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DIPHTHERIA ANTIBODIES AND T LYMPHOCYTE COUNTS IN PATIENTS INFECTED WITH HIV-1

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ABSTRACT

We assessed the IgG levels anti-diphtheria (D-Ab) and T cell counts (CD4+ and CD8+) in HIV-1 infected subjects undergoing or not highly active antiretroviral therapy (HAART). Approximately 70% of all HIV-1 patients were unprotected against diphtheria. There were no differences in D-Ab according to CD4 counts. Untreated patients had higher D-Ab (geometric mean of 0.62 IU/ml) than HAART-patients (geometric mean of 0.39 IU/ml). The data indicated the necessity of keeping all HIV-1 patients up-to-date with their vaccination.

Key-words: HIV-1, antibody to diphtheria toxin, vaccination.

Human immunodeficiency virus-1 (HIV-1) infection leads to a progressive loss of immune functions involving both T and B lymphocytes (7, 9). Phenotypic and functional alterations of memory B cells are only partially recovered upon highly active antiretroviral therapy (HAART) (11). Serum antibodies against measles, tetanus toxin, and HIV-1 antigens are significantly reduced in patients with low memory B-cells (2).

The Brazilian Immunization Program provides special recommendations and offers free immunization for HIV-1 infected patients (8), but this recommendation is not regularly enforced (3). Accordingly, HIV-1-infected patients should be vaccinated against diphtheria/tetanus, pneumococcal disease, influenza, and hepatitis (8).

Adequate immunization against diphtheria/tetanus consists of receiving 3 injections at 2, 4 and 6 months of age followed by booster doses every 10 years (8). Several studies have shown that healthy adult populations may not be fully protected against diphtheria (1, 5, 6). A considerable proportion of susceptible adults to diphtheria were formerly observed in different healthy population of Rio de Janeiro, Brazil. Diphtheria toxin IgG levels in a group of Brazilian blood donors showed that a greater percentage (71%) of young military (18 to 30 years) was protected when compared to civilians (54%) of the same age group. These differences were possibly due to a higher rate of booster vaccination in young military group compared to civilians (1, 10).

In the present study we assessed the immune status concerning diphtheria in civilian or military HIV-1 infected subjects undergoing or not HAART. The levels CD4 and CD8 T lymphocytes subsets were also evaluated.

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The participants in the study, were volunteers, HIV-1-infected patients aged 11– 78 years (mean and median of 44 and 41 years, respectively), 63 military (3 females) and 42 civilians (35 females), recruited from Instituto de Biologia do Exército (IBEx), Rio de Janeiro, between July of 2006 and May of 2008. The research procedures were approved by the Ethical Committee of Hospital Universitário Pedro Ernesto, Universidade do Estado do Rio de Janeiro (HUPE/UERJ), register number 1548- CEP/HUPE dated October 30, 2006, and written informed consent was obtained from all volunteers during interview.

Diphtheria toxin IgG-specific antibody titers were determined by means of a commercial ELISA kit (Immuno-Biological Laboratories, Hamburg, Germany) with titers (IU/ml) calibrated against the WHO standard NIBSC 91/534, as previously standardized (1). The titers were classified into three groups: < 0.1 IU/ml, between 0.1 IU/ml and 0.9 IU/ml and $\geq$ 1.0 IU/ml indicating unprotected, partially protected, and fully protected individuals, respectively.

CD3+/CD4+ and CD3+/CD8+ T lymphocyte counts were determined in conformity with the National Program for Sexually Transmitted Diseases and AIDS of the Brazilian Health Ministry. Peripheral blood lymphocyte phenotypes were determined by two-color flow cytometry using a commercially available kit with fluorochrome-labeled monoclonal antibodies (BD FACSCount™ Reagents; Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). All analysis was done automatically within the FACSCount. Absolute cell counts (cells/µl) were recorded.

The ELISA results were expressed as geometric mean (geomean) in attempt to give a normal distribution of data allowing the use of standard statistical tests. The levels of significance of the differences between means were examined by the Unpaired t test (parametric test). The Mann-Whitney test was used for data that did not follow the normal distribution. Statistical differences between proportion values were evaluated by the Chi Square test. These analyses were performed with GraphPad-Prism software, version 4.02 and EpiInfo version 3.5.1. $P<0.05$ was taken as significant.

Table 1 shows the distribution of IgG anti-diphtheria toxin levels in military and civilian individuals classified by age. Four (3.8%) military had less than 0.1 IU/ml (unprotected) of IgG anti-diphtheria toxin and were not included in Table 1. Depending on the age group, 25 to 39% of civilians and military were considered to be protected (IgG levels $\geq$ 1 IU/ml) against diphtheria. Most (69 to 71%) of the patients were partially protected (IgG levels $\geq$ 0.1 < 1.0) against the disease. A similar ($P>0.05$) geomean of specific IgG concentration was detected in civilian (0.43 - 0.45 IU/ml) and military patients (0.36 – 0.45 IU/ml) of different age groups (data not shown). Only 7 (6.7%) out of 105 individuals reported that they had been recently vaccinated against diphtheria, with a mean of 10.6 months (varying from 27 days to 30 mo.) before blood collection. Five (71%) of these vaccinated individuals were military.

Table 1. Percentage of HIV-1 infected patients with antibodies to diphtheria toxin stratified by age.

Age group (years)	IgG (IU/ml) anti-diphtheria toxin			
	$\geq$ 0.1 < 1		$\geq$ 1	
	Civilian	Military	Civilian	Military
11 – 30[a]	75 (6/8)[b]	75 (12/16)	25 (2/8)	25 (4/16)
31 – 40	64 (7/11)	61 (8/13)	36 (4/11)	39 (5/13)
41 – 78	74 (17/23)	70 (21/30)	26 (6/23)	30 (9/30)
Total: 11 - 78	71 (30/42)	69 (41/59)	29 (12/42)	31 (18/59)

[a] There was only one individual (civilian) aged 11 years. All the others were older than 16 years of age.
[b] % (6 individuals out of 8 presenting IgG levels $\geq$ 0.1 < 1)

There were no significant differences in antibody levels or in the proportion of protected individuals between military and civilian HIV-1 patients. Comparing with previous data from Brazilian blood donors (10) we detected that military HIV-1 patients had lower ($P < 0.05$) IgG anti-diphtheria (geomean of 0.43 IU/ml) than military blood donors (geomean of 0.61 IU/ml). This fact was due to a high specific IgG levels in military blood donors aged from 18 to 30 years (geomean of 0.82 IU/ml) who may have a higher rate of compliance with booster vaccination regimens (10). However, civilians from the HIV-1 group and Brazilian blood donor group had similar antibody levels (0.45 IU/ml and 0.55 IU/ml, respectively) against diphtheria.

Protection against diphtheria is associated with the development of toxin-neutralizing antibodies (4). Due to the inherent technical difficulties in determining neutralizing antibody titers, in many opportunities commercially available ELISA kits are used to investigate IgG antibody levels to diphtheria toxin (12). Neutralizing antibody levels are categorized according to internationally accepted ranges: < 0.01 IU/ml (non-protective), between 0.01 to 0.09 IU/ml (basic protection), $\geq$ 0.1 IU/ml (full protection) (12). We have previously compared the antibody titers detected by the ELISA kit used in this study and the neutralization assay using a sampling of serum collected of blood donors from Rio de Janeiro (1). The ELISA kit revealed a high specificity and good predictive value for evaluation of full protection (1). The levels of protection against diphtheria based on the concentration of ELISA antibodies against the toxin determined by this kit are about 10 times higher than the corresponding neutralization antibody levels. Despite the significant positive correlation between the two assays, the neutralization assay has a greater sensitivity compared to the ELISA. Thus, the percent of individuals not protected, as detected by ELISA, could be even higher than the present report.

As expected for this patient population, lymphocyte subset distribution showed an inverse distribution of CD4 and CD8 T cells in HIV-1 patients. Significantly low CD4 count values were detected, varying from 13 to 930 cells/µl (geomean of 348 cells/µl), without significant differences between age groups (data not shown). CD8 T cell counts were significantly higher than CD4 numbers ranging from 262 to 2879 cells/µl (geomean of 933 cells/µl). Consequently, the CD4:CD8 ratio was greatly reduced with a geomean value of 0.37 (data not shown). Our reference values for CD4 and CD8 numbers were 825 and 505 cells/µl, respectively, based on the T lymphocyte counts detected in blood donors from IBEx (unpublished data). Concerning lymphocyte subset distribution and specific antibodies according to gender, we found a similar profile for male and female HIV-1 patients (data not shown).

The geomean of IgG anti-diphtheria among individuals grouped by different CD4 numbers ($\geq$ 500, $\geq$ 200 and < 500 and < 200 cells/µl) varied between 0.41 and 0.47 IU/ml ($P > 0.05$). The percentage of individuals protected against the disease was similar ($P>0.05$) for all groups and varied between 25 and 34% (data not shown). In addition, there were no significant correlations between CD4 counts and specific IgG levels of HAART- treated patients and those not being treated (data not shown). These data suggest that the number of T lymphocyte subsets does not correlate significantly with lymphocyte function as previously described elsewhere (7).

In spite of the fact that the sample size of untreated patients was small (n = 18) compared to HAART-treated patients (n = 85), data in Table 2 showed that untreated HIV-1 patients had higher ($P < 0.05$) IgG anti-diphtheria levels (geomean of 0.62 IU/ml) and viral load (log mean of 3.9) compared with HAART-treated patients (geomean of 0.39 IU/ml) and log mean viral load of 2.5. About 26% of HAART-patients had protective levels of antibodies against diphtheria toxin ($\geq$ 1.0 IU/ml) compared to 39% of the untreated patients ($P>0.05$). Comparing with previous published data (12), untreated patients had a similar antibody response to diphtheria toxin to civilian blood donors from the IBEX (geomean of 0.55

IU/ml). In contrast, HAART- patients had a significant lower specific IgG levels compared with IBEX blood donors, suggesting the persistence of B and/or T cell deficiency despite the specific treatment to HIV-1 infection. There were no significant differences of CD4 or CD8 counts and CD4:CD8 ratio between patients, whether or not undergoing HAART (Table 2).

Table 2. Geometric mean (95% CI; n) of IgG anti-diphtheria, CD4 and CD8 counts (cell/µl) in HIV-1 patients treated by highly active antiretroviral therapy (HAART) or not (No HAART)

	HAART	**No HAART**
IgG (IU/ml)	0.39 (0.32 – 0.47; 85)	0.62 (0.48 - 0.79; 18)*
% individuals IgG >= 1 IU/ml	26	39
CD4 count	339 (288 – 400)	399 (328 – 486)
CD8 count	910 (813 – 1019)	1043 (894 – 1217)
Viral load (log mean)	2.5	3.9*

* $P < 0.05$ comparing HAART-treated patients and no-HAART patients by Mann-Whitney test.

The reasons for the difference in antibody response between treated and untreated groups are unknown but some considerations should be done: i) the distribution of civilian and military individuals was similar in HAART-treated patients (61% were military) and non-treated patients (58% were military); ii) the mean and median ages of treated patients were 46 and 42 years, respectively, while for the untreated ones it was 34 and 30 years. As tetanus and diphtheria antibodies tend to decrease with time, the difference in age between HAART-treated patients and those not being treated might introduce a bias in the study. iii) Based on the time elapsed between blood sample collection and HIV-1 diagnostic, untreated patients had a lower duration of disease (mean of 4.1 years) compared to HAART-patients (mean of 6.5 years). The mean duration of the HAART regimen was 3.7 years, indicating that HAART therapy was initiated at a mean time of 2.8 years after HIV-1 infection diagnostic. A critical question is whether the introduction of HAART recovers and/or maintains the B cell compartment fully functional. Initiation of HAART in HIV-1 vertically-infected children within the first year of life permits the normal development and maintenance of the memory B cell compartment. On the contrary, memory B cells from patients treated later in time are remarkably reduced and their function is compromised regardless of viral control (9). Finally, iv) for HAART-patients CD4 cell counts varied from 13 to 930 cell/µl, with 21% of individuals showing less than 200 cells/µl. For untreated patients, CD4 cell counts varied from 153 to 751 cell/µl, with 11% of individuals showing less than 200 cells/µl ($P > 0.05$ compared with HAART-patients). Therefore, the age of the patient, the duration of disease, the time of HAART-therapy onset and the CD4 cell number variations may have influenced the antibody differences between treated and untreated groups.

A recent study (3) reported that in relation to diphtheria and tetanus, the majority of Brazilian HIV-1 patients have inadequate immunization (30.6% have received an incomplete scheme, and 33.3% have never been vaccinated). Only 36.1% of patients showed up-to-date immunization. The present study also indicated that most of the HIV-1 patients are unprotected against diphtheria suggesting that knowledge about protective antibody levels against pathogenic microorganisms in HIV-1 patients will be beneficial to review the vaccination schedules used in this particular group of patients. The data shown here reinforce the necessity of keeping not only HIV-1-infected individuals, but the whole population, up to date with their vaccinations against diphtheria, including in Rio de Janeiro.

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