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Cellular Immune Status of Herpes simplex virus type- 2 Infected Pregnant Women in Warri, Nigeria

Favour Osazuwa ^{*1,2}, Nosa Odeh Eghafona ², Rachael O. Okojie ³

Department of Laboratory services, Lily Hospitals Limited (Echolily-healthcare), Warri, Nigeria ¹, Department of Microbiology, University of Benin, PMB 1154, Benin City ², Immunology Unit, Department of Microbiology, Faculty of Life Sciences, University of Benin, Benin City.
Corresponding Author: favour.newlife@gmail.com / +234-810-079-2725

Abstract

Herpes simplex virus type- 2 (HSV-2) is a major cause of genital ulcer disease and has been hypothesized to cause increase in inflammatory markers that contribute to atherogenic process. The aim of this study was to determine the cellular immune status and levels of systemic inflammatory markers in HSV-2 sero-positive pregnant women attending the antenatal clinic of Central Hospital, Warri, Nigeria. This study included three hundred and sixteen (316) pregnant women. The seroprevalence of Herpes simplex virus type-2 was determined using enzyme linked immunosorbent assay while levels of cellular immune status (CD_4^+) and inflammatory markers (C-reactive protein, total leukocyte count, total lymphocyte count and plasma viscosity) were determined using standard procedures. The prevalence of HSV-2 was 192 (60.8%). Mean C-reactive protein and lymphocyte concentration were significantly higher in HSV-2 sero-positive subjects than in sero-negative counterpart (15.63 versus 11.02, $p < 0.001$) and (41.90 versus 33.71, $p < 0.001$) respectively, but CD_4^+ count did not differ in both subject group ((1114.39 versus 988.21, 95% C.I 55.493, 196.86, $p=0.076$). Also the levels of total white cell count and plasma viscosity were not significantly different in HSV-2 sero-positive and sero-negative subjects. Increased levels of C-reactive protein and total lymphocyte count were associated with HSV-2 infection. Increased CRP levels are known to contribute to atherogenic process; its routine quantification in patients managed for herpes infection is however advised

Key words: Herpes simplex virus type-2, Cellular immune status, CD_4^+ , C-reactive protein.

Introduction

Herpes simplex virus type-2 (HSV-2) is now recognized as a major cause of genital ulcer disease

worldwide (Herbst-Kralovetz and Pyles, 2006). Genital herpes may be caused by either herpes simplex virus type 1 (HSV-1) or type 2 (HSV-2) but, globally, the large majority (over 90%) of cases are caused by HSV-2 (Herbst-Kralovetz and Pyles, 2006).

The immune response against HSV-2 involves both innate and adaptive immune mechanisms. The innate antiviral response is thought to play a pivotal role in determining the outcome of an HSV-2 infection. Accordingly, the production of type I interferon (IFN), comprised largely of IFN- α and IFN- β , has been linked to protection against disease in both mouse models and in human studies (Erikson et al., 2004). In addition, the adaptive immune response has been shown to play important roles in disease progression, latency and control of virus spread. Neutralizing antibody levels have been negatively correlated with disease severity (Erikson et al., 2004).

Studies have shown that CD_4^+ T cells are very essential in controlling HSV-2 infection (De la Calle-Martin et al., 2001). This is evident from the fact that CD_8^+ deficient humans do not suffer from any abnormal viral infections, including those caused by members of the family of *Herpesviridae*, whereas CD_4^+ deficient AIDS patients do (Shinkatu et al., 2003). CD_4^+ T-cell responses to HSV-2 appear to be directed against envelope glycoprotein's, capsid proteins and regulatory elements within the tegument (De la Calle-Martin et al., 2001).

There is accumulating evidence that some agent of sexually transmitted diseases notably HSV-2 can cause the pancreas multiple small foci of haemorrhagic necrosis in humans (Epstein, (2002) and could induce the production of cytokines and inflammation response both locally and systemically with chemo tactic cytokine release into the

peripheral circulation with the production of acute phase response (pyrexia, leukocytosis etc.) and mobilization of lymphocytes and neutrophil which may indirectly influence the course of atherosclerosis (Kerry et al., 2001). This could raise levels of inflammatory markers in systemic circulation with consequent deleterious activity on cardiovascular system and a possibility of coronary artery disease (CAD) (Hedwin, 2008). Progressive depletion in polymorphonuclear cells has been hypothesized to be a significant risk factor for genital herpes acquisition (Hedwin, 2008). Also, a low white blood cell count may lead to reactivation of genital herpes (Hedwin, 2008).

The aim of this study is to determine the cellular immune status (CD_4^+ T cell count) and levels of systemic inflammation markers (C-reactive protein (CRP), Total lymphocyte count, Plasma viscosity and Total white cell count) in Herpes simplex virus type-2 sero-positive pregnant women in Warri, Southern, Nigeria.

Materials and Methods

Study Area

This study was conducted at the antenatal clinic of the Department of Obstetrics and Gynaecology, Central Hospital, Warri, Delta state, Nigeria. This hospital is the major government owned tertiary health facility in Delta Central Senatorial district, it also house the antenatal clinic annex of Delta State University Teaching Hospital, Oghara.

Study Design

This study was a cross-sectional descriptive study to evaluate cellular immune status and inflammatory markers in Herpes simplex virus type -2 positive pregnant women (15 to > 40 Yrs) attending antenatal clinic of Central Hospital, Warri, Delta state, Nigeria. The subjects used for this study was chosen by stratified random sampling technique at the antenatal clinic.

Study Population and Selection Criteria

The study population included three hundred and sixteen (316) pregnant women between the ages of 15 and >40 years. Inclusion criteria included being pregnant, no history of drug use, absence of chronic disease, absence of pregnancy induced hypertension, non-smoking and non- high risk pregnancy

Ethical Approval

Verbal informed consent was obtained from subjects before recruitment into this study. The protocol for this study was approved by the ethics committee of Delta State Hospitals Management Board, Warri

Medical Zone (Central Hospital, Warri) in the letter referenced (CHW/ECC/VOL1/48).

Sample Collection and Preparation

About 10 ml of blood was obtained by vein-puncture; equal volumes were dispensed into EDTA bottles and plain containers. Samples were securely and clearly labeled with codes corresponding to a subject name to prevent mismatch and misinterpretation of result. Grossly haemolysed and hyperlipaemic samples were discarded as they could generate false positive result. Samples containing residues of fibrin or heavy microbial filaments and bodies were also discarded because of risk of false positive result. In general, phlebotomy errors that could undermine final results were minimized.

Total white cell, CD_4^+ and lymphocyte count were determined in the EDTA samples within 2 hrs of collection. Blood samples were allowed to settle down and Serum and plasma was separated using a bucket centrifuge calibrated to run at a speed of 2000 Revolutions per minute (RPM). . Remainder plasma was stored frozen for diagnosis of HSV-2 IgG and IgM. Serum samples were used to estimate levels of C-reactive protein and plasma viscosity.

Sample Analysis

Analysis of samples was carried out at the Institute of Human Virology Nigeria (IHVN) laboratory located at Central Hospital, Warri and Immunology Laboratory of Department of Chemical Pathology, University of Benin Teaching Hospital, Benin City, Nigeria.

Herpes simplex virus type 2 IgG and IgM ELISA (Enzyme linked immunosorbent assay)

Antibodies to HSV type 2 (IgG and IgM) was determined using type specific (HSV glycoprotein G) Herpes simplex virus type 2 ELISA IgG and IgM kits (Dia Pro Diagnostics, Milan Italy), and the results was interpreted following manufacturers specifications.

Principle of HSV-2 ELISA Test

The type specific HSV 2 IgG and IgM ELISA is based on a solid phase enzyme-linked immunosorbent assay (ELISA). The assay uses affinity purified HSV type 2 antibodies for solid phase (microtiter wells) immobilization and horseradish peroxidase (HRP) conjugated anti-HSV type 2 antibodies for detection. The test sample is diluted and incubated in the microtiter wells for 45 minutes. The microtiter wells are subsequently washed and HRP conjugate is added and incubated for 30 minutes. This result in HSV type 2 molecules

being sandwiched between the immobilization and detection antibodies. The wells are then washed to remove unbound HRP-labeled antibodies and TMB Reagent is added and incubated for 20 minutes at room temperature. This results in the development of a blue colour. Colour development is stopped by the addition of Stop Solution, changing the colour to yellow, and optical density is measured spectrophotometrically at 405 nm. The concentration of HSV type 2 is proportional to the optical density of the test sample. A calibration curve, calibrated against an internal Gold standard, makes possible a quantitative determination of the antibodies in the patient.

CD₄⁺ T Cell Count

Blood samples were analysed for CD₄⁺ T cell estimation using flow cytometry. (Partec, GmbH, Germany). The Partec Cyflow SL_3 is a single platform, three-parameter (SCC plus two colour fluorescence) desktop flow cytometer. It contains a solid laser for green excitation. It analyses concentrations of any particle or cell subpopulation of interest, using true volumetric absolute counting. The Partec CD₄% reagent kit used contained direct immunofluorescence reagents for enumeration of mature CD₄⁺ lymphocytes. Reference values for CD₄ T lymphocyte count in apparently healthy humans is > 450 CD₄⁺ lymphocytes/ μ l.

Procedure for CD₄⁺ T cell estimation

20 μ l of CD₄⁺ PE antibody was placed into a Partec test tube and 20 μ l of well mixed whole blood was added, mixed gently and incubated in the dark for 15 minutes at room temperature. The mixture was agitated during incubation every 5 minutes. CD₄ buffer (800 μ l) was added to the mixture of antibody and sample and mixed gently. This was then plugged to the counter for counting.

Total Leukocyte and Lymphocyte count

Total leukocyte and lymphocyte counts were determined using EDTA anticoagulated blood using the Sysmex KX-21N Haematology auto analyzer (Plastaris and Kampessi, 2009). Reference value for total leukocyte count and total lymphocyte count in humans is 4.0- 10.0 x 10⁹/L and 25-40 % respectively.

C- reactive protein (CRP) estimation

The Turbitest AA Line (Wierner Laboratories, S.A.I.C, Argentina). C reactive protein test kit based on immunoturbidimetry was used for the quantitative determination of serum levels of C reactive protein in subjects studied. The detection limit of this kit

(the minimum analyte amount capable of being detected as a sample, different than zero, and corresponds to the concentration of 0.5 mg/l). Its linearity (Assay range) is from 2 mg/l to 200 mg/l CRP approximately. Assay conditions: wavelength (340nm), reaction temperature (37^oc), reaction time (10 minutes), sample volume 80 μ l) and final reaction volume (1.28 ml). Provided reagents: Calibrator (Calibrador en serie, 1;3;5;6;7 and 8), Reagent A (Saline buffer solution, Ph 7.6) and Reagent B (monospecific anti-CRP antibodies). Chemwell 2910 (Automated EIA and chemistry analyser) (Manufactured by Awareness Technology, Incorporated, Palm City, United States of America) was used for measurement of absorbance of test and calibrators. The reference range for CRP in apparently healthy individuals is <10 mg/L.

Determination of Plasma Viscosity

A modification of the method of Reid and Ugwu (1987) was used. A 1ml syringe with a hypodermic needle (21.6 x 0.8 x 4mm) was used. Briefly, plasma was drawn into the syringe, avoiding air bubbles, till the 1.0ml mark. The plunger was carefully, removed and the time taken for the entire plasma to drain was noted. This was done twice for each sample and the average time taken for that sample to drain was noted. The entire process was repeated using distilled water. The plasma viscosity is the ratio of the flow rate of plasma to that of water.

Data analysis

Data were analyzed using Statistical Packages for Social Sciences (SPSS version 16). Sero-prevalence was reported in percentages and comparison of prevalence with cellular immune status and inflammatory markers was done using chi square test, ANOVA and Fisher's exact were necessary.

Result

The Mean age of the study participants at enrolment was 29 \pm 4.8 years. The obstetric characteristics of the study population is presented in Table 1. Among the enrolled subjects, 44.3% (n=140) were in their 2nd trimester, 43.0 % (n=136) was in their third trimester while subjects in their 1st trimester had the least numbers of participants in this study 12.7 % (n=40). The sero-prevalence of antibodies to Herpes simplex virus type-2 is presented in Table 2. The total prevalence of HSV-2 was 192 (60.8%). Serological antibody positive latent infection was the most common infection (50.6 %), with the least being new infections (IgM only) (4.1 %). Table 3 summarizes the mean concentration of cellular immune parameters (CD₄⁺cell count, Total

Leukocyte count, Total Lymphocyte count, C-reactive protein and Plasma viscosity) of study participants. The mean CD₄⁺ and total white cell count were within reference range (400- 1800 /μl and 4000-10000/l respectively). The association between Herpes simplex virus type-2 sero-positivity and inflammatory markers is presented in Table 4. Mean C-reactive protein and lymphocyte concentrations were significantly higher in Herpes simplex virus type-2 positive subjects than in sero-negative subjects (15.63 versus 11.02, 95% CI= -

5.997,-3.220, p<0.001) and (41.90 versus 33.71, 95%, p<0.001) respectively, but there was no statistically significant difference between total white cell count in Herpes simplex virus type-2 sero-positive and sero-negative subjects (Mean: 5600.00 versus 5618.75, p=0.895). CD₄⁺ count were higher in Herpes simplex virus type-2 sero-negative subjects when compared to their sero-positive counterparts but this was not significantly different (1114.39 versus 988.21, 95% CI=55.493, 196.864, p=0.076) as shown in Table 5.

Table 1: Obstetric characteristics of pregnant women attending the antenatal clinic of Central Hospital, Warri

Parameters	Frequency	Percentage (%)
Marital Status		
Married	276	87.3
Single	32	10.1
Divorce	8	2.5
Trimester		
1st	40	12.7
2nd	140	44.3
3rd	136	43.0
Parity Status		
Primigravidae	155	48.7
Multigravidae	165	52.2
Coitarchal Age (Age at first debut) (Years)		
<16	103	32.5
>16	213	67.5
Symptomatic (Clinical) Genital Ulcer Disease		
Yes	36	11.4
No	280	88.6
History of Genital Ulcer Disease		
Yes	49	15.5
No	267	84.5
History of Spontaneous Abortion		
Yes	28	8.9
No	288	91.1

Table 2: Prevalence of latent, reactivated and new Herpes simplex virus type-2 infection among pregnant women attending the antenatal clinic of Central Hospital, Warri

Herpes simplex type 2 sero status	Number	%
Latent Infection (IgG only)	60	50.6
Reactivated Infection (IgG + IgM)	19	6.0
New Infection (IgM only)	13	4.1
No Infection	124	39.2

Table 3: Concentration of cellular immune parameters among pregnant women attending the antenatal clinic of Central Hospital, Warri

Parameter	Mean ± SD
CD ₄ ⁺ Count	1037.72 ± 317.394
Total White cell count	5611.39 ± 1224.925
Total Lymphocyte count	38.68 ± 11.880
Plasma Viscosity	1.66 ± 0.134
C reactive protein	13.82 ± 6.517

Table 4: Association between HSV- 2 status and systemic inflammatory markers among pregnant women attending the antenatal clinic of Central Hospital, Warri

Parameter HSV-2 status	Mean± S.E	p- value
White cell count		
Negative 124	5600.00± 93.199	0.294
Positive 192	5618.75 ± 96.307	
Lymphocyte count		
Negative 124	33.71± 0.684	0.020*
Positive 192	41.90± 0.938	
C- reactive protein		
Negative 124	11.02± 0.310	0.024*
Positive 192	15.63± 0.530	
Plasma Viscosity		
Negative 124	1.62± 0.010	0.267
Positive 192	1.69± 0.010	

*Statistically significant

Table 5: Association between HSV- 2 status and cellular immune status among pregnant women attending the antenatal clinic of Central Hospital, Warri

Parameter	Mean± S.E	95%C.I	p- value
CD₄⁺			
Negative 124	1114.39± 21.172	55.493, 196.864	0.076
Positive 192	988.21± 25.420		

Discussion

This study determined the association between Herpes simplex virus type -2 sero-positivity and cellular immune parameters with focus on CD₄⁺ T lymphocyte count and inflammatory markers among pregnant women. Increased lymphocyte count in HSV-2 sero-positive subjects in this study agrees with the report of an earlier study (Zhu et al., 2000). Lymphocytes are made by the immune system to fight disease. An increase in circulating lymphocytes is a sign of viral infection and is most commonly seen in infectious mononucleosis and pertussis, or lymphoproliferative disorders such as acute and chronic lymphocytic leukaemia or in recurrent or new Herpes simplex virus infection (Zhu et al., 2000).

In this study C- reactive protein was significantly higher in HSV-2 sero-positive subjects than those

without infection. C-reactive protein (CRP) is an acute phase protein which is known to typically increase in blood as a result of inflammation, tissue injury, or disease and can often be used as surrogate biomarkers of disease progression. It has been observed that pathogen induced inflammation may contribute to atherogenic process that may lead to coronary heart disease (CAD) (Kerry et al., 2001). A significant association between increasing burden of antibodies to persistent viral infections known to establish persistent lifelong infections or produce a lifelong immune response and increased levels of CRP and CAD was found in a study that tested the association of a five panel of pathogens (Cytomegalovirus, HSV-1, HSV-2, *Chlamydia* and Hepatitis A virus) to CAD risk (Zhu et al., 2001).

Other studies (Rupprecht et al., 2001; Schaftenaar et al., 2014), have also shown that increasing burden of

persistent viral infection such as HSV-2 leads to increased endothelial dysfunction which is one of the earliest signs of atherosclerosis. Epstein (Kerry et al., 2001), summarized that apart from the ability of persistent viral infections to contribute to atherosclerosis, they can also trigger plaque rupture and acute thrombotic occlusion which are major factors in acute myocardial infarction and for sudden deaths in patients with CAD.

The CD₄⁺ cell count in HSV- 2 sero-positive pregnant women was lower than their sero-negative counterparts but were not statistically different. Our study shows that HSV-2 does not lead to CD₄⁺ cell decline unlike the Human immunodeficiency virus (HIV) with tropism for CD₄⁺ cells. In a recent study, there was no association between CD₄⁺ T cell counts and HSV-1 or HSV-2 sero-status in a proof of concept study among pregnant women in primary health care facilities across the Mopani district, Limpopo province, South Africa (Rupprecht et al., 2001). Similarly, a study among postpartum women in Kenya did not find any significant association between HSV-2 infection and CD₄⁺ T cell decline (Schaftenaar et al., 2014). Our result does not agree with previous reports (Roxby et al., 2011; Barnabas et al., 2011), which reported a marginally slower rate of CD4 count decrease among HSV-2 infected women . The non- association between HSV-2 infection and CD4+ cell decline has also been proven in a United States epidemiology study (Hoots et al., 2011), but there was a significant association between HSV-2 infection and HIV acquisition.

Total white cell count and plasma viscosity were not associated with HSV-2 sero-positivity. This indicates that they may not be useful indicators for non-specific evaluation for HSV-2. Plasma viscosities have been hypothesized to be a useful non-specific marker for inflammation in HIV (Omoriegie et al., 2008). This assertion was however not proven in this study for its utility in HSV-2. The low concentration of plasma viscosity among pregnant women in this study is thought to be influenced by packed cell volume which has been found to be generally low among pregnant women in sub-Saharan Africa (Imoru et al., 2009).

Conclusion

In conclusion, there were no significant association between HSV-2 sero-positivity and CD₄⁺ T cell counts levels, plasma viscosity and total white cell count. It can be hypothesized that HSV-2 does not cause CD₄⁺ cell decline and its routine measurement in management of HSV-2 mono-infection in a resource limited setting such as ours might be of no

diagnostic value except when there is HIV co-infection. C-reactive protein and total lymphocyte count were significantly associated with HSV-2 sero-positivity indicating a causal role of HSV-2 in inflammation. Since increased CRP levels are known to contribute to atherogenic process, its routine quantification in patients managed for herpes infection is advised.

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Conflict of interest

We declare that we have no conflict of interest.

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