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Effect of Immunomodulator Dzherelo on CD4 + T-Lymphocyte Counts and Viral Load in HIV Infected Patients Receiving Anti-Retroviral Therapy

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Abstract: The phase II, randomized, clinical trial was conducted in 40 HIV infected patients to evaluate the effect of oral immunomodulator Dzherelo on immune and viral parameters. The arm A (n = 20), received standard Anti-Retroviral Therapy (ART) consisting of zidovudine, lamivudine and efavirenz (AZT/3TC/EFV) and arm B (n = 20) received 50 drops of Dzherelo twice per day in addition to ART. After 2 months the total CD3 T-lymphocytes increased in ART recipients from 664 to 819 cells μL^{-1} (p = 0.06), whereas in Dzherelo recipients they rose from 595 to 785 cells μL^{-1} (p = 0.03). The population of CD4 T-cells expanded by 57.3% in ART (218-343; p = 0.002) and by 93.5% in Dzherelo arms (184-356; p = 0.004). The accrual in absolute and relative number of CD8+ lymphocytes in ART and Dzherelo recipients was 43.2% (2.7%) and 50.4% (-0.5%) , respectively. The CD4/CD8 ratio in Dzherelo recipients had increased from 1.495 to 1.940 (p = 0.03) but in the control group the increase was not significant, i.e., 1.418-1.613 (p = 0.14). About three-quarters (14/19) of patients on ART displayed the decrease in viral load (1718-1419 copies mL^{-1}; p = 0.008), while 95% of patients on Dzherelo had a reduction in the number of viral copies (1793-1368 copies; p = 0.001). Dzherelo has a favorable effect on T-lymphocyte subsets and viral burden in HIV patients when given as an immunomodulating adjunct to ART.

Key words: AIDS, antiviral, ART, HAART, immunotherapy, phytotherapy

INTRODUCTION

The antiretroviral drug resistance, drug toxicity and adherence are major concerns in clinical management of HIV infection (Pokrovskii, 2001). The combination of antiviral drugs, such as Highly Active Antiretroviral Therapy (HAART), may prevent HIV from mutating and spreading, allowing patients to rebuild their immune system to the same levels as in normal individuals (Mocroft *et al*., 2007). On the other hand, the immune activation caused by enhanced immune reaction to HIV, is now recognized as a cause of depletion of CD4 T-cells and resulting immunodeficiency (Appay *et al*., 2005). These problems are driving force for research on new therapies that can provide an answer. Most optimal therapeutic solution is an effective and safe immunotherapy that could regulate the immune response in a manner favorable to a host. There are many types of immune modulators that have been used clinically for viral infections, but for HIV the choice of immune interventions is limited (Ershov, 2003). Ukraine has the highest prevalence of HIV infection in Eastern Europe (Kelly and Amirkhanian, 2003; Vander werf *et al*., 2006). Oral immunomodulator Dzherelo is used in Ukraine for the management of HIV infections, including patients co-infected with TB (Chkhetiany *et al*., 2006, 2007; Kutsyna *et al*., 2003, 2005; Prihoda *et al*., 2006; Zaitseva, 2006). Clinical studies have indicated that Dzherelo can significantly increase CD3 and CD4 T-lymphocyte populations and helps to achieve better clinical response when combined with standard Anti-Retroviral Therapy (ART) consisting of zidovudine (AZT); lamivudine (3TC) and Efavirenz (EFV) (Chkhetiany *et al*., 2007, 2006; Kutsyna *et al*., 2003, 2005; Prihoda *et al*., 2006). Dzherelo has been found to decrease the incidence of opportunistic infections and reverse AIDS-associated wasting (Chkhetiany *et al*., 2007; Kutsyna *et al*., 2003, 2005).

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Dzherelo has also been found to decrease the hepatotoxicity associated with ART (Chkhetiany *et al*., 2006, 2007; Kutsyna *et al*., 2003, 2005; Prihoda *et al*., 2006; Zaitseva, 2006).

Dzherelo was approved in 1997 by the Ministry of Health of Ukraine as immunomodulatory preparation, which so far has been used by over 150,000 individuals for various indications including chronic bacterial and viral infections such as TB and HIV, autoimmune diseases and malignancy. Dzherelo contains concentrated aqueous-alcohol extract from medicinal plants such as Aloe, Common knotgrass, Yarrow, Purple coneflower, St. John's Wort, Centaury, Snowball tree berries, Nettle, Dandelion, Sweet-sedge, Oregano, Marigold, Seabuckthorn fruit, Elecampane, Tormentil, Greater plantain, Wormwood, Siberian golden root, Cottonweed, Licorice, Fennel, Birch tree fungus, Thyme, Three-lobe Beggarticks, Sage, Dog rose fruit and Juniper fruit. Our study was aimed at evaluating the effect of Dzherelo on immune cell subsets and viral load among HIV patients treated with standard ART in comparison to a control population which received ART alone.

MATERIALS AND METHODS

Patients: The patients, aged 20-59 years, have been selected and divided into arms A and B, each consisting of 20 patients randomized by their disease progression. The average (median) age in arms A and B was 33.9 (31.5) and 32.8 (31) years. The proportion of males and females was 15/5 and 17/3 in arms A and B, respectively. Patients were in advanced clinical stage of HIV infection with average baseline CD4+ T-cell count below 200 cells/microliter. Another inclusion criterion was the lack of any form of anti-retroviral therapy prior to the trial. The diagnosis of HIV infection was established by standard ELISA test further confirmed by Western blot analysis. The participation in this trial was voluntary and patients were enrolled only after signing the written consent indicating that they were free to withdraw from the study at any time. The conduct of the trial was approved by the advisory board of the AIDS center.

Treatment regimens : None of the patients received anti-retroviral therapy prior to the trial. After initial screening, qualifying patients were randomly divided in two arms: Arm A was prescribed: zidovudine (AZT) at 300 mg doses twice-daily; lamivudine (3TC) 150 mg tablets twice-daily and Efavirenz (EFV) 600 mg dose once-daily. The arm B received, in addition to ART, twice per day dose of Dzherelo which was given as 50 drops diluted in 100 mL of water.

Immunophenotyping of lymphocyte subpopulations: The samples of peripheral blood of patients with HIV were analyzed using commercially available Clonospectr panel of monoclonal antibodies against surface antigens of lymphocytes (MedBioSpectr, Moscow, Russia). Assays were carried out at study entry and after 1 and 2 months on the therapy. The absolute and percent values of the following subpopulations were assessed in a blinded fashion by fluorescent microscopy: Total T lymphocytes (CD3+), helper T lymphocytes (CD3+CD4+) and cytotoxic T lymphocytes (CD3+CD8+). In addition the changes in the ratio between CD4 and CD8 cells were evaluated as a part of assessment of the immune status of patients. The samples of the blood from 19 healthy blood donors were analyzed as a reference for normal values.

PCR analysis: Stored frozen samples of plasma were processed in bulk by using commercially available PCR kit (AmpliSense HIV-1, Central Research Institute of Epidemiology, Moscow, Russia) designed for quantitative analysis of HIV-RNA copies. Tests were carried out at baseline and after two months of the therapy.

Statistical analysis: The obtained results were analyzed with the aid of statistical software STATMOST (Datamost, South Sandy, UT). The baseline cell numbers relative to 1st and 2nd months of follow-up were evaluated by paired Student t-test. The non-parametric values of viral load were analyzed by Wilcoxon signed-rank test. All statistical calculations were per intent-to-treat basis or the total number of available patients without subgrouping them into responders and non-responders. The resulting probability values were considered as significant at the cut-off levels of $p \leq 0.05$.

RESULTS

After one month on the therapy there was a clear distinction between recipients of ART alone and those who received ART along with the daily dose of Dzherelo. This disparity became even more evident at the end of 2nd month of therapy. The changes in viral load among HIV patients of both groups have also reached statistical significance. These findings are described in detail below.

CD3+ total T Lymphocytes: After one month on ART alone the absolute and percent (%) values of total CD3+ lymphocytes per microliter of blood have changed in a statistically discordant manner, i.e., 664 (36.5%) vs

743(38.4%) with p = 0.13 (p = 0.03), as analyzed by paired Student t-test. Similarly, at the end of the first month, in the group receiving Dzherelo there was a statistical discordance between absolute and percent CD3+ values: 595(34.2%) vs 664(40.5%); p = 0.14(p = 0.0003). After 2 months the number of total CD3+ lymphocytes increased further to 785 (43.9%) in arm B, i.e., p = 0.034 (p = 0.0002), whereas in the control it increased to 819 (39.8%) cells, with probability values p = 0.06 (p = 0.03) , respectively. The accrual in total lymphocytes from baseline to the end of follow-up, was 23.3 and 31.9% for absolute and 9 and 28.4% for relative numbers in control and Dzherelo arms, respectively (Fig. 1).

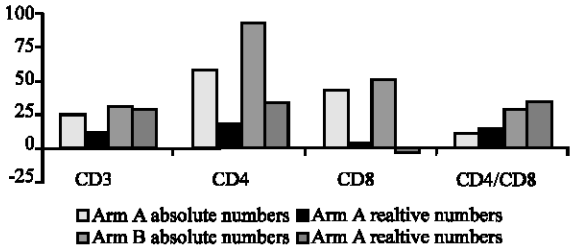

Fig. 1: Changes in absolute and relative numbers of T-lymphocyte subsets at 2 months post-therapy as expressed in percentage values relative to their respective baseline levels

CD4+ T Lymphocytes: The trends similar to those of total CD3+ lymphocytes were observed when CD3+CD4+ lymphocyte subsets were analyzed. Significant changes were seen in ART alone arm after one month, i.e., 218(30.3%)-295(35.2%); p = 0.007 (p = 0.02). Similarly in Dzherelo arm the helper T-cell counts have risen in a significant manner from 184 (28.4%)-254 (34.8%) cells; p = 0.03 (p = 0.0002). At the end of 2nd month lymphocyte subsets have risen to 343 (35.7%) and 356 (38%) with probability values p = 0.002 (p = 0.01) and p = 0.004(p = 0.0005) for arms A and B , respectively. When study completion results of ART and Dzherelo recipients were calculated in terms of accrual in CD4+ lymphocytes relative to entry levels there was an increase of 57.3% (17.8%) and 93.5% (33.8%) in absolute and relative values.

CD8+ T Lymphocytes: The changes observed in CD3+CD8+ cytotoxic T-cell population are different from those seen with helper cells. In ART alone group absolute but not relative numbers of CD8+ cells increased in a significant manner from 155 (22.6%)-203(24%); p = 0.014 (p = 0.21), while in Dzherelo group the changes were insignificant in both categories, i.e., from 123 (19.8%)-152 (20.3%); p = 0.08 (p = 0.28). At the end of the 2nd month the CTL population in ART group was still above baseline, i.e., 222 cells (23.2%), an accrual that was statistically significant for absolute numbers p = 0.009 but not significant when relative numbers were evaluated (p = 0.39). Similarly, among Dzherelo recipients the 2nd month absolute but not relative numbers of CD8 cells have also increased in a significant manner, from baseline levels 123(19.8%)-185(19.7%), with P values being 0.013 and 0.39, respectively. When study completion results of ART and Dzherelo recipients were calculated in terms of accrual in CD8 + lymphocytes as compared to baseline levels there was an accrual corresponding to 43.2% (2.7%) and 50.4% (-0.5%) of absolute and relative values, respectively.

CD4/CD8 ratio: The differential changes in CD4 and CD8 lymphocyte numbers had affected the CD4/CD8 ratio in patients on ART alone regimen as early as one month after treatment initiation. Their ratio had increased from baseline value 1.418-1.532 but without reaching significance (p = 0.23). The CD4/CD8 ratio among Dzherelo recipients had increased from 1.495-1.671, which was also above the cut-off value (p = 0.09). The disparity between CD4 and CD8 lymphocytes had progressed further by the end of 2nd month. Among ART alone patients the ratio had increased to 1.613(p = 0.14), while in Dzherelo group the ratio had risen to 1.940 (p = 0.03). When study end results of ART and Dzherelo recipients were calculated in terms of relative accrual from baseline levels there was a gain of 13.8 and 29.3% , respectively.

Lymphocyte subsets in normal blood donors: Samples of the peripheral blood of 19 healthy individuals were analyzed to obtain the normal distribution values of peripheral blood subsets. The average number of absolute and relative (%) CD3 lymphocytes were 1,370±169 cells μL^{-1} (52.9±6.8). The values of CD4 and CD8 lymphocytes were 622±89 (35.9±4.3) and 349±42(19.9±2.1), respectively, with ratio being 1.76±0.19.

Viral load: The viral load, as measured by plasma RNA-PCR at baseline and at the end of 2nd month, decreased in ART group (1718-1419 copies mL^{-1}, p = 0.008), as analyzed by Wilcoxon signed rank test. In Dzherelo arm the viral load decreased from 1793-1368 copies; p = 0.001). About three-quarters (14/19) of patients on ART alone had displayed the decrease in viral load, while the 18 out of 19 of patients on Dzherelo (95%) had a reduction in their number of viral copies (Table 1).

Table 1: Effect of 2-month ART without or with Dzherelo on HIV-RNA plasma levels

Arm A			Arm B		
HIV patients on ART alone (N=20)			HIV patients on ART + Dzherelo (N=20)		
HIV-RNA copies mL^{-1} at baseline Mean±SD = 1717±1660 Geometric mean = 1199 Median = 1045	HIV-RNA copies mL^{-1} at 2nd month Mean±SD = 1419±1650 Geometric mean = 876 Median = 1124	Difference compared to baseline Mean±SD = -299±448 Geometric mean = 272 Median = -208 Wilcoxon signed rank test; p = 0.008	HIV-RNA copies mL^{-1} at baseline Mean±SD = 1792±2202 Geometric mean = 1222 Median = 974	HIV-RNA copies mL^{-1} at 2nd month Mean±SD = 1368±2208 Geometric mean = 762 Median = 788	Difference compared to baseline Mean±SD = -425±507 Geometric mean = 353 Median = -355 Wilcoxon signed rank test; p = 0.001

DISCUSSION

In the prior studies Dzherelo has been shown to influence positively CD3 and CD4 lymphocyte numbers (Chkhetiany *et al*., 2006, 2007; Kutsyna *et al*., 2003). Dzherelo reduced the incidence of opportunistic infections and reversed body weight loss associated with HIV (Chkhetiany *et al*., 2006, 2007; Kutsyna *et al*., 2003, 2005). It had also reduced the toxic side effects of ART, the hepatotoxicity in particular (Chkhetiany *et al*., 2006, 2007; Zaitseva, 2006). For example, elevated liver aminopeptidase ALT and AST levels caused by ART have been shown to return back to normal levels. However, these studies have not dealt with the effect of Dzherelo on other immune markers and viral load.

Our 2-month study conducted in prior anti-retroviral drug-naïve population reveals that when Dzherelo is added to ART there are significant benefits associated with this intervention. In our hands Dzherelo appears to display the same effect as reported by independent investigators. Our results indicate that administration of Dzherelo along with ART can produce significant increase in total CD3+ lymphocytes, CD4+ helper cells, better CD4/CD8 ratio, higher number of CD3+HLA-DR+ activated lymphocytes and NK cells. Dzherelo appears to increase absolute but not relative numbers of CD8+ T lymphocytes and reduce significantly CD20+ B lymphocyte subpopulation. Furthermore, Dzherelo appears to contribute to inhibitory effect of ART on viral replication resulting in statistically significant lower viral load in a higher proportion of patients (Table 1).

It is well established that elevated CD3 and CD4 counts and higher CD4/CD8 ratio are associated with better prognosis in patients with HIV (Bonger and Goebel, 1991). For this reason Dzherelo is likely to influence positively the outcome of treatment and disease progression in our study population. Similarly, the viral load is a predictor of HIV disease progression, its persistent elevation in HIV infected patients is indicative of poor prognosis (Arduino *et al*., 2001; Dybul *et al*., 2002). While, there were earlier indications that Dzherelo may reduce the viral burden, our study is the first to report this phenomenon in a systemic fashion. Despite the fact that the HIV RNA levels had decreased by less than a log the difference between baseline and outcome levels was highly significant (Table 1). It is likely that the observed effect on viral load is mediated by immune cells since Dzherelo does not have the direct effect on HIV replication (Chkhetiany *et al*., 2007).

Many studies have been conducted aimed at determining the phenotype of immune cells in HIV infection. While there is a consensus that the immune response plays a critical role in determining the clinical outcome much more has to be learned in order to have a clear picture of cellular events during the course of disease. The understanding of the immune mechanism controlling HIV may result in design of better vaccines and immunotherapies. Currently available anti-retroviral therapy is far from ideal, requiring multiple drugs to be taken in combination for the rest of life of a patient (Pokrovskii, 2001). The extended duration of therapy, coupled with the side effects, often results in poor patient adherence, treatment failure and the emergence of drug resistance with major social and economic implications. We believe that the immunotherapy is the indispensable part of therapeutic strategies against HIV. The development of novel immune-based therapies is an urgent objective for anti-HIV drug discovery. Many immune interventions are available against bacteria, protozoa, fungi and viruses (Ershov, 2003). While often effective the mechanism of many immunomodulators is poorly understood. This downside should be balanced against clinically confirmed benefits. Our study provides an early glimpse into the putative immune mechanism of Dzherelo, which has been successfully used as an immune adjunct to HIV therapy in Ukraine during last ten years (Chkhetiany *et al*., 2006, 2007; Kutsyna *et al*., 2003, 2005; Prihoda *et al*., 2006; Zaitseva, 2006) Additional studies need to be conducted to develop better understanding of Dzherelo's properties and to enlarge the current arsenal of HIV therapies.

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