric HIV-seropositive individuals treated with AZT, soluble CD4-IgG, or ddI (Clerici et al., 1992). We observed that all three compounds were capable of temporarily restoring IL-2 production independently of major variations in CD4 counts. That this results could have clinical implications was suggested by the fact that, in ddI-treated children, in vitro restoration of IL-2 production by antigen-stimulated PBL was associated with a significant reduction in the incidence of opportunistic infections during follow-up (Clerici et al., 1989).

Whereas the effect of AZT + ddI and AZT + 3TC on CD4 increase at the end of the study period was comparable, different dynamics were observed. Thus, changes in CD4 counts in AZT + ddI-treated individuals were detected early into therapy and were followed by a subsequent steady and subtle decline. In contrast, a constant increase in CD4 counts throughout the study period was observed in patients receiving AZT + 3TC. CD4 increase might not have been as prompt in AZT + 3TC-treated patients because changes in HIV viral load followed a slower, more gradual slope in these individuals. On the other hand, a roughly specular pattern was detected when susceptibility to in vitro stimulated ACD was analyzed (early and time-limited decline in AZT + ddI-treated patients; sustained trend toward reduced susceptibility in patients receiving AZT + 3TC). CD4 decline in HIV infection was suggested to be provoked by different mechanisms amongst which accelerated and extensive viral replication (Ho et al., 1995) has an essential role; increased susceptibility of lymphocytes to ACD (Ameisen and Capron, 1991; Gougeon et al., 1991; Meyaard et al., 1992) was also shown to contribute to this phenomenon. Because (1) it was suggested that in HIV infection type-1 cytokines reduce susceptibility to ACD whereas type-2 cytokines have either no effect or enhance susceptibility to ACD (Clerici et al., 1994; Raddrizzani et al., 1995; Estaquier et al., 1995); and (2) increases in CD4 counts were paralleled by increases in the type-1/type-2 ratio and a reduction in HIV viral load (which was achieved in both treatment groups) it is possible to speculate that an association might exist between improved production of type-1 cytokines and improved CD4 counts.

In conclusion, we analyzed two of the combinations of antiretroviral compounds most commonly used in the therapy of HIV infection. Even if it has to be underlined that ex vivo data might not necessarily reflect in vivo immunomodulation, our results showed that even in the face of comparable effects on suppression of HIV viral load and augmentation of CD4 counts, AZT + 3TC might be more powerful than AZT + ddI in stimulating potentially beneficial immune functions. These data could be useful in the therapeutic decision process. Double therapy is considered to be substandard by current guidelines; it will therefore be of interest to examine whether the differences observed in this study can be translated into immunological efficacy of different triple therapy combinations.

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