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Evaluation of the Immunological Status in Occult Hepatitis B Virus-infected Patients Attending Rivers State University Teaching Hospital

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Authors' contributions

This work was carried out in collaboration among all authors. Author BBDK designed the study. Author BD carried out the laboratory experiments. Authors ZAJ, BBDK and TPM managed the literature searches, performed the statistical analysis, wrote the protocol. Author BD and BBDK wrote the first draft of the manuscript and managed the analyses of the study. Author ZAJ ensured integrity of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Background: Occult hepatitis B infection (OBI) is a rare form of infection that is characterized by the presence of replication-competent HBV DNA in the liver but without detectable HBsAg in the serum.
Aim: This study aimed to determine the comparative levels of immunological variables particularly CD4 counts and differential white blood cell (WBC) counts in occult hepatitis B and HBsAg positive subjects among prospective blood donors in Port Harcourt Metropolis in Nigeria.
Methods: The CD4 count and total and differential WBC counts were analyzed with automated

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techniques using BD Fascount and Sysmex XP-300 respectively. Data were analyzed as mean (standard deviation) and significance was assumed at 95% confidence interval using student t-test and one-way ANOVA on GraphPad prism.

Results: The mean values for CD4, WBC, LYM (lymphocyte), MXD (differential mixed cells), and NEU (neutrophils) were 607±286 µ/l, 16±24 /l, 1.8±0.55 %, 0.46±0.15 % and 3.1±1.1 % respectively for occult hepatitis B subjects; 609±222 µ/l, 5.4±1.7 /l, 2.3±1.2 %, 0.54±0.31 % and 2.7±1.2 % respectively for HbsAg positive subjects and 823±256 µ/l, 10±5.4 /l, 2.4±1.6 %, 0.69±0.49 % and 6.4±4.7 % respectively for hepatitis B negative subjects. CD4 levels for male and female occult hepatitis B subjects are 729±309 µ/l and 461±190 µ/l respectively; 582±210 µ/l and 643±250 µ/l respectively for HBsAg positive subjects while that of hepatitis B negative subjects were 824±305 µ/l and 821±199 µ/l respectively. OBI showed a significant negative association between differential mixed cells and lymphocytes (r= -0.89 and p= 0.001) alone signifying that increment in former could reduce the latter. Only the HBsAg positive subjects showed a significant positive correlation of age to differential mixed cells (r=0.94 and p=0.000) which imply as the age increase the level of differential mixed cells will rise. Again, the HBsAg positive subjects showed a significant positive correlation between the differential mixed cells against neutrophil and WBC (r=0.53 and p=0.050), (r=0.56 and 0.036) respectively. Smoking and alcohol consumption caused raised levels of CD4 cells in OBI.

Conclusion: This study revealed a significant decrease in CD4 count, increase in total WBC and neutrophil counts while lymphocyte counts were decreased in occult hepatitis B subjects. Gender difference affect the level of CD4 cells and significant correlation were observed especially with the differential mixed cells.

Keywords: Viral hepatitis; immunological variables; occult hepatitis; CD4 cells.

1. INTRODUCTION

Occult Hepatitis B virus (OBI) infection is a condition defined by the presence of replication-competent hepatitis B virus (HBV) DNA in the liver or blood of individuals that are negative for the viral surface antigen [1]. It is characterized by the persistence of cccDNA in the nucleus of hepatocytes. Quantitatively, OBI is classified as a reduced level of HBV DNA in the serum below 200 IU/ml, immune cells, and/or liver tissues in an individual serological marker of prior HBV infection [2]. Approximately two billion of the world population have been infected, of which 250 million live with hepatitis B infection [3]. Different regions across the globe have a different prevalence of OBI. This differs from 1 to 87% across the different parts of the world [4-5]. Minuk et al. [3] also demonstrated the presence of OBI even in regions with reduced prevalence in hepatitis B infections. The level of prevalence across the globe is proportional to the endemic nature of the infection. For instance, regions of low endemicity (such as the USA and UK) have been shown to have a reduced prevalence rate of the infection while highly endemic regions (such as Asia-Pacific regions and Sub-Saharan African regions) has higher prevalence [5-9].

The exact mechanism for the development of OBI has not yet been elucidated hence, there are different proposed phenomena responsible for the infection. Most reports tend to support the idea that OBI is due to the permanent intrahepatic persistence of the wild-type of the viral genomic DNA [10]. Some scientists have suggested that its development could be due to the use of the immunoassay technique in the detection of the virus instead of polymerase chain reaction (PCR) [11]. However, this explanation does not elucidate the slower rate of replication of HBV noted OBI patients. A more precise suggestion associated this development with both viral and host factors [12-13]. Mutation observed in the X region of the HBV has been noted to suppress the transactivation of proteins needed for viral replication [14]. This leads to the suppression of the HVB replication. Again, the persistent synthesis of a very small undetectable amount of covalently closed circular DNA (cccDNA) and other viral proteins keeps the detection at a very low level [15].

The most commonly noted means of transmission of OBI occurs during blood transfusion [11]. This mode of transmission is very common in the developing nations that still adopt the immunoassay method as a diagnostic tool during the screening of blood donors [16-17]. This observation is in contrast to major means of transmission in the developed countries that use nucleic acid amplification test (NAT) in the

detection of most infections including HBV infection to ensure safety during the screening of blood before transfusion [18-19]. This technique detects the HBV DNA before the appearance of HBsAg and together with polymerase chain reaction-based amplification of the viral genes are used as the gold standard for the diagnosis of OBI. The detection range is usually less than ten copies of the viral DNA per reaction [20].

There is a paucity of data on the link between OBI and immunological variables in patients. However, OBI reactivation usually occurs in patients given immunosuppressive therapy [21-22]. The role of CD8 positive cells in the suppression of HBV infection has been previously recorded while data on the level of association of CD4 positive cells and the infection has not been widely reported. Hence, this study aimed to determine the immunological indices particularly CD4 counts as well as total and differential white blood cell (WBC) counts amongst hepatitis B and occult hepatitis B patients in the Port Harcourt metropolis.

2. METHODS

2.1 Study Area

The study was carried out in the Port Harcourt metropolis. Port Harcourt is the headquarters of Rivers state, Nigeria. It is located in the Niger Delta with a population of 1,148,665. The Rivers State University Hospital is a government hospital owned by the government of Rivers State and is located at 5-8 Harley Street, Old GRA Port Harcourt, Rivers State of Nigeria. It is made up of 375 beds with 731 medical staff.

2.2 Study Design

The research was an analytical cross-sectional study on 100 participants. It utilized a random stratified sampling method in the selection of subjects into subgroups: gender (male and female), lifestyle (smoking and drinking), and age (10-65 years). The study was done from January to May 2019. The individuals used in this study were blood donors in a tertiary hospital. The inclusion criteria in the study were subjects that were positive for HBsAg and occult hepatitis B but negative for any other chronic diseases. The exclusion criteria were known to be suffering from chronic conditions, pregnant women, subjects with difficulties in obtaining samples, and those below 10 years and above 65 years. Those persons that had been taken therapy within the last 2 months were also excluded from the study. Written consent was obtained from the subject prior to sample collection. For minors, below 18 years of age, consent was obtained from their parents or guardians. Oral informed consent was obtained from the subjects before enrolment and ethical approval of the study was obtained from Rivers State Ministry of Health with the number MH/PRS/391/Vol.2/524. The study was done on one hundred (100) subjects attending the Braithwaite Memorial Specialist Hospital in Rivers State.

2.3 Sample collection

Four (4) milliliters of blood were collected under aseptic technique using the venipuncture method into an anticoagulated container. These samples were analyzed and screened for occult hepatitis B infection, CD4, total and differential white blood cells

2.4 Detection of Occult Hepatitis

2.4.1 Hepatitis B surface antigen (HBsAg)

A drop of serum was added to the specimen pad of the test device (Rapid Diagnostic Test Kit using SKYTEC (USA)) and allowed to stand for 15 minutes and the results were subsequently.

2.4.2 Hepatitis B surface antigen, surface antibody, core antibody, E antigen, and E antibody panel

Equal drop of serum and antigens containing HBsAg, HBsAb, HBeAg, HBeAb, and HBcAb were mixed on a test board. Results were read 15 minutes later. This test was done using the rapid diagnostic test kit from Swe-Care (Sweden).

2.5 Total and Differential White Blood Cell Count

The blood sample for the study was mixed properly with the aid of the bench-top mixers for 3-5 minutes and the total and differential WBC counts were determined using an auto-analyzer.

2.6 CD4 Lymphocyte Count

Whole blood was mixed by inversion and 50 µl of the patient's whole blood sample was pipetted into the reagent tube and vortexed upright for 5 seconds. The sample was incubated at room

temperature in a dark chamber for 60-120 minutes. After incubating the tube, it was then uncapped and 50 µl of the fixative solution was pipetted into it and vortexed for 5 seconds and then run using the BD Fascount machine. The Sample was aspirated and after about a minute the result was shown.

2.7 Statistical Analyses

The data obtained in this study were represented as mean ± SD (standard deviation) and bar chart. The statistical analysis used was a t-test for comparisons between two variables and ANOVA for more than two variables together with the corresponding post analyses. P-value was considered to be statistically significant at $P<0.05$.

3. RESULTS

3.1 Comparative Analyses of the Levels of Immune Parameters in Subjects

Table 1 shows the mean values for CD4 positive cells, WBC, lymphocytes, differential mixed cells and neutrophils were 607±286 µ/l, 16±24 /l, 1.8±0.55 %, 0.46±0.15 % and 3.1±1.1 % respectively for occult hepatitis B subjects; 609±222 µ/l, 5.4±1.7 /l, 2.3±1.2 %, 0.54±0.31 % and 2.7±1.2 % respectively for HbsAg positive subjects and 823±256 µ/l, 10±5.4 /l, 2.4±1.6 %, 0.69±0.49 % and 6.4±4.7 % respectively for hepatitis B negative subjects.

Table 1. Comparisons of immunological parameters among subjects

	CD4 (µ/l)	WBC x10^{12}/l	LYM (%)	MXD (%)	NEUT (%)
Neg (n=46)	822.7±256.3	10.1±5.4	2.4±1.6	0.7±0.5	6.4±4.7
Pos (n=32)	609.0±222.4	5.4±1.7	2.3±1.2	0.5±0.3	2.7±1.2
Occult (n=22)	607.2±285.7	15.6±24.5	1.8±0.6	0.5±0.2	3.1±1.1
ANOVA	0.0184	0.1063	0.4658	0.2824	0.0039
Tukey's Multiple Comparison (p-values)					
N vs P	0.0350*	0.4688	0.9684	0.5128	0.0057*
N vs O	0.0645	0.4346	0.4451	0.3065	0.0465*
P vs O	0.9998	0.0882	0.6108	0.8639	0.9491

*Key: Keys: Bold – significant level of comparison (p<0.05). Neg – negative control, Pos – HbsAg positive, occult – occult hepatitis, WBC – white blood cell, LYM – lymphocytes, MXD – differential mixed cells, and NEUT – neutrophil. *Results that showed significant level of comparison with P<0.05*

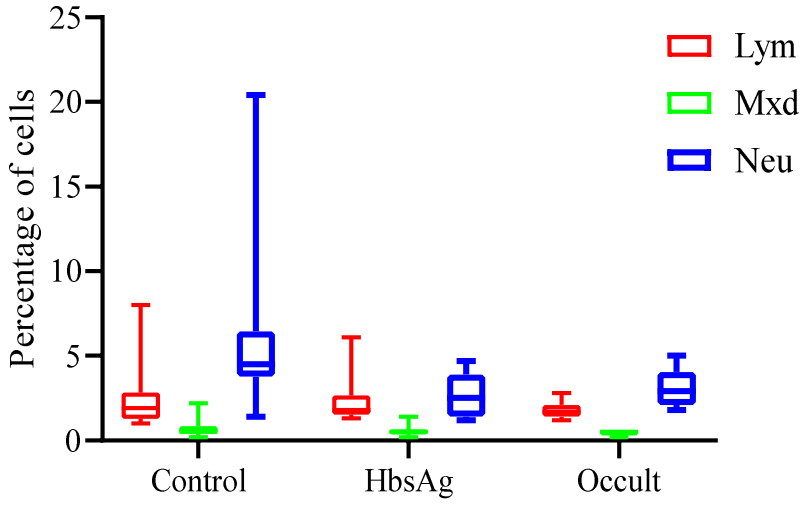

Fig. 1. Levels of WBC among subjects. Key: Control – subjects without hepatitis infection
Keys: HbsAg – subjects positive for hepatitis surface antigen, Occult – occult hepatitis patients, Control – hepatitis B negative subjects. LYM – lymphocytes, MXD – differential mixed cells, and NEUT – neutrophil

3.2 Age-dependent Analyses of the Levels of CD4 Cells in Subjects

The Mean values for CD4 count amongst occult hepatitis B subjects within the age groups of ≤20, 21–30 and ≥ 31 years were 610±292 µ/l, 1022±0.71 µ/l and 500±240 µ/l respectively; 678±305 µ/l, 584±212 µ/l and 567±149 µ/l for HbsAg Positive subjects while that of Hepatitis B Negative subjects were 774±108 µ/l, 863±332 µ/l and 812±261 µ/l as shown in Table 2.

3.3 Effect of Gender on the Levels of CD4 Cells in Subjects

The mean values for CD4 for male and female occult hepatitis B subjects are 729±309 µ/l and 461±190 µ/l respectively; 582±210 µ/l and 643±250 µ/l respectively for HbsAg positive subjects while that of hepatitis B negative subjects were 824 ± 305 µ/l and 821 ± 199 µ/l respectively as shown in Table 3.

3.4 Effect of Smoking and Alcohol Consumption on the Levels of CD4 Cells in Subjects

The mean values for CD4 for drinkers, smokers, neither smokers nor Drinkers and both smokers and drinkers for occult hepatitis B subjects are 658±278 µ/l, 864±138 µ/l, 547±314 µ/l, and 864±138 µ/l, respectively; 598±181 µ/l, 515±143 µ/l, 628±298 µ/l, and 515±143 µ/l respectively for HbsAg positive subjects while that of hepatitis B negative subjects were 759±176 µ/l, 682±166 µ/l, 875±306 µ/l, and 682±166 µ/l respectively as shown in Table 4.

Table 2. Comparison of CD4 among subjects based on age

Age Groups (Years)	Subjects CD4 cells (µ/l)		
	Occult hepatitis B	**HBsAg positive**	**Hepatitis B negative**
≤ 20	610 ± 292	678 ± 305	774 ± 108
21 – 30	1022 ± 1	584 ± 212	863 ± 332
≥ 31	500 ± 240	567 ± 149	812 ± 261
p-value	0.3857	0.7309	0.8125
Significance	NS	NS	NS

Keys: NS – not significant

Table 3. Effect of age on CD4 levels among subjects in both genders

Sex	Subjects CD4 cells (µ/l)		
	Occult hepatitis B	**HbsAg positive**	**Hepatitis B negative**
Male	729 ± 309	582 ± 210	824 ± 305
Female	461 ± 190	643 ± 250	821 ± 199
P-value	0.1250	0.6039	0.9822
Significance	NS	NS	NS

Keys: NS – not significant

Table 4. Effect of smoking and alcohol consumption on CD4 levels among subjects

Lifestyles	Subjects CD4 cells (µ/l)		
	Occult Hepatitis B	**HbsAg Positive**	**Hepatitis B Negative**
Drinkers	658 ± 278	598 ± 181	759 ± 176
Smokers	864 ± 138	515 ± 143	682 ± 166
Both	864 ± 138	515 ± 143	682 ± 166
Non	547 ± 314	628 ± 298	875 ± 306
P-value	0.2705	0.6578	0.2504
Significance	NS	NS	NS

Keys: NS – not significant

3.5 Correlation Matrix of the Variables

Tables 5-7 show the correlation matrixes between hematological variables of control, HBsAg positive and, OBI subjects respectively. For the control subjects, the comparisons of the differential mixed cells against neutrophils, and wbc demonstrated significantly positive association; (r=0.51 and p=0.019), (r=0.62 and 0.003) respectively (Table 5). Also, comparing the neutrophil against wbc showed a significantly positive correlation and (r=0.94 and p=0.000) (Table 5). For the HBsAg positive subjects, the comparisons of differential mixed cells against neutrophils, wbc, and age show significantly positive correlation (r=0.53 and p=0.050), (r=0.56 and 0.036), and (r=0.94 and p=0.000) respectively (Table 6). For the OBI subjects, only the differential mixed cells and lymphocyte shows significant negative correlation (r=-0.89 and p=0.001) (Table 6).

Table 5. Correlation matrix of immunological parameters in control subjects

	Age		CD4		WBC		LYM		MXD		NEU	
	r	p	r	p	r	p	r	p	r	p	r	p
Age	1.00	0.000										
CD4	-0.11	0.642	1.00	0.000								
WBC	-0.21	0.358	0.07	0.767	1.00	0.000						
LYM	-0.17	0.474	0.17	0.458	0.32	0.162	1.00	0.000				
MXD	0.03	0.900	0.21	0.369	0.62	0.003	0.21	0.354	1.00	0.000		
NEU	-0.16	0.487	0.14	0.532	0.94	0.000	0.00	0.998	0.51	0.019	1.00	0.000

Key: Bold – significant level of comparison (p<0.05). WBC – white blood cell, LYM – lymphocytes, MXD – differential mixed cells, and NEUT – neutrophil

Table 6. Correlation matrix of immunological parameters in HBsAg positive subjects

	Age		CD4		WBC		LYM		MXD		NEU	
	r	p	R	p	r	p	r	p	r	p	r	p
Age	1.00	0.000										
CD4	-0.20	0.487	1.00	0.000								
WBC	0.09	0.771	0.26	0.362	1.00	0.000						
LYM	-0.42	0.134	0.42	0.133	0.26	0.378	1.00	0.000				
MXD	0.69	0.007	0.01	0.967	0.56	0.036	-0.16	0.596	1.00	0.000		
NEU	0.21	0.468	0.04	0.880	0.85	0.000	-0.27	0.350	0.53	0.050	1.00	0.000

Keys: Bold – significant level of comparison (p<0.05). WBC – white blood cell, LYM – lymphocytes, MXD – differential mixed cells, and NEUT – neutrophil

Table 7. Correlation matrix of immunological parameters in OBI subjects

	Age		CD4		WBC		LYM		MXD		NEU	
	r	p	R	p	r	p	r	p	r	p	r	p
Age	1.00	0.000										
CD4	-0.47	0.202	1.00	0.000								
WBC	0.47	0.200	0.05	0.891	1.00	0.000						
LYM	-0.24	0.540	0.42	0.265	0.33	0.386	1.00	0.000				
MXD	0.14	0.713	-0.60	0.091	-0.29	0.449	-0.89	**0.001**	1.00	0.000		
NEU	-0.01	0.979	0.26	0.494	0.45	0.229	0.23	0.550	0.17	0.656	1.00	0.000

Keys: Bold – significant level of comparison (p<0.05). WBC – white blood cell, LYM – lymphocytes, MXD – differential mixed cells, and NEUT – neutrophil

4. DISCUSSION AND CONCLUSION

The findings in this study revealed a significant ($P<0.05$) decrease in CD4 count of occult hepatitis B subjects when compared to apparently healthy control subjects which agree with Arababadi et al. [23] suggesting that some aspects of T-lymphocyte function, such as migration (chemokine expression), antigen recognition (MHC expression), activation (intracellular signaling pathway), and proliferation are defective to some degree in occult hepatitis B infected patients. A general comparison shows that the levels of CD4, lymphocytes, and differential mixed cells and neutrophils are lowest in HBsAg positive and OBI subjects and highest in the control subjects (Table 1). The implication of this observation is that the presence of the virus is either in the active (i.e. positive subjects) or latent state (i.e. OBI subject) reduces the level of some immunological parameters such as CD4, lymphocytes, and differential mixed cells. Recent studies have suggested the antiviral role of naïve CD4 cells in recognizing viral peptides on the major histocompatibility complex of antigen-presenting cells to produce T_{H1} cells due to the presence of interferons and interleukin-12 [24-27]. This underscores the significance of the immunological variables selected for this study. As expected in a healthy individual, higher values of hematological variables were seen in the hepatitis B negative control subjects.

The study further explored the effect of age on the levels of CD4 cells in OBI, HBsAg positive, and control subjects. Subjects within the age range of below 20 years and above 31 years exhibited the same trend in the level of CD4 which was highest in the negative control participants followed by HBsAg positive subjects then OBI subjects. However, a contrary observation was noted in the subjects of age range between 21 and 30 which has the highest level of CD4 in the OBI subject followed by the negative control subjects then HBsAg positive subjects. These observed phenomena suggest that OBI infection could be inducing a higher level of CD4 higher than negative control subjects. A proposed mechanism that could cause this is the presence of the viral antigens stimulating cytokine production that can potentially activate CD4 proliferation and differentiation. Constant et al. [27] reported that the amount of antigens, duration of exposure to antigens, and the nature and kinds of cytokine produced to determine the extent of T cell production and the subtypes of its differentiation. OBI by definition has a persistent presence of cccDNA of the virus which could be causing constant stimulation of the immune system, thereby causing the higher level of CD4 cell production that is observed in this study.

In addition, the research investigated the impact of sex on the level of CD4 among the study subjects. The male showed higher levels of CD4 cells in negative control and OBI subjects but lowest in HBsAg positive subjects than the female. In both genders, the negative control subjects had the highest level of CD4 than both virally infected cases. It is a worthy of note in the OBI, that the CD4 level of the male subject is approximately two-fold higher than the female which implies a higher level of lymphocytosis in males than females as suggested by some authors [28-29].

The study further examined the effect of smoking and alcohol consumption on CD4 levels in OBI, HBsAg positive, and control subjects. Smoking and alcohol consumption caused raised levels of CD4 cells in OBI. A similar observation on the effect of smoking has been noted by Malenia et al. [30] whereby they established that smoking caused elevation in lymphocyte levels compared to non-smokers. Their study used healthy subjects while the current study used hepatitis B infected individuals. Tobacco the major constituent of smoking contains over four thousand chemical compounds with potentially adverse effects on human health. Nicotine, free radicals, and carbon (II) oxide are among the important compounds noted to show the observed pharmacological effect [31]. These compounds cause thrombosis, activation of the coagulation cascade, and adhesion of leukocytes to blood vessels. This leukocyte adhesion is a hallmark in inflammation which causes an increased level of leukocytosis.

Approximately 86 percent of all the comparisons that showed significant correlation are linked to differential mixed cells. OBI showed a significant negative association between differential mixed cells and lymphocytes alone signifying that increment in the former could reduce the latter. Only the HBsAg positive subjects showed a significant positive correlation of age to differential mixed cells which implies as the age increase the level of differential mixed cells will rise. Again, the HBsAg positive subjects showed significant positive correlation between the differential mixed cells against neutrophil and WBC. Differential mixed cells are mainly

granulocytes that act as antigen-presenting cells, hence sends cytokines for the stimulation of the immune system. Their modulation by the lymphocytes in OBI as noted demonstrates that their functions could be impaired during hepatitis B infection.

CONSENT AND ETHICAL APPROVAL

Oral informed consent was obtained from the subjects before enrolment and minors' consent were obtained via their parents or guardians. The ethical approval of the study was obtained from Rivers State Ministry of Health with the number MH/PRS/391/Vol.2/524.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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