International Journal of Health Research, June 2011; 4(2): 69-74



Available at http://www.ijhr.org

# Original Research Article

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Online Journal

# Humoral Immunological Factors and Nitric Oxide Levels in HIV Patients with Low CD4+ T-Lymphocytes Count

## *Abstract*

**Purpose:** This study determined the plasma levels of nitric oxide (effector molecule in intracellular killing by phagocytes), regulators (C3 inactivator and C1 inhibitor) and factors (C4 and C3c) of Complement system and immunoglobulin classes (IgG, IgA and IgM) in HIV patients compared with HIV un-infected controls. The aim is to provide additional explanation for long persistence of human immunodeficiency virus (HIV) in HIV patients.

**Methods:** The plasma levels of Complement regulators (C3 activator and C1 inhibitor), Complement factors (C3c and C4), immunoglobulin classes (IgG, A and M) were estimated using immunoplates in controls, HIV patients with <200 CD4+ T lymphocytes per microliter of blood and HIV patients with 200–499 CD4+ T lymphocytes per microliter blood. Also the level of plasma NO was determined using Griess reagent.

**Results:** The levels of C1 inh, C3 act, C3c, NO, IgG, IgA and IgM were significantly higher in all HIV patients compared to controls. The mean values of C1 inh and C4 were significantly higher in HIV patients with a CD4+ T lymphocytes <200 cells/ µl blood compared with HIV CD4+ T lymphocytes 200–499 cells/ µl blood.

**Conclusion:** It may be concluded that plasma levels of C1 inh and C4 might be used to distinguish HIV severity.

**Keywords:** Immunodeficiency virus, Complement system, Immunoglobulin Classes, Reactive Oxygen Species.

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## Introduction

HIV patients showed abnormal patterns of serum protein electrophoresis [1], polyclonal hyper-gammaglobulinemia, hyperproteinemia, and plasma cell dyscrasias [2, 3]. This may suggest that autoimmune responses or plasma cell neoplasia is associated with HIV infection. Few autoantibodies (anti-Protein S autoantibodies, anti cardiolipin autoantibodies and anti-C1q autoantibodies) have been reported in HIV

patients [4, 5], thus increased concentrations of immunoglobulin classes obviously reflect abnormal underlying humoral immunological process of HIV. Therefore, it is possible that there are many other yet unrecognised autoantibodies in HIV patients. One of the aims of this study was to confirm polyclonal hypergammaglobulinemia and possible autoimmune disorder in HIV patients with low CD4 counts.

Patients infected with HIV have high levels of circulating immune complexes [2, 6, 7], which are potent activators of the complement system. Reports on the levels and activities of Complement system in HIV patients are inconsistent. Complement activation in HIV patients was reported to be via classical pathway [6, 7, 10] but one study reported [8] alternate pathway of complement activation in HIV subjects. Despite these activations of Complement System in HIV patients, HIV survives in the patients. The virolysis of HIV was reported [11] to be prevented by complement regulatory molecules, which either are included in the virus membrane upon budding from infected cells (e.g. DAF/CD55) or are secondarily attached to HIV envelope glycoproteins (Factor H) [12].

Another possible mechanism by which HIV escape complement lysis was reported to be mutated HIV envelope proteins [10, 13]. Thus complement-activating anti-HIV antibodies present in the plasma may be unable to bind and lyse HIV [10, 13]. The present study therefore provides additional information to intrinsic resistance of HIV to complement-mediated virolysis in HIV patients. This was done by measuring C1 inhibitor and C3 activators as regulators of classical and alternate pathways of complement activation respectively while the levels of C4 and C3c were measured as factors of classical and alternate pathways of complement system respectively.

Polymorphonuclear leucocytes produce reactive oxygen radical during intracellular killing of ingested particles. In HIV subjects, phagocytotic activity was reported to be reduced due to down regulation of IL-12, impaired phagosome-lysosome fusion by gp 120 [14], inhibition of chemotaxis by Nef protein [15]. This impairment allows for establishment and reactivation of opportunistic infections [16]. However, it was reported that in the early stages of HIV infection, phagocytosis by monocytic/polymorphonucleated cells and release of reactive oxygen products were increased [17]. The present study determined plasma level of nitric oxide in HIV patients.

# Methods

A total of 36 consecutive HIV patients aged 22–69 years were recruited for this case control study in Haematology Department, University College Hospital, Ibadan, Nigeria. Twenty-nine age-/sex-matched healthy HIV-negative health workers (23-65years) were recruited as controls. Informed consent was obtained from each subject before collection of blood samples. A sample of 5 ml of venous blood was collected from the antecubital vein without venous stasis from each patient into bottle with anticoagulants. The blood samples were centrifuged in MSE centrifuge for 10 min at 3,500 rpm. The plasma was immediately placed in a freezer until analysis. The main outcome measures were IgG, IgA, IgM, C3c,

The plasma levels of immunoglobulin classes and Complement factors/regulators were measured using immunoplates. The method is based on the principle of single radial immunodiffusion as previously described [1]. Radial immunodiffusion involves radial diffusion of a specific antigen through an agarose gel containing the appropriate mono-specific antibody. During radial diffusion, stable antigen-antibody complexes are formed at equilibrium which appears as a visible ring. The diameter of the ring is a proportional to the concentration of the antigen. The level of NO was determined using Griess reagents and method [18]. Structured questionnaire was used to obtain socio-demographic data while Body Mass Index (BMI) was calculated using the values of height and weight.

Those with presence of confounding illnesses were excluded. Also excluded are cigarette smokers or those on locally brewed drinks were excluded. Others excluded were those with high

abnormal liver functions, pathogenic infections and abnormal renal functions as presented by blood, urine and stool tests. Other exclusion criteria were subjects with rheumatic fever, rheumatoid arthritis, subjects who received oral contraceptives, nonsteroidal anti-inflammatory drugs, corticosteroids, anticon-vulsants and antidepressants. Those that declined their consents were also excluded. These exclusion criteria lead to the numbers of alcohol consumers (n = 36) and controls (n = 29) in the study.

The laboratory investigations that were carried out and the reasons are provided in Table 1.

**Table 1:** Laboratory tests

| Test | Purpose |
|---|---|
| Complete blood count | To exclude anaemia, leucopenia, leucocytosis, eosinophilia or any other abnormal figures in blood count |
| Thyroid function tests | To exclude increased T3 and T4 serum levels or patients with low serum T3 and T4 levels |
| Renal function tests (blood urea and serum creatinine) | To exclude renal impairment |
| Liver function tests | To exclude liver affection, especially those with high liver enzymes or those with diminished albumin levels or high globulin levels |
| Urine and stool analysis | To exclude urinary tract infection or parasitic infestations. |

**Data analysis**

The biochemical indices were determined as means with their respective standard deviations. The significances of the differences between mean values were determined using Students (t) test. Chi-square was used to test for significance between socio-demograhic and clinical variables of patients and control subjects. At 95% confidence interval p values below 0.05 were regarded as significant.

# Results

The levels of C1 inh, C3 act, C3c, NO, IgG, IgA and IgM were significantly higher in all HIV patients or HIV patients with <200 CD4+ T lymphocytes per microliter blood or HIV patients having between 200–499 CD4+ lymphocytes per microliter blood compared with controls. C4 was significantly reduced in HIV subjects having 200‑499 CD4 cells/µl blood compared with controls or HIV subjects having <200 CD4 cells/µl blood (Table 2). However, C4 was not significantly different in all HIV patients or in HIV subjects having <200 CD4 cells/µl blood compared with the controls. The mean values of C1 inh was significantly higher in HIV patients with a CD4 <200 CD4 cells/µl blood compared with 200‑499 CD4 cells/µl blood while the levels of C3act, NO, C3c, IgA, IgG and IgM were not significantly different in HIV patients with a CD4 count of <200 CD4 cells/µl blood compared with 200‑499 CD4 cells/µl blood (Table 2).

Most of the HIV patients were unemployed and had lower BMI than other subjects (Table 3).

# Discussion

Immunoglobulin classes were significantly raised in HIV patients compared with controls but there were no significant difference in the levels of Ig classes when patients with CD4+ T cell count below 200 were compared with patients having CD4+ T cell 200-400 per microliter of blood. The implication is that plasma concentrations of Ig classes might not be useful in differentiating severity of HIV. The present observation of raised Ig classes support non-specific polyclonal B cell activation in HIV subjects as previously reported [2,3]. Polyclonally raised immuno-globulins are common with many infective and inflammatory conditions [2,3]. HIV infection is inflammatory in nature as reported by high concentrations of certain acute phase proteins.

Other possibilities for raised levels of Ig classes in HIV patients might be abnormalities of lymphokines or other signals from co-infections

**Table 2:** The plasma levels of NO, Immunoglobulin classes, Complement Regulators and Complement Factors in HIV patients and control

| | Control (n = 29) | HIV (n = 36) | <200 CD4cells/µl blood (n= 26) | 200-499 CD4cells/ µl blood (n= 10 ) |
|---|---|---|---|---|
| NO (µM/L) | 4.02 ± 1.69 | 5.34 ± 1.96* | 6.45 ± 2.64* | 5.98 ± 2.18* |
| IgG (mg/dL) | 547.1 ± 188 | 871 ± 176.1* | 880 ± 164* | 859 ± 187* |
| IgA (mg/dL) | 107.3 ± 26 | 153 ± 41* | 155 ± 31* | 150 ± 51* |
| IgM (mg/dL) | 45.4 ± 17 | 99.8 ± 33.7* | 92 ± 30* | 111.3 ± 35.3* |
| C4 (g/L) | 0.15 ± 0.07 | 0.15 ± 0.05 | 0.15 ± 0.05 | 0.12 ± 0.04*▪ |
| C3c (g/L) | 0.63 ± 0.16 | 0.77 ± 0.27* | 0.84 ± 0.28* | 0.84 ± 0.34* |
| C3 activator (g/L) | 0.34 ± 0.11 | 0.51 ± 0.31* | 0.61 ± 0.45* | 0.54 ± 0.21* |
| C1 inhibitor (g/L) | 0.48 ± 0.2 | 0.77 ± 0.37* | 0.97 ± 0.43* | 0.56 ± 0.30*▪ |

**Significantly different from controls at p <0.05 (2-tailed); ▪Significantly different from HIV <200CD4cells/µl blood at p <0.05 (2-tailed)*

**Table 3:** Socio-demographic characteristics and clinical variables of study subjects

| Characters | HIV Positive n=36 (%) | Control n=29 (%) |
|---|---|---|
| **Age group** | | |
| 20–29 | 9 (25) | 5 (17) |
| 30–40 | 18 (50) | 24 (83) |
| 41–50 | 7 (19) | 0 |
| 51–60 | 1 (3) | 0 |
| 61-70 | 1 (3) | 0 |
| **Sex** | | |
| M | 22 (61) | 17 (59) |
| F | 14 (39) | 12 (41) |
| **Marital status** | | |
| Married | 25 (69) | 21 (72) |
| Single | 6 (17) | 8 (21) |
| Widow | 3 (8) | 0 |
| Seperated | 2 (6) | 0 |
| ***BMI** ($kg/m^2$) | 21.3±2.0 | 28.1±2.1 |
| **Employment Status:** | | |
| Employed | 20 (56) | 26 (90) |
| *Unemployed | 16 (44) | 3 (10) |
| **Educational Status:** | | |
| Below Sec School | 19 (53) | 6 (21) |
| Above Sec School | 17 (47) | 23 (79) |
| **Sexual Orientation:** | | |
| Heterosexual | 36 (100) | 29 (100) |
| Homosexual | 0 | 0 |
| **Mode of Transmission:** | | |
| Unknown | 36 (100) | Not applicable |

such as bacterial lipopolysaccharide and similar bacterial products. Studies of unregulated or abnormal regulation of B cell functions and / or T cell functions usually result in autoantibody formation [3-5]. This therefore supports the possibility of autoimmune responses or auto-antibody formation in HIV patients, which requires further investigation. There is evidence of a slightly increased risk of multiple myeloma occurring in HIV patients [3], and there have been a number of case reports of unequivocal multiple myeloma in HIV patients. Amara et al [19] reported that 28% of their HIV patients with monoclonal gammopathy developed malignancy (usually a B-cell/plasma cell malignancy) after a mean follow-up of 21 months.

Complement system functions in interactive sequence and it serves a very important role in host defense but can be detrimental when not regulated [7]. One of the regulators of classical pathway is C1 inhibitor. C1 inhibitor (C1-INH) inhibits C1r and C1s by binding covalently to them, causing disassembly of C1 macromolecular complex. The inhibitor is synthesized in the liver and blood monocyte. The alternative pathway is regulated by C3 activator (among others), which cleaves C3 into anaphylatoxin (C3a) and C3b.

There were significantly raised levels of C3c, C3 activator and C1 inhibitor in HIV patients compared with controls. This observation is comparable to previous studies [6, 9] that reported significantly higher levels of other complement factors such as C4d, Ba and C3d in HIV patients. The implication of the present

results is that activation of classical pathway of complement system in HIV patients was reduced but alternative complement pathway was increased as a compensatory mechanism. Other researchers [6,9,10] have reported that activation of complement system in HIV patients was mainly via classical pathway. The present study is in line with Eitan et al [8] that reported alternative complement activation pathway in HIV patients.

NO is produced during intracellular killing by phagocytes and inflammation [16]. It might be speculated that elevated NO in HIV patients was in response to inflammation. NO was reported to induce tissue damage especially after conversion into peroxynitrite radical (ONOO·) [20], thus elevated NO in HIV patients might have contributed to tissue damage and wasting in HIV patients.

# Conclusion

C1 inh and C4 appear to be good candidates that can be included in the indices for distinguishing HIV severity.

# Acknowledgements

We are grateful to subjects that participated in this study and Prof CP Muller of the Department of Immunology, National Health Laboratory, Luxembourg for the antisera provided for this study.

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