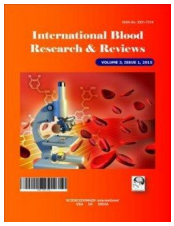

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Microbicidal Activity of Neutrophils Isolated from HIV Patients

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Authors' contributions

This work was carried out in collaboration between all authors. Authors ARTP and SL designed the study, wrote the protocol, and wrote the first draft of the manuscript. Authors JBB and TSJ managed the literature searches, managed the experimental process. Authors NCSS and NAL performed the spectrophotometry analysis. All authors read and approved the final manuscript.

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ABSTRACT

HIV infection is associated with a progressive loss of T cell functional capacity and reduced responsiveness to antigenic stimuli. Neutrophils are crucial cellular components of the innate immune system. Current study evaluates the functional activity of neutrophils isolated from HIV/AIDS patients with similar clinical laboratory parameters differing only in the use of antiretroviral therapy (HAART). Two patients HIV/AIDS patients, a female and a male, were selected for this study, based on clinical history, general physical examination and laboratory tests. Further, two apparently healthy volunteers of the same age and gender former de control group. Neutrophils isolated from human peripheral blood were placed in contact with the yeast in a proportion of 1:10 leukocytes for 1 hour. PMN Fluorescence was detected in FL1 on a flow cytometer and results were recorded as fluorescence intensity and percentage of positive cells in

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the sample. HOCl formation was monitored by spectrophotometry based on the resulting taurine chloramine-forming reaction of hypochlorous acid with taurine. The experiments revealed that patient I with HAART had a 17.3% lower response activity of neutrophils when compared with the control in the production of hypochlorous acid with PMA stimulation. Patient II, who did not use HAART, was 81% less active than the control in the production of hypochlorous acid. The two patients had similar clinical laboratory parameters differing only in the number of CD4 cells, which was higher in Patient II. Results show that the patient submitted to antiretroviral treatment had a better quality of functional response of neutrophils although with fewer CD4 cells.

Keywords: AIDS; neutrophils; microbicidal activity; HOCl.

1. INTRODUCTION

Despite of 30 years of intensive research, our understanding of how HIV virus undermines the ability of the immune system against common infections is limited. Although we know that T cells, a key cell population that normally invading pathogens lose their function capacity in HIV infected individuals, the reason they do so is unknown. It has been discovered that HIV virus activates another type of cells, called neutrophils, the most common type of white cell in the blood. Activated neutrophils negatively affect the function of T cells and prevent them from producing cytokines, protective proteins that serve as messengers orchestrating the immune response to bacteria and viruses [1].

Neutrophils, the most abundant leukocyte population, are traditionally recognized as essential effector cells of the innate immune system in host defense against invading pathogens [2]. A new appreciation has recently emerged on the role of neutrophils in their interaction with and regulation of the adaptive arm of the immune system [2,3]. Neutrophils co-localize and actively communicate with T cells at sites of infection and migrate to the draining lymph nodes where they are involved in the induction and regulation of cellular and humoral immune responses by exerting a pro-inflammatory or anti-inflammatory function [4]. Accumulated evidence supports the role played by neutrophils in the negative regulation of T cell function via production of reactive oxygen species (ROS) [5-7].

Due to the functional importance of neutrophils in infection by microorganisms particularly with HIV, current study evaluates the microbicide activity of peripheral blood neutrophils of patients with HIV/AIDS when compared to the activity of a control group of healthy volunteer donors.

2. MATERIALS AND METHODS

2.1 Subjects

HIV/AIDS patients treated at the Center for Studies and Support to HIV Patients of the State University of Maringá (Department of Basic Health Sciences) were clinically evaluated and laboratory tests were performed prior to their participation in the projects.

2.2 Inclusion Criteria

Inclusion criteria comprised age between 35 and 45 years and 10 years infection time. After the explanation of the project, patients signed an informed consent approved by the Ethics and Research on Human Experimentation of the State University of Maringá.

Considering their clinical history, general physical examination and laboratory tests, two patients HIV/AIDS, a female and a male were selected for current study. Composite control group of two apparently healthy volunteers of the same age and gender of patients was also selected.

2.3 Clinical and Laboratory Analysis

The clinical history of each patient was obtained by the epidemiological record. The clinical aspects of several other systems (musculoskeletal, neurological, respiratory, cardiovascular, genitourinary and digestive) were evaluated during the general physical examination.

Laboratory evaluation comprised the number of total leukocytes and differential count of neutrophils and lymphocytes obtained by automated cell counter (MINDRAY BC-3000 Plus) coupled to microscopic evaluation. Evaluation of plasma levels of liver enzymes AST, ALT and GGT assessed liver function, total

cholesterol, triglycerides and fasting glucose. All biochemical laboratory measurements were performed by specific commercial kits.

2.4 Isolation of Peripheral Neutrophils

2.4.1 Blood collection

Three experiments were performed for each patient, at an interval of at least 30 days. Samples for the HIV/AIDS patients and volunteers (control group) were drawn in experimental day. Samples were collected by anterior-ulnar venipuncture using vacuum collection system. During collection HIV testing was also carried out on patients and volunteers.

2.4.2 Isolation of neutrophils

The blood samples were collected in heparinized tubes (10 U/ml) and then diluted 1: 1 with 10 mM PBS. pH 7.4. The dilution was placed in 10 ml of Histopaque® (BOYUM 1968).

The material was centrifuged at 2500 rpm at room temperature for 20 minutes. An infranatant was added with 15 mL of 5% Dextran diluted in 10 mM PBS pH 7.4 for sterile sedimentation of erythrocytes. The material was kept in an ice bath (with a 45° inclination) for 45 minutes, the supernatant was collected (volume made up to 30 ml of 10 mM PBS) and centrifuged at 2500 rpm at room temperature for 5 minutes. The supernatant was discarded and the infranatant subjected to hemolysis in 10 ml of cold distilled water with constant stirring for one minute. Isotonicity was restored with 5 ml of 2.7% NaCl and 15 mL of sterile 10 mM PBS. The material was centrifuged at 2500 rpm at room temperature for 5 minutes, the supernatant was discarded and the infranatant resuspended in 1 ml RPMI.

2.5 Evaluation of Microbicidal Activity of Neutrophils

2.5.1 Determination of HOCl

HOCl formation was monitored by spectrophotometry based on the resulting taurine chloramine-forming reaction of hypochlorous acid with taurine. Neutrophils ($2x10^6$ cells/mL), activated or not with the standard strain of microorganisms in 10 mM phosphate buffer (pH 7.4) containing 140 mM NaCl, 1 mM CaCl2, 0.5mM $MgCl_2$ and 1 mg/mL glucose were incubated with 15 mM taurine at 37°C under gentle agitation. The reaction was stopped after 30 minutes by adding of 20 ug/ml catalase (10 minutes, 2000 rpm). The concentration of taurine chloramine-present in the supernatant was estimated by acid oxidation of 5-thio-2-nitrobenzoic acid (TNB) with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) which measured the decrease in absorbance at 412 nm as described [8].

2.6 Statistical Analysis

Group-comparing statistics were performed with Graph Pad Prism 6.0 (Graph Pad, San Diego, CA, USA) with Student´s *t* test at $p < 0.05$ considered statistically significant.

3. RESULTS

Two patients were selected for the study after clinical and laboratory examination. Patient I (M.E.D) 45 years old, CD4 cells levels 246 cells/μL, viral load < 50 copies/mL, with no history of liver disease, heart or hypertension. Clinical parameters were within normal levels. He was diagnosed for HIV 10 years ago with levels of CD4 cells 3 cells/μL, once diagnosed treatment began with antiretroviral therapy. Patient II (SRO) 38 years old, 10 years of infection without significant symptoms during the course of the disease, had no history of liver disease, diabetes and hypertension. Viral load 180 cells/μL and the amount of CD4 cells about 400 cells/μL. Post after diagnosis for HIV patient did not use antiretroviral therapy at the moment.

Two healthy volunteers were included in our study after confirmation they had no sign of acute inflammatory and infectious process such as sore throats, fractures, bacterial, fungal, viral and parasitic infections, or suffering from chronic inflammatory diseases.

Table 1 shows clinical laboratory parameters and levels of CD4 + lymphocytes of patients.

Table 2 shows levels of total leucocytes, neutrophils and lymphocytes of HIV patients.

Fig. 1 shows the production of HOCl (hypochlorous acid) of neutrophils (PMN) in healthy patients and HIV-positive patients.

Results show lower activity neutrophils obtained from Patient I in response to the stimulus compared to control, which is 17.3% lower than the control response in the production of hypochlorous acid prior to PMA stimulation.

Table 1. Clinical and laboratory parameters of HIV patients

HIV patient	Gender/Age	Infection time (years)	CD4 cells/mm^3/viral load	HAART	clinical laboratory changes
I	Male/45	10	246 cells /< 50 copies/mL	For 10 years	no
II	Female/38	14	400 cells/180 copies/mL	Not use	no

Table 2. Levels of total leucocytes, neutrophils and lymphocytes of HIV patients

HIV patient	Total leucocytes cells/mm^3	Neutrophils cells/mm^3	Lymphocytes cells/mm^3
I	3,400	1,496	1,394
II	3,500	1,620	1,295

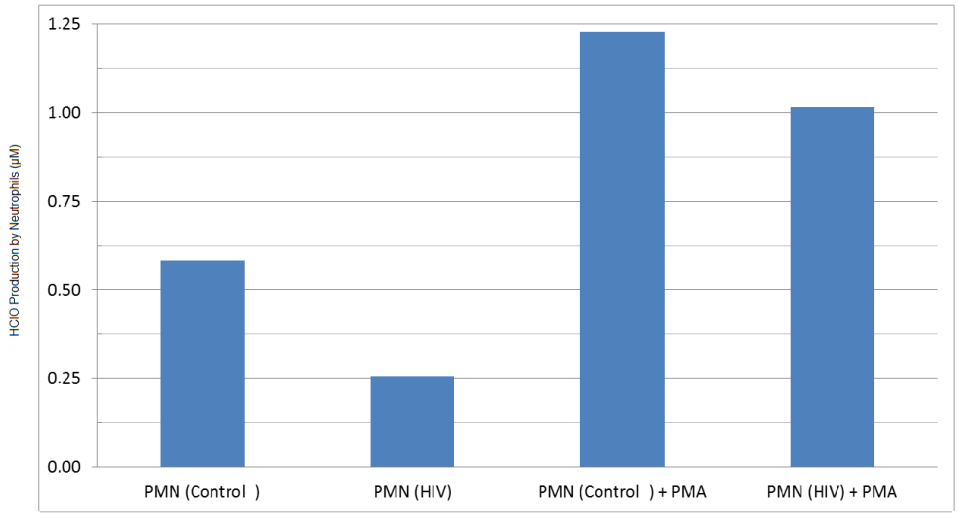

Fig. 1. Determination of the formation of HOCl (hypochlorous acid) accompanied by spectrophotometry of patient I (HIV/AIDS) compared to control, a male without disease

Fig. 1 Production of HOCl (hypochlorous acid) of neutrophils (PMN) in healthy patient and HIV patient I. Comparison of HOCl production of neutrophils (2.0 x 10^6 cells / ml) from healthy patients and in patients with to virus or not stimulated with PMA was determined by spectrophotometry at 655nm

Fig. 2 shows the results of lower activity neutrophils obtained from Patient II in response to stimulus when compared to control, which is 81% lower than the control response in the production of hypochlorous acid prior to PMA stimulation.

Table 3 shows the hypochlorous acid production by neutrophils isolated from the HIV patients compared to healthy controls.

4. DISCUSSION

Neutrophils are crucial cellular components of the innate immune system. They are the first cells recruits to sites of microbial challenges or injury. An essential function of neutrophils include their ability to promptly generate and release copious amount of reactive oxygen species (ROS) in a process referred to as oxidative burst. The production of ROS is critical to neutrophil antimicrobial activities. A deficiency in oxidative metabolism may result in immune impairment, as may be seen in chronic granulomatous disease [9].

People infected with HIV become progressively immunodeficient, a process that exposes infected individuals to an escalating risk of opportunistic infections. Even though HIV immunodeficiency is generally etiologically likened to CD4 lymphocytes, opportunistic infections organisms observed during HIV disease (i.e. *Pneumocystis carinii, Candida albicans* or *Mycobacterium avium)* are suggestive of immunodeficiency in other immune

cell type such as neutrophil [10,11]. *Ex vivo* peripheral neutrophils isolated from HIV infected subjects display a dysfunctional phenotype of impaired chemotaxis and deregulated production of ROS [12,13]. ROS production in HIV individuals has been reported to be either exaggerated or reduced when compared to con troll HIV infected subjects. Deregulated responses to stimulation and/or inhibition of neutrophil oxidative metabolism may therefore contribute towards immune dysfunction and oxidative stress in HIV disease [14].

Consequently, repeated microbial challenges and/or inflammatory conditions in the course of HIV disease is likely to result in disproportionate neutrophil oxidative activity, which in turn would aggravate oxidative stress resulting in infections, increased HIV viremia and cardiovascular disease. Consequently, infection and inflammation are a crucial aspect in the management of HIV disease [14].

So that important functions of leukocytes, such as phagocytosis and microbicidal activity, could be maintained, their chemotactic function is preserved [15]. In patients with infections and sepsis to reduce, the neutrophil chemotactic function compared to healthy volunteers. It may be suggested that dysfunction contributes towards the development of infection [16]. In Infectious diseases such as AIDS, the role of neutrophils as a defense cells is well documented In other words, the antiretroviral drugs increase the chemotaxis of neutrophils and monocytes and reduce the incidence of infections [17].

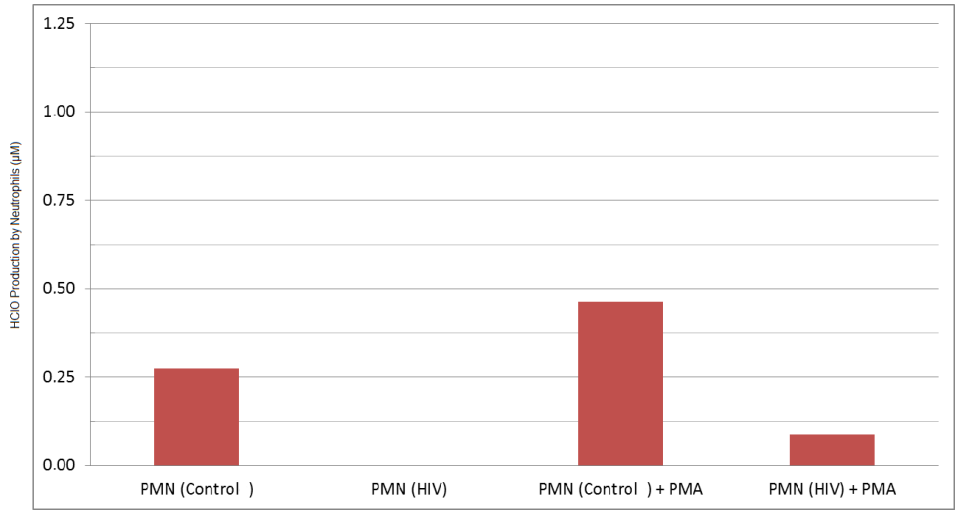

Fig. 2. Determination of the formation of HOCl (hypochlorous acid) accompanied by spectrophotometry of patient II (HIV/AIDS) compared to control, a female did not have the disease

Fig. 2 production of HOCl (hypochlorous acid) of neutrophils (PMN) in healthy patient and HIV patient II. Comparison of HOCl production of neutrophils (2.0 x 10^6 cells / ml) from healthy patients and patients with virus or not stimulated with PMA was determined by spectrophotometry at 655nm

Table 3. Production of hypochlorous acid by neutrophils isolated from HIV patients compared to controls

HIV Patient	PMN	PMN (HIV)	PMN + PMA	PMN(HIV) +PMA
I	4.495±0.37	3.228±0.24	5.268±0.39	4.110±0.13*
II	0.275±0,15	- 0.1±0.001	0.463±0.06	0.088±0.03*

*Results were expressed as mean ± SD from at least three independent experiments, with *p≤0.05 considered significant%.PMN (control) = leukocytes isolated from control patient PMN (HIV) = polymorphonuclear leukocytes isolated from HIV-infected patient, PMN (control) + PMA = polymorphonuclear leukocytes isolated from control patients with PMA stimulation to evaluate the production of hypochlorous acid leukocytes PMN (HIV) + PMA = polymorphonuclear leukocytes isolated from HIV patient infected with PMA stimulation to evaluate the hypochlorous acid production by leukocytes*

With the evolution of HIV infection, some individuals have physiological and functional changes such as oxidative stress (OS) which enhance the immune dysfunction and promotes an increase in viral replication [18]. In addition, OS may promote apoptosis of T cells and is involved in the mechanism of induction of tumor necrosis factor (TNF) -α [19].

Several studies indicate that human immunodeficiency syndrome is associated with morphological abnormalities in the bone marrow and a decrease of progenitor blood cells. The depletion of these cells could be explained by the same mechanism of apoptosis, including neutrophils, changes in cytokine or other immune factors [20]. Research reveals that progenitor cells from bone marrow of HIV-infected patients undergo apoptosis. Further, the apoptosis of these cells occurs through the Fas-L, cytokine produced by activated T cells, a mechanism by which T cells activated fighting cells infected with HIV [21]. This suggests a mechanism in reducing the chances number of neutrophils and, other cells in HIV-infected patients.

In the late phase of HIV infection, there is a decrease in serum levels of IL-2 and IFN-γ and IL-4 and IL-10 increased with decrease of the adaptive immune response. HAART may lead to an increase in pro inflammatory cytokines such as TNFα. The balance of cytokines such as IL-6, TNFα and IL-10 contributes to the establishment of equilibrium which can be determinant of disease progression.

The above changes may result from the evolution of the infection itself or induced by the use of some drugs of antiretroviral therapy presenting myelosuppressive activity. Results comprise progressive decrease in blood cellularity [22] when compared to healthy patients without HIV.

Although current study presents different results similar the production of HOCl by patients with the same infection period the difference lies in the fact that Patient I uses antiretroviral therapy and patient II does not (Table 3, Figs. 1 and 2). Table 2 also demonstrate that the patients have very similar numbers of total leukocytes, lymphocytes and neutrophils.

On the other hand, as Table 1 shows the number of CD4 cells is higher in Patient II (400 cells/mm^3) when compared to that Patient I (246 cells/mm^3). This fact serves as a laboratory parameter to indicate the non-use of antiretroviral by Patient II.

Results obtained in current study with patients isolated neutrophils indicate that Patient I had a better functional response of neutrophils when compared to Patient II although with a smaller number of CD4 cells. Table 1 shows the number of CD4 cells, or rather, Patient II (400 cells/mm^3) shows highest number with respect to the patient I (246 cells/mm^3). The cells CD4 rate serves as a laboratory parameter to indicate the use of antiretroviral drugs by patients, this patient mode II has no laboratory indication for use of antiretroviral

Results obtained in current study of patients with isolated neutrophils indicate that the Patient I has a better functional response of neutrophils when compared to the Patient II although it has a smaller number of CD4 cells. Thus, the importance of the functional capacity of neutrophils and not merely the number of cells (total leukocytes, lymphocytes, neutrophils and CD4 cells) should be underscored, a fact evidenced in this study.

Despite recent advances in the understanding of HIV infection, major scientific incognita remain and HIV infection is still a global challenge for mankind. Viruses exclusively depend on the host's cellular machinery for their propagation and survival and therefore need to invade the host's cell. HIV infection is associated with a progressive loss of T cells functional capacity and reduced responsiveness to antigenic stimuli. The mechanisms underlying T cell dysfunction in HIV/AIDS are not completely understood. Current author have indicate that HIV virus activates another type of cells, neutrophils, the most common type of white cell in the blood. Activated neutrophils negatively affect the function of the T cells and prevent them from producing cytokines, protective proteins that serve as messengers orchestrating the immune response to bacteria and viruses [1].

HIV establishes persistent infection in human subjects. Although antiretroviral therapy prevents AIDS-related complications and prolongs life expectancy. HIV-1 infected patients have several comorbidities that are usually observed during the human aging process. PMNs are a key component of the early innate response to bacterial and fungal pathogens. In response to pathogens, PMNs rapidly migrate from the blood to inflamed tissues, where their activation

triggers microbicidal mechanisms as rapid production of reactive oxygen species (ROS) in oxidative bursts [23]. After they kill microbes, PMNs die spontaneously, mainly through apoptosis. Although they have a very short lifespan their activation by circulating microbial products, as well as by proinflammatory mediators, promotes their survival. In fact, it is a critical mechanism in their effectiveness against pathogens. Nevertheless, inappropriate PMNs survive might lead to a chronic persistent inflammatory mediators and damage associated molecular patters through PMN necrosis [24]. Although PMNs are primarily protective, their inappropriate, excessive or prolonged activation presents the risk of tissue injury and organ dysfunction. It has been involved in various inflammatory diseases, including cardiovascular and osteoarticular disorders [25,26]. The results of this study corroborate these studies, as demonstrated that the patient (II) which is not used HAART showed a neutrophil response less than the patient (I) who used HAART confirming a direct effect of HIV on the quality immunological response of the host even with acceptable number of CD4 cells did.

Finally, further investigation should concentrate the causes for the differences in neutrophil responses observed in HIV disease, at cellular and molecular level. The identification of these causes and the normalization of neutrophil responses may improve the overall immune status and prognosis of HIV infected individuals.

5. CONCLUSION

HOCl is considered the most bactericidal oxidant produced by neutrophils. The production of ROs, as HOCl has a critical role in bactericidal and fungicide activity, and enhances the inflammatory reaction.

During the experiments, which included the isolation of neutrophils and the monitoring the formation of the HOCl isolates, it was possible to compare two HIV/AIDS patients whose antiretroviral treatment was the basic difference. Through experiments, the authors observed that the Patient I with HAART had a response activity of neutrophils 17.3% lower when compared to control in the production of hypochlorous acid before the PMA stimulation. On the other hand, Patient II, who did not use HAART, was active 81% less than control in the production of hypochlorous acid. The two patients had similar clinical laboratory parameters and differed only in the number of CD4 cells, which were higher in Patient II. Results show that the patient submitted to antiretroviral treatment had a better quality of functional response of neutrophils although with fewer CD4 cells.

ETHICAL APPROVAL

The study was conducted in accordance with the ethical standards set out in Resolution No. 196/96-CNS Ministry of Health on research involving human beings in Brazil, after being approved by the Ethics Committee on Human Research of the State University of Maringa, Maringá PR Brazil. The protocol was approved according to Resolution n^{o} 196/96 and additional CNS/MS in deliberative meeting of COPEP. CAAE n° 10718612.6.0000.0104/2012.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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