A study on the association between parvovirus B19 infection, serum tumour necrosis factor and C-reactive protein levels among Nigerian patients with sickle cell anaemia

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INTRODUCTION Microbial burden involving parvovirus B19 infection has been recognised as a major cause of morbidity and mortality in sickle cell anaemia (SCA) patients. Given the recent reports of parvovirus B19 infection in Nigeria and the role of inflammation in sickle cell crisis, knowledge of the relationship between the two may be essential for deploying appropriate interventions in infected patients. This study determined the serum levels of tumour necrosis factor alpha (TNF-α) and C-reactive protein (CRP) as inflammatory markers in Nigerian SCA patients with and without parvovirus B19 infections.

METHODS A total of 64 SCA patients aged 5–25 years and 41 age-matched apparently healthy volunteers with haemoglobin genotypes AA or AS were enrolled with consent into the study. Parvovirus B19 infection and serum levels of TNF-α and CRP were determined by the ELISA method.

RESULTS The overall prevalence rate of parvovirus B19 infection in the study subjects was 13.3%. This rate further showed gender variation and negative correlation with age. Significant (p < 0.05) increases in serum CRP and TNF-α levels, with further elevation in unsteady state SCA patients, were observed in comparison with the control. Unlike the control, 29.6% and 21.9% of the SCA patients elicited TNF-α and CRP above threshold levels, respectively. Parvovirus B19 infection was found to elicit greater increases in these inflammatory markers than in infected non-SCA controls.

CONCLUSION We conclude that parvovirus B19 infection is common in this environment, and that serum TNF-α and CRP are predictors of clinical inflammatory episodes in infected SCA patients.

Keywords: inflammation, Lagos, Nigeria, parvovirus B19, sickle cell anaemia


INTRODUCTION Microbial infections remain a major cause of morbidity and mortality among sickle cell anaemia (SCA) patients worldwide. They have been associated with steady-state period reduction, higher frequency of crisis and longer hospital stays compared to uninfected patients. In Nigeria, with an average of 2% of population infected by SCA, bacterial infections due to Streptococcus (S.) pneumoniae, S. pyogenes, Haemophilus influenzae, Klebsiella spp., Staphylococcus aureus, Salmonella spp., Proteus spp., Escherichia coli, Helicobacter pylori and protozoal infections such as Plasmodium falciparum have been reported by many investigators. These infections have been shown to be the aetiologies of complications such as sepsis, pneumonia, avascular necrosis of the femoral head, osteomyelitis, severe haemolysis and opiate-required acute pain crisis, to mention just a few. In spite of the benefits of penicillin prophylaxis and vaccines, crisis remains the bane to quality of life and life expectancy in SCA patients throughout the world, including Nigeria.

Continuous monocyte activation, chronic haemolysis, expression of adhesion molecules and the presence of a small population of dense cells remain the hallmark of the pathophysiology of subclinical endothelial dysfunction and inflammation during the steady state of SCA. Baseline leukocytosis, higher platelet counts and systemic coagulation stress due to overproduction of systemic coagulation factors such as fibrinogen, platelet factor 4 and von-Willebrand factor, which promote crisis, have also been linked to the presence of microbial infections.

Tumour necrosis factor-alpha (TNF-α), produced by activated monocytes and tissue macrophages, is involved in both subclinical and clinical inflammation in a concentration-dependent manner. Its pleiotropic functions include the triggering of oxidative stress, causing cell membrane lipid peroxidation, and promotion of the expression of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), platelet endothelial adhesion molecule (PECAM), vascular adhesion molecule (VCAM), E-selectin, P-selectin, β1-integrins, and very late antigen 4 (VLA4) in cell-mediated immunity cells, such as neutrophils, monocytes, macrophages, lymphocytes, mast cells, as well as reticulocytes, sickled erythrocytes and platelets.
C-reactive protein (CRP) is an acute-phase protein synthesised and secreted by liver hepatocytes following activation by interleukin-6 (IL-6) from activated monocytes and the liver itself. Like TNF-α, CRP is a marker of low-grade systemic inflammation and is also pleiotropic in function. Among its numerous functions are the exacerbations of dyserythropoiesis leading to severe anaemia, promotion of monocyte chemotaxis to damaged endothelium to promote further damage to the vasculature, alteration of the complement pathway to intensify inflammation, induction of adhesion molecule expression on the endothelial cell and as positive feedback regulator of inflammatory cytokines, including IL-6. Raised plasma levels of CRP at baseline have also been associated with vaso-occlusive crisis in SCA. Parvovirus B19 (Parvoviridae) as a viral infection of clinical relevance in sickle cell disease (SCD) among endemic populations has recently been reported in Nigeria as a risk factor for hydrops foetalis among pregnant women attending antenatal care. Recently, we found significant titres of anti-parvovirus B19 IgM and IgG antibodies to suggest acute infection and previous exposure to this virus among a cohort of SCA patients who live in Lagos in steady and unsteady states of the disease (unpublished). Diagnosis of parvovirus B19 viraemia in a patient is based on the detection of a significant level of anti-parvovirus B19 IgM antibody, which indicates acute infection.

Given the subclinical nature of inflammation in SCA patients, which progresses to clinical episodes characterised by elevated levels of inflammatory markers such as TNF-α and CRP, the link between parvovirus B19 infections and inflammation remains unclear. This study was carried out to gain information concerning the relationship between parvovirus B19 and the levels of TNF-α and CRP in Nigerian SCA patients. Knowledge of this is expected to provide strategies and targets for improved management of SCA patients with parvovirus B19 infection in the country.

**METHODS**

The study population included 64 SCA patients aged 5–25 years who were enrolled at the time of their presentation for care at four healthcare facilities in Lagos, Nigeria, between September 2009 and August 2010. The SCA status of the patients was confirmed by sickling test and celluloate acetate electrophoreses using standard haemoglobin genotypes AA (HbAA), AS (HbAS), SS (HbSS) and AC (HbAC) as control. 41 gender- and age-matched volunteers of HbAA and HbAS blood statuses within the health facilities were enrolled as control. In line with the declaration of Helsinki, informed consent was obtained from each participant prior to blood sample collection at the time of enrolment into this study. Participants who declined consent or had an indeterminate electrophoresis outcome were excluded from the study. The study protocol was approved by the Lagos State Ethics Review Committee. Clinically, the SCA patients were classified into steady and unsteady states of disease, as previously described.

In brief, a patient was said to be in a steady state of SCA if he was afebrile and asymptomatic at presentation or at ten days prior to the date of blood draw.

Aliquots of venous blood samples from each study participant were separately submitted for routine haematological assays, which included total leukocytes, platelet and reticulocyte counts using standard haematological methods. Blood Hb level was determined using Drabkin’s reagent at 540 nm. Each assay was run in duplicate. Another aliquot of venous blood sample from each study participant collected in plain tubes were processed into serum by centrifugation at 3,000 rpm for 10 minutes. Each serum was diluted 1:100 using sample diluent, and 100 µl of aliquot was used in duplicate to determine the TNF-α and CRP concentrations by enzyme-linked immunosorbent assay (ELISA) using 96-well microtitre plates coated with mouse TNF-α (Anogen, Mississauga, ON, Canada) and CRP (mp Biochemical, Solon, OH, USA) monoclonal antibodies according to the manufacturers’ directives. The serum levels of these parameters were determined from the standard curve, 15.6–1,000 pg/ml for TNF-α (intra-assay coefficient of variation 2.5%–3.5%; detection limit 2.4 pg/ml) and 0.05–0.10 mg/L for CRP (coefficient of variation 2.3%–7.8%). Absorbance readings were taken at 450 nm for both assays. Serum TNF-α and CRP levels ≥ 40 pg/ml and ≥ 10 mg/L, respectively, were considered to be above the normal values and thus indicate a significant inflammatory response.

Samples with TNF-α and CRP values above the inflammatory threshold were further diluted 1:100 and re-assayed.

The detection and quantitation of parvovirus B19 IgM was done using the solid-phase ELISA technique, which was based on the sandwich principle, using the purified recombinant parvovirus B19 VP2 protein as an antigen to coat the IgM (Biotrin International, Dublin, Ireland). Each sample was diluted in the ratio 1:101 (v/v) with a diluent buffer, and the influence of interfering substances was annulled by mixing the diluted serum (200 uL) with the RF-absorbent solution (10 uL) provided by the manufacturer. The assay was carried out according to the manufacturer’s directive using peroxidase-labelled rabbit anti-human IgM as the secondary antibody, tetramethylbenzidine as a substrate and 1 M H2SO4 as a stop solution. Absorbance was read at 450 nm using an ELISA reader within 15 minutes of colour development. Assays were standardised using serial dilutions of parvovirus B19 IgM standard solutions (10–50 U/ml) and run twice per sample. The absorbance of anti-parvovirus B19 IgM standard level of 20 U/ml was taken as the cutoff value. Index value was calculated as the ratio of absorbance of sample to the cutoff value. Index value of 0.8–1.2 was taken as an equivocal result. Samples below this range were taken as negative, while samples above this range were considered positive for IgM. A positive IgM result was considered to be an indication of acute parvovirus B19 infection.

Data were reported as mean ± standard error of mean for continuous variables, and numbers and percentages for categorical variables. Continuous variables were compared...
between the study groups using the Student’s t-test and multiple mean analyses with Bonferroni test, as an ad hoc test. Categorical variables were compared using chi-square (χ²) and Fisher’s exact tests, where appropriate. Statistical outcomes with p < 0.05 were taken to be significant.

RESULTS

Table I presents data on haematological and inflammatory variables determined in the SCA patients and control. Overall, SCA patients had significant (p < 0.05) increases in platelet (411.5 ± 04.8 vs. 251 ± 7.7 cells/mm³ × 10³), reticulocyte (4.7% ± 0.3% vs. 1.4% ± 0.1%) and white blood cell (WBC) (10.9 ± 0.2 vs. 5.4 ± 0.1 cells/mm³ × 10³) counts, but decreased Hb level (8.3 ± 0.1 vs. 11.9 ± 0.7 g/dL) compared with the control. Alterations in these parameters were further intensified in unsteady-state patients compared with patients under steady conditions. With regard to the inflammatory makers, higher overall mean serum levels of TNF-α (34.1 ± 2.1 vs. 7.9 ± 0.9 pg/mL) and CRP (6.0 ± 0.3 vs. 2.3 ± 0.1 mg/L) were observed in the SCA patients compared with the control. Of the 64 SCA patients, 29.6% and 21.9% of them had TNF-α ≥ 40 pg/mL and CRP ≥ 10 mg/L, respectively, suggesting clinical inflammation. These percentages increased significantly (p < 0.05) to 56.5% for TNF-α and 43.5% for CRP in unsteady-state patients compared to 9.8% for each inflammatory marker in steady-state patients. None of the patients in the control group had TNF-α ≥ 40 pg/mL and CRP ≥ 10 mg/L. The mean age of the unsteady-state patients (13.2 ± 0.7 years) was found to be significantly (p < 0.05) lower than that of the steady-state patients (16.8 ± 0.8 years).

Of the 64 SCA patients, eight (12.5%) had parvovirus B19 infections, while the infection rate in the normal control population was 14.6%. Overall, a parvovirus prevalence rate of 13.3% was found in the study population. This was further observed to decrease with age, from 42.9% for patients aged 5–11 years to 35.7% for those aged 21–18 years and 21.4% for those aged ≥ 19 years. Viral burden was found to show gender variations, with higher infection rates in males (35.7 vs. 7.2%; p < 0.05) aged 5–11 years and females (28.6 vs. 7.1%; p < 0.05) aged 12–18 years (Fig. 1). Parvovirus B19 infection was further found to result in significant (p < 0.05) increases in TNF-α, by 59.1% in SCA patients and 298.5% in apparently healthy control volunteers. Significant (p < 0.05) elevation in CRP by 61.8% and non-significant (p > 0.05) elevation by 29.4% in the SCA patients and control, respectively, were observed (Table II).

DISCUSSION

Parvovirus B19 infections – acute or chronic – remain a cause...
for concern in SCA patients, as they are increasingly being linked to complications beyond their deleterious effects on the haematopoietic system.\textsuperscript{27-29} In this study, we investigated the effect of parvovirus B19 infection on inflammatory responses, with the focus on TNF-\(\alpha\) and CRP as inflammatory markers, in a cohort of 64 SCA patients and 41 apparently healthy individuals with AA and AS genotypes. As a routine check, we screened our SCA patients for haematological parameters such as Hb, WBC, platelet and reticulocyte counts, and found these parameters eliciting significant elevations, with the exception of Hb, which showed a reduction compared with the control. These patterns are consistent with findings reported previously by several investigators, including the current authors in Lagos and other states in Nigeria,\textsuperscript{14,20} as well as in other countries such as the USA,\textsuperscript{31} Ghana\textsuperscript{32} and Saudi Arabia,\textsuperscript{33} where SCA remains a public health burden. Leukocytosis, platelet activation and marked reduction in Hb level are well-established risk factors for crisis, longer hospital stays and deaths in SCA patients.\textsuperscript{34} Leukocytosis increases the number of leukocytes that are vulnerable to endothelial adhesion, expression of adhesion molecules, and enhanced synthesis and release of pro-inflammatory cytokines such as TNF-\(\alpha\), while platelet activation in number increases the coagulability of blood in the intravascular vascular compartment.\textsuperscript{16,17}

Although SCA patients can adapt to life with a lower Hb level compared to genotypes AA and AS individuals, further reduction in this parameter arising from acute haemolysis or sequestration in SCA may evoke cardiac dysfunction and exacerbate oxidative stress, leading to crisis in SCA patients.\textsuperscript{35} Therefore, monitoring these parameters is highly important in optimising care for steady-state sustenance and in improving the quality of life of patients with SCA. Our observed lower mean age of unsteady-state patients compared with their steady-state counterparts is a further attestation of age as a risk factor of crisis in SCA, as reported in previous studies.\textsuperscript{18,19,30,31} As part of care for SCA patients, the use of penicillin prophylaxis and pneumococcal vaccines in clinical trials has been found to be beneficial in terms of frequency and duration of crisis, risk of acute haemolytic anaemia and severe haemodynamic alterations.\textsuperscript{5,9,10,30}

The circulation of parvovirus B19 virus in Lagos, based on significant parvovirus B19 IgM antibody titre, was recently observed by the authors (Iwalokun et al, personal communication) and also reported recently by Emiasegen et al\textsuperscript{20} in Jos, Nigeria. However, the present study not only validates these findings but has also shown the distribution of this virus by gender and age-group in the overall population. We found an overall parvovirus B19 infection rate of 13.3% (12.5% in SCA patients; 14.6% among control volunteers), eliciting the finding that the rate of infection increases with decreasing age, and also showed the variations in infection rate by gender in our study cohort. Firstly, our observed rate of 13.3% is comparable to the 13.2% reported for pregnant women in Jos\textsuperscript{20} and the 11.3% found among SCD patients in the USA.\textsuperscript{31} In the setting of SCA, the observed parvovirus B19 infection rate in this study is higher than that found among Saudi Arabian (3.89%) and Tunisian (0.7%) patients.\textsuperscript{31,36} In addition to portraying differences in the endemicity of parvovirus B19 and its associated risk factors in these populations, the varied parvovirus B19 infection rates may be due to differences in sample size and enrolment criteria. For instance, in the US study, patients with other haemoglobinopathies were included in the study population,\textsuperscript{31} while in Saudi Arabia, crisis-modifying factors such as \(\beta\)-globin gene mutations, SC and \(\beta\)S genotypes are commonly found,\textsuperscript{37} and these factors may influence anti-parvovirus B19 IgM levels.

On the basis of absorbance index \(\geq 12\) for IgM as an indicator for parvovirus B19 infection, lower infection rates have been reported in Asian populations, where blood disorders such as thalassaemia and haemophilia are common.\textsuperscript{18} In terms of age, our observations agree with those of previous epidemiological studies of parvovirus B19 infections in other endemic settings of the world.\textsuperscript{39,40} On the basis of gender, studies have found an association of parvovirus B19 infection with the female gender, attributing this to the fact that women serve more often as caregivers and thus have more contact with children in the households.\textsuperscript{18} Several studies have also provided contradictory or gender-independent results in relation to parvovirus B19 infections.\textsuperscript{31,33,34} In this study, we found a higher occurrence of parvovirus B19 in males aged 5–11 years and females aged 12–18 years. Our findings may be due to the age distribution of our SCA cohort and the fact that the highest distribution of parvovirus B19 infection was observed in the 5–11 years age group, in which there was a higher proportion of males.

In relation to inflammation, we found that parvovirus B19 infection causes significant increases in TNF-\(\alpha\) (by 59% and 298% in SCA patients and the control, respectively). When this inflammatory marker was not stratified by parvovirus B19 infection, a higher mean value was found in our SCA patients compared with the control, thus reflecting the chronic

| Table II. Parvovirus B19 infection and serum TNF-\(\alpha\) and CRP levels among the SCA patients and control. |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| Defect                          | SCA patients                   | Control                         | SCA patients                   |
| Parvovirus B19 [+]              | Parvovirus B19 [-]             | Parvovirus B19 [+]              | Parvovirus B19 [-]             |
| TNF-\(\alpha\) (pg/mL)          | 51.2 ± 8.2\textsuperscript{a}  | 32.1 ± 2.2\textsuperscript{b}  | 19.7 ± 1.9\textsuperscript{c}  |
| ∆ (%)                          | 59.1                           | 298.5                           | 298.5                           |
| CRP (mg/L)                     | 8.9 ± 0.7\textsuperscript{a}  | 5.5 ± 0.5\textsuperscript{a}  | 2.2 ± 0.1\textsuperscript{c}  |
| ∆ (%)                          | 61.8                           | 29.4                           | 1.7 ± 0.04\textsuperscript{c}  |

Data is presented as mean ± standard error of mean of \(n\) determinations. Figures with different superscripts per row are significant (\(p < 0.05\)). ∆ denotes increase in parameter measured in percentage (parvovirus B19 [+] vs. parvovirus B19 [-]).

SCA: sickle cell anaemia; TNF-\(\alpha\): tumour necrosis factor-beta; CRP: C-reactive protein.
by the liver hepatocytes, following activation by cytokines such as TNF-α. Meanwhile, a systemic TNF-α threshold level of 40 pg/ml has been defined as the level beyond which inflammation episodes become significant and clinical in manifestations. In this study, 9.8% of our steady-state SCA patients had an above-normal TNF-α level. This is still lower than the 33% rate reported by Kubdívila et al(15) for steady-state SCA patients in the USA. On this basis, 9.8% of our steady-state SCA patients who elicited TNF-α at clinical level can be said to be asymptomatic, thereby mimicking a ‘pseudo’ subclinical state. Given the endemity of other inflammatory diseases, such as malaria, and their high risk of being comorbid factors in SCA patients, as observed in previous studies,(4,7,10) our observations suggest the possibility of raising the normal TNF-α threshold of SCA patients in this environment. While this may reflect an adaptation to the chronic inflammation state that is characteristic of SCA patients, it also has implications for early detection of patients at risk of crisis in the nearest future, thereby delaying interventions that could avert and shorten the crisis duration in this group of steady-state patients.

In unstratified data, similar patterns of CRP increase were observed between our SCA patients and the control, and between unsteady-state and steady-state patients, confirming the chronic inflammation state of SCA and exacerbation of this inflammation during crisis. However, when stratified according to parvovirus B19 infection, persistent CRP elevation, by 61.9% and 29.4% in SCA patients and control, respectively, was only significant in the former. This puts CRP at a disadvantage as an inflammatory correlate of parvovirus B19 infection in apparently normal individuals with AA and AS genotypes. Several studies conducted in other endemic populations have reported higher rates of asymptomatic illness in immunocompromised humans infected with parvovirus B19.(19,40) On the basis of genotypes on which the participants’ recruitment was based, our control volunteers can be said to be immunocompetent. This may explain our observations of zero rate of clinical inflammation in relation to TNF-α and CRP among the control population as well as the nonsignificant CRP level between infected and non-infected volunteers.

In pathophysiological terms, CRP is a nonspecific inflammatory marker that is synthesised and released majorly into circulation by the liver hepatocytes, following activation by cytokines such as IL-6 in the acute state of infection, and it may persist at a higher level in chronic inflammatory episodes.(15,18,19) Although TNF-α is also nonspecific, this inflammatory marker is synthesised by many cell types, including blood monocytes, tissue macrophages and activated/damaged endothelial cells.(16,17) Recent studies have demonstrated the ability of parvovirus B19 to infect these nonerythroid cells and induce cytotoxicity based on the nonstructural protein, NP1 expression. Given the fact that SCA patients are under a chronic inflammatory state, which is characterised by chronic activation of cells involved in cell-mediated immunity and inflammation, our observed elevations in serum TNF-α and CRP levels, as reported in studies conducted in other endemic populations,(15) are to be expected. To the best of our knowledge, data on systemic TNF-α and CRP levels in relation to parvovirus B19 infection are scarce. Therefore, this study has provided additional biochemical information for monitoring cellular events associated with parvovirus B19 infection, particularly in the setting of SCA, and in validating early findings concerning the asymptomatic situation of this viral infection in immunocompromised humans.

In conclusion, the results of this study indicate that parvovirus B19 infection is associated with the intensification of inflammatory response in SCA patient, which is characterised by significant elevations in serum TNF-α and CRP levels. Both parameters were also elevated in infected non-sicklers, but with only TNF-α reaching a significant level. On this basis, both CRP and TNF-α in SCA patients and TNF-α alone in non-sicklers have the potential to serve as inflammatory correlates of parvovirus B19 infections in this environment.

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