THE ROLE OF BLOOD GROUPS IN PREVENTING OR ENHANCING HIV INFECTION IN BOTSWANA

MODISA SEKHAMO MOTSWALEDI

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The role of blood groups in preventing or enhancing HIV infection in Botswana

By

Modisa Sekhamo Motswaledi

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Supervisor: Professor Oluwafemi O Oguntibeju

Co-supervisor: Professor Ishmael Kasvosve

Bellville

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DECLARATION

I, Modisa Sekhamo Motswaledi declare that the contents of this dissertation/thesis represent my own unaided work, and that the dissertation/thesis has not been previously submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of Cape Peninsula University of Technology.

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ABSTRACT

Knowledge of population vulnerabilities to infectious diseases is key in managing many public health problems and for mapping appropriate strategies for prevention or intervention. A number of genes associated with resistance to HIV infection, such as the double deletion of 32 base pairs in the CCR5 gene , have been described and potentially account for lower HIV infections in some populations. The magnitude of the HIV pandemic in Sub-Saharan Africa warrants an investigation of the peculiar genetic factors that may have exacerbated its spread. An understanding of the genetic factors that are involved may aid in the development of specific strategies for prevention such as vaccine development, genetic counselling as well as gene therapy. The aim of this project was therefore to study the relationship between blood groups and HIV-infection in Botswana. HIV infection in Africa has not been linked to particular blood groups.

The project was undertaken in two phases from December 2012 to December 2017. In the first phase, 346 subjects of known HIV status (negative or positive) were phenotyped for 23 erythrocyte antigens via standard scientific procedures. A Chi-square analysis was used to determine those antigens associated with increased or reduced risk of HIV infection. In the second phase, 120 samples were phenotyped for the protective blood group (RhC) and the risk-associated groups (Lu^b and P_1). The samples were also characterized according to their laboratory results for viral load, lymphocyte sub-populations, complete blood count and blood chemistry, including total cholesterol. Some of the samples were also assessed for erythrocyte-associated viral RNA.

Generally, the prevalence of the blood groups in the general population in Botswana did not differ with the known prevalence for Africans broadly. Three novel findings were established. First, the blood group Rh(C) was associated with a 40% risk reduction for HIV infection. Immunologically, carriage of the C antigen was associated with a more robust cell-mediated immunity as evidenced by enhanced cytotoxic T cell counts. Moreover, this antigen occurred with a frequency lower than 30% in all countries where HIV prevalence was high. There was therefore an inverse relationship between Rh(C) frequency and HIV prevalence. An examination of reports from previous studies revealed that the pattern was consistent in Africa, Europe, Asia, South America and Caribbean countries. It appears that the population frequency of this antigen explains, at least in part, a genetic factor that puts some African populations at higher risk for HIV infection. These results are novel in that Rh antigens have not been previously associated with immunity in any reports.

Novel findings regarding the P_1 blood group was its association with a double risk for HIV infection. While the plasma viral load did not differ between P_1-positive and P_1-negative subjects, P_1-positive erythrocyte lysate yielded more viral RNA than P_1-negative cells, implying more intracellular HIV RNA. Intra-erythrocytic viral RNA was detected even in patients with an undetectable plasma viral load. Glycosphingolipids, of which P_1 is an example, have been documented to promote viral fusion to cells independent of CD4 receptors or other ligands. In at least one report, the presence of sphingolipids in lipid rafts was considered to be

sufficient for viral fusion. The presence of viral RNA even in erythrocyte lysates corroborates this phenomenon and potentially explains the double risk of HIV infection observed. The occurrence of HIV RNA in erythrocyte lysate is a novel finding that suggests a new viral reservoir. Apparently, P_1 has a high frequency among Africans and low in other races.

The Lu^b blood group was associated with triple risk for HIV infection and a lower absolute count of circulating monocytes. This monocytopenia was accentuated in the presence of higher cholesterol levels and in the presence of the Lu^b antigen. The occurrence of monocyte depletion in a setting of high cholesterol plus an adhesion molecule suggests conditions that favour atherosclerotic plagues. The increased risk of HIV-infection in Lu^b positive individuals is therefore likely linked to tethering and emigration into tissues, thus disseminating the virus to distant organs.

Our results suggest that blood group C protects against HIV infection by enhancing cytotoxic T-cell counts. Furthermore, the P_1 blood group potentially promotes HIV infection by creating viral reservoirs within the erythrocytes that can later be released when aging erythrocytes are trapped in the spleen and engulfed by splenic macrophages. The Lutheran blood group appears to enhance monocyte depletion in a setting of hypercholesterolemia and chronic inflammation created by HIV infection. So, while it is generally accepted that cardiovascular disease has a genetic predisposition, the results of the current study suggest that Lutheran blood group antigens may be a novel part of that genetic milieu of atherogenic factors in HIV-positive persons. The results further suggest the prophylactic possibility of suppressing endothelial expression of Lutheran antigens or modulating the expression of integrins on monocytic cells to reduce the adhesion risk. Finally, the ability of HIV to fuse to glycosphingolipids independent of CD4 receptors raises the possibility that HIV virions could be targeted by use of RNase-laden glycosphingolipid liposomes in post-exposure prophylaxis or where there is need to rapidly reduce the plasma viral load.

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DEDICATION

To my dear wife Agnes and the children who endured my substantial absence in pursuit of this qualification and to my students, past, present and future who need to know that perseverance and hard work are normally and ultimately rewarded. Above all, to God the Creator of all things, in whom are hidden all the treasures of wisdom and knowledge.

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GLOSSARY

ABBREVIATION	DEFINITION / EXPLANATION
ACHAP	African Comprehensive HIV/AIDS Partnerships
AHG	Antihuman Globulin antibody
ART	Anti-retroviral Therapy
BHHRL	Botswana-Harvard HIV reference Laboratory
CCL5	Cysteine-Cysteine Ligand Type 5
CCR5	Cysteine-cysteine Receptor Type 5
CCR5Δ32/Δ32	CCR5 mutation with double deletion of 32 base pairs
CHD	Coronary Heart Disease
CR1	Complement Receptor Type 1
CTL	Cytotoxic T-Lymphocytes
CVD	Cardio-vascular Disease
CXCR4	Cysteine-X-Cysteine Receptor Type 4
DARC	Duffy Antigen Receptor for Chemokines
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine-tetraacetic acid
EGF	Epidermal Growth Factor
E-HIV	Erythrocyte-associated Human Immunodeficiency Virus
Fy^a or Fy^b	antithetical Duffy "a" or "b" antigens
GSL	Glycosphingolipid
HAART	Highly-active anti-retroviral therapy
HDL	High Density Lipoprotein (Good cholesterol)
HIV	Human Immunodeficiency Virus
IQR	Interquartile Range
ISBT	International Society for Blood Transfusion
Jk^a/Jk^b	Antithetical antigens of the Kidd blood group system
LDL	Low Density Lipoprotein (Bad cholesterol)

Lea/Leb	Lewis "a"/Lewis "b" antithetical blood groups
LU1	Lu^a
LU2	Lu^b
Lu^a	Lutheran $^{\text{"a"}}$ blood group antigen antithetical to Lu^b
Lu^b	Lutheran antigen antithetical to Lu^a
Lu/BCAM	Lutheran/Basal Cell Adhesion Molecule
LW	Landsteiner-Weiner
MCP-1	Monocyte Chemo-attractant Protein-1
MHC-I (II)	Major Histocompatibility Complex Class I (or II)
PCR	Polymerase Chain Reaction
RANTES	Regulated on Activation Normal T-Cell Expressed and Secreted
RBC	Red Blood Cell
rh'/rh"	Weiner designation for Rh (C) and (E) antigens and genes
RhAG	Rh-associated Antigen
RhC	"C" antigen in the Rhesus blood group
Rh2	ISBT name for Rh(C)
RHCE*C	Another designation of the "C"antigen in the Rh system
RNA	Ribonucleic acid
SIRPα	Signal-regulatory Protein α
TC	Total Cholesterol
UNAIDS	Joint United Nations Programme on HIV/ Acquired Immune Deficiency Syndrome
VL	Viral Load
X4 variants	HIV strains that use the CXCR4 co-receptor
X5 variants	HIV strains that utilize CCR5 co-receptor

PREFACE

This dissertation is presented in article format. The chapters appear in the format of the journals in which they have been published or submitted for publication.

Chapter 1 is the general Introduction, which basically puts the problem in perspective. Here the attention of the reader is drawn to the magnitude of the HIV and AIDS problem globally as well as in Botswana, which is the particular setting of the current research project. Epidemiologic data from UNAIDS on both the global and local situations is presented to underscore the importance of the subject. The role of erythrocyte antigens as binding sites for infectious agents is discussed with examples of other infectious agents that specifically bind to blood group antigens. The chapter then makes a brief reference to the role of red blood cells (erythrocytes) in HIV-infection and singles out red cell antigens as potential binding entities in the transmission of HIV to susceptible cells, thus justifying the relevance of the blood groups in HIV research.

Chapter 2 is a more detailed review of literature on the role of the erythrocytes in HIV and other diseases. A review article from this thesis was published in BioMed Research International journal in 2013. The paper points to the fact that frequencies of blood groups differ among populations partly due to breeding practices as well as disease-induced selection pressure. Individuals whose cell antigens provide binding sites for pathogenic microorganisms will naturally be susceptible to negative selection. The paper further discusses a number of erythrocyte antigens that have been linked to diseases, prototype of which is the Duffy antigen that has been eliminated to a significant level in individuals living in endemic malaria areas. Controversies in this area of research are also addressed.

The physiologic function of erythrocyte antigens is discussed to highlight relevance to normal function in general, and to innate immune responses. A detailed discussion of the role of blood groups in specific diseases, including potential mechanisms of cytopathic effects in HIV-infection concludes the paper.

Chapter 3 (This part of the research work has been published as an article in the journal: PLOS One). The paper reports the screening of 346 HIV-infected and uninfected controls for 23 antigens, these being A, B, "O", D, C, c, E, e, Fy^a, Fy^b, Jk^a, Jk^b, Kp^a, Kp^b, Le^a, Le^b, Lu^a, Lu_b, M,N, P_1, S, and s. In this study, one blood group, Rh(C), also designated as rh' or Rh2, was found to be protective while two blood groups, P_1 and Lu^b, were associated with significantly increased risk for HIV-infection. Our data also contributed to the hotly contested role of the Duffy blood group as risk factor for HIV infection as none was found in our study. The rest of the blood groups investigated were not associated with significant risk or protection. Another important finding of this investigation was that the protective blood group Rh(C) occurred much less frequently among Africans than it did among Caucasians and Northern Indians while the risky blood groups, P_1 and Lu^b had prevalence in excess of 90%. Information available in the literature was helpful in relating the biochemical nature of the risky blood groups to the study observations. However, the protective mechanism for Rh(C) appeared inexplicable in the

absence of information on the biological role of this antigen. Mechanisms were therefore suggested for the risk-association of these antigens.

Chapter 4 is an article that is under review by the African Journal for Laboratory Medicine. The substance of this article sought to gain insight into the mechanisms accounting for the risk observed in the Lutheran blood group. The Lutheran blood group antigens are members of the immunoglobulin superfamily and act as adhesion molecules expressed on monocytes and endothelial cells. Carriage of the Lu^b antigen (also designated LU2) was associated with a lower monocyte count, and the count diminished with increasing cholesterol levels. This cholesterol-associated monocyte depletion was accentuated in Lu^b-positive individuals but virtually absent in Lu^b-negative subjects. The depletion of monocytes in a setting of high cholesterol and chronic inflammation points to an atherogenic environment and potentially explains why HIV-infected individuals are prone to cardiovascular disease. Moreover, if this depletion truly represents extravasation of monocytes, this could explain the increased risk of infection as infected monocytes migrate and introduce the virus to other anatomical sites. Novel findings here include both the increased risk of infection in LU2 individuals as well as the monocyte-depleting effect of this blood group.

Chapter 5 presents the findings as they relate to blood group C (or Rh2). The main finding here was the association of Rh2 expression with both a lowered risk for HIV-infection in the context of enhanced cytotoxic T-cells counts. These cells are important for the control of viraemia and are known to confer resistance in long term progressors and non-progressors. This is the first report linking an antigen in the Rh system with immune function.

Chapter 6 reports on the findings relating to the P_1 blood group. Since P_1 is a glycosphingolipid capable of promoting viral fusion with cell membranes, evidence for intra-erythrocytic viral RNA was sought. The study confirmed the presence of erythrocyte-associated HIV RNA in patients with or without detectable plasma viral load. However, this viral pool was not detectable in every patient with a detectable or even high viral load. P_1 blood group was associated with increased intra-erythrocytic HIV-1 RNA. This too was consistent with the role of this blood group, a glycosphingolipid, which enhances the fusion of the virus to cells, thus promoting infection.

Based on these studies, it can be concluded that there is adequate scientific evidence for the role of blood groups C, P_1 and Lu^b in enhancing or preventing HIV-infection, thus the purpose of this study was achieved.

Chapter 7 presents a general discussion and conclusions. Here all the main novelties are highlighted with potential mechanisms. The prevalence data is compared with HIV prevalence in various countries to show that the findings in this study are consistent with severity of the disease in those countries or communities. Uganda and Botswana stand out in that respect.

Chapter 8 provides recommendations for further enquiries to elucidate specific research questions emanating from this study.

CHAPTER ONE

INTRODUCTION

The HIV Pandemic in Botswana

The HIV and AIDS pandemic is one of the world's worst health-related catastrophes in recent history. UNAIDS estimates that since its start in the early 1980's, the pandemic has infected more than 70 million people and claimed at least half the lives of those infected as at the end of 2016 (UNAIDS, 2017a). It is further estimated that 36.7 million people are living with the virus world-wide, with 1.8 million new infections experienced in 2016. The report further takes cognizance of the notable variation between countries and regions, but Sub-Saharan Africa is the most severely affected region and bearing 2/3 of global infections (UNAIDS, 2017a).

On the economic front, the virus has prompted the commitment of massive resources for prevention and treatment programmes, with US$19.1 billion having been committed to support low and middle income countries in 2016 alone (UNAIDS, 2017a).

Botswana is one of the worst-affected countries by the HIV pandemic. During the period 1992 to 1994, the country experienced a dramatic increase in the HIV prevalence with urban areas such as Gaborone and Francistown jumping from 15% to 28% and from 23% to 30% (Macdonald, 1996), respectively.

In 2002, the Government of Botswana introduced the Masa Project, in which anti-retroviral treatment was offered to eligible individuals (Farahani et al., 2014). Initially, the criterion for initiation of combination anti-retroviral treatment (cART) included a cut-off CD4 cell count (initially 200 cells/μL). This programme radically reduced mortality rates from 63% in 2002 to 0.8% in 2010 (Farahani et al., 2014). In 2004, the government of Botswana scaled up the anti-retroviral (ARV) programme and began to offer HIV-testing at health service points as well as voluntary testing and counselling centres around the country. The clinical efforts were also accompanied by intense clinical trials and other forms of research that developed robust interventions such as the Prevention of mother-to-child (PMTCT) study (Shapiro et al., 2013) and the micronutrient studies that added to the quality of the clinical interventions. Based on continuous research, strategies were modified to obtain best practices (Zash et al., 2016). Through sustained and focused clinical and laboratory-based studies (Novitsky et al., 2010), the Botswana Programme ultimately graduated to a "Treatment as Prevention" or "Treat-all" programme (Botswana Ministry of Health, 2016) based on the observation that individuals who achieved viral suppression were less likely to transmit the virus to their partners(Hayes et al., 2011).

In 2010, the country was reported to be the world's second worst hit country with prevalence rates then at 17.6% and as high as 20% in women (Kandala et al., 2012). The current UNAIDS reports indicate that at least 350,000 individuals in Botswana are living with the virus in a population barely exceeding 2 million, with a prevalence of 22.2% in the age group 15-49 years of age (UNAIDS, 2017b). This astounding spread of the virus may have been due to peculiar genetics of the population in addition to other socio-economic factors.

Statement of the Problem

Population genetics is an important consideration in studying the epidemiology of infectious diseases. Blood groups are genetically-determined antigens carried on red blood cells or erythrocytes that are inherited as co-dominant Mendelian traits. Blood groups are mainly important in blood transfusion because they determine compatibility of donor and recipient cells. However, some blood group antigens function as receptors for infectious agents such as malaria (Hadley and Peiper, 1997), parvo virus (Brown et al., 1993) and *Helicobacter pylori* (Anstee, 2010, Aspholm-Hurtig et al., 2004). In recent years, the binding of the human immunodeficiency virus to erythrocytes and the apparent increased infectivity of such bound virus has sparked interest in this potential viral reservoir (Puri et al., 1998, Hammache et al., 1999, Beck and Alving, 2011). Blood groups therefore emerge as logical binding sites for the virus on erythrocytes and candidate molecules for scientific enquiry.

The aim of this research project was therefore to identify blood group antigens associated with increased or decreased HIV infection and, where possible, elucidate the underlying mechanisms. The results of the study were intended to help clarify the genetic basis of the HIV epidemic as it relates to blood groups in the Botswana population. The discovery of HIV-resistant blood groups could open a number of frontiers for prevention and treatment of HIV. Couples seeking artificial fertilization may have the option of semen of the appropriate blood group to produce HIV-resistant offspring. Furthermore, research efforts could be focussed on the use of hematopoietic stem cells as these are better tolerated in the developing foetus. Consideration could also be made for using blood transfusion to treat infections or as pre- or post-exposure prophylaxis.

The Aim of the Study

The overarching aim of this study is to identify candidate antigens that may predispose to or impact on the risk for HIV infection, and if any are identified, to investigate potential mechanisms by which protection or risk is conferred.

Specific objectives

The objectives of the study were:

a. Estimate the population frequencies of 23 blood group antigens in Botswana.
b. Compare the occurrence of blood groups in HIV-infected versus uninfected individuals.

    c.   Investigate the mechanism responsible for increased or reduced susceptibility to HIV infection.

Hypothesis:

Ho: Blood groups are not associated with HIV infection.

Ha: Some blood groups are associated with HIV infection.

In the light of the above, the research sought to answer the following questions:

1. What are the frequencies of the 23 blood group antigens in the Botswana population?
2. Is there any difference in occurrence of HIV among the different blood groups?
3. What mechanisms account for the difference in susceptibility to HIV infection among the blood groups?

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CHAPTER TWO

LITERATURE REVIEW

THE ROLE OF RED BLOOD CELLS IN ENHANCING OR PREVENTING HIV INFECTION AND OTHER DISEASES

[1,2]Modisa S Motswaledi, [1]Ishmael Kasvosve & [2]Oluwafemi O Oguntibeju

[1]University of Botswana, Department of Medical Laboratory Sciences, Faculty of Health Sciences, Gaborone, Botswana. [2]Oxidative Stress Research Centre, Department of Biomedical Sciences, Faculty of Health & Wellness Sciences, Cape Peninsula University of Technology, Faculty of Health and Wellness Sciences, South Africa, Bellville, 7535.

Address correspondence to: oguntibejuo@cput.ac.za, bejufemi@yahoo.co.uk Tel: +27219538495, Fax: +27219538495

Modisa S Motswaledi, MT (ASCP), MSc, BSc, (modisa.motswaledi@mopipi.ub.bw)

Ishmael Kasvosve, PhD, MSc, BSc (ishmael.kasvosve@mopipi.ub.bw)

Oluwafemi O Oguntibeju, DTech, FIMLS, FACBS, FIBMS, CSci (oguntibejuo@cput.ac.za, bejufemi@yahoo.co.uk)

Summary:

AIM: To highlight the apparently neglected role of red blood cell antigens in the epidemiology of infectious diseases, with particular focus on HIV, with the prime objective of stimulating research in this area.

METHOD: PubMed database was searched for relevant articles from 1984 to 2013, this being the era covering active HIV research. However, the review was limited by the relative scarcity of articles on the subject, and only articles written in English were reviewed.

RESULTS: Substantial documentary evidence attests to the ability of infectious agents to bind to erythrocyte-borne molecules, thereby facilitating infection.

CONCLUSION: Mature erythrocytes, by their lack of a nucleus are not candidates for HIV infection. Emerging information, however, suggests that red blood cell antigens may be important in the pathogenesis of many diseases, including HIV as they bind free viruses and viral immune complexes and are instrumental in transmitting the virus to susceptible cells. The role of erythrocyte surface molecules acting as viral binding sites is discussed in light of current scientific knowledge.

Key Words: Blood groups, HIV infection, Erythrocyte-bound HIV, chemokines, disease associations

Introduction

Blood groups have been known to exist for centuries ever since man entertained the possibility of replacing blood loss through transfusion. They are responsible for determining compatibility of blood in transfusion medicine and are also responsible for foetal loss in haemolytic disease affecting the foetus or new born (Bowman, 1996).

Blood groups are inherited as Mendelian co-dominant(Neil et al., 2003) traits and should be expected to occur in somewhat comparable frequencies in the human race. However, differences do exist in the distribution of blood groups in various human populations. This has been largely attributed to selection pressure as endemic diseases appear to have a predilection for selected blood groups leading to the demise of individuals bearing those susceptible blood group antigens(Anstee, 2010, Le Pendu et al., 2006). But certainly, there must be more to blood groups than just causing problems in blood transfusion and predisposing to myriad self-annihilation prospects.

On the other hand, HIV has emerged as one of the major public health concerns of the 21st century. Some genetic factors have been cited as contributors to HIV susceptibility or resistance, among them blood groups such as ABO/Rh(Sayal et al., 1996, Arendrup et al., 1991, Abdulazeez et al., 2008, John et al., 2011, Neil et al., 2003), Duffy(Bolton and Garry, 2011, Lachgar et al., 1998, Ramsuran et al., 2011, He et al., 2008) and P^k(Lund et al., 2009). However, the matter has not been without controversy as some investigators have reported findings to the contrary(Ukaejiofo and Nubila, 2006, Winkler et al., 2009) against what appears to be conclusive evidence of red cell interaction with the virus(Bolton and Garry, 2011, Beck

et al., 2009, Puri et al., 1998, Hammache et al., 1999). Blood groups therefore appear to have a contribution to public health, at least in the area of infectious disease, which makes it imperative to synthesize available knowledge in an attempt to decipher the extent to which red blood cell (RBC) antigens are involved in HIV epidemiology and to unveil avenues for future research; here follows a synthesis of current research on the beneficial and detrimental effects of RBC antigens with probable molecular explanations.

Methods:

A literature search was performed on the PubMed database. This was achieved by using the phrases "erythrocyte blood groups AND HIV" (81 articles) or "red cell antigen, blood groups AND HIV" (60 articles). A manual search was also done using Google Scholar and supplemented by other original articles referenced by various authors.

Physiologic Functions of RBC Antigens

The physiologic functions of most blood group antigens are unknown (Greenwell, 1997). Few antigens, however, such as the Kidd (urea transporter), Diego (anion exchanger), Colton (water channel), Cromer (Decay Accelerating Factor) and Duffy (chemokine receptor) have had their physiologic functions somewhat elucidated (Mudad and Telen, 1996, Telen, 1995). The importance of such functions remains a matter of debate though, since individuals who are negative for these blood group antigens live an apparently normal life (Daniels, 1999).

A lot has been documented about the Duffy blood group as a chemokine receptor, a function that has led to its designation as the Duffy antigen receptor for chemokines (DARC), and to the investigation of its potential role in modulating the inflammatory response (Hadley and Peiper, 1997, Lachgar et al., 1998, Nibbs et al., 2003, Gardner et al., 2004, Bolton and Garry, 2011). Chemokines are important for recruiting white blood cells to the site of infection or inflammation by binding to their receptors. However, HIV also uses these chemokine receptors as co-receptors for its entry into permissive cells. It is known that R5 viruses utilize CCR5 while R4 viruses use CXCR4 as a co-receptor. The binding of HIV to the co-receptor and its subsequent entry into the cell is inhibited by chemokines which act as competitors for the binding site (Lachgar et al., 1998, Bolton and Garry, 2011). The Duffy antigen receptor for chemokines binds a wide range of chemokines and thus competes with white blood cells for chemo-attractants as well as increase cell exposure to HIV infection. DARC also binds other inflammatory mediators such as interleukin 8 (IL-8), RANTES and monocyte chemo-attractant protein-1 (MCP-1) (Telen et al., 1990, Reich et al., 2009, Cartron, 2010), all of which are important in contributing to a successful defence against pathogens.

Red blood cells also bear the CD44 antigen, a ligand for hyaluronic acid. Binding of hyaluronic acid to this RBC antigen would have the significance of dampening its inflammatory effect. As in the case of the Duffy antigen binding to chemokines, this erythrocytic property may be important in absorbing excess stimulant signals intended for inflammatory cells, thereby modulating immune responses (Telen, 1995). In cases where the stimulant is produced in small amounts, it is plausible that erythrocyte antigens, by their sheer numbers, may out-compete the intended cells, leading to an immunological hypo-response.

Human red blood cells not only transport oxygen and carbon dioxide, but are important in the elimination of many molecules from circulation, including products of immunologic reactions. RBCs possess adhesion molecules that bind complement fragments(Horakova et al., 2004) and are therefore important for capturing and delivering such complexes to the reticulo-endothelial system for elimination from circulation. In this regard, Horakova et al(Horakova et al., 2004) investigated the binding of HIV immune complexes to erythrocytes. Their investigations led to the conclusion that at least three mechanisms are involved, these being; the binding of complement-opsonized immune complexes via CR1, direct binding of the virus in a complement-dependent manner but in which specific antibodies were not required, and a third mode in which complement was not required at all. Their conclusion was that the third mode of binding might be instrumental in the spread of infection at the time of primary infection when specific antibodies would not have been formed yet.

Some RBC antigens are also known to bind inflammatory mediators with potential to affect innate immune responses (Telen, 1995, Hadley and Peiper, 1997). These RBC antigens absorb and limit the amount of chemokine stimulant activity (Reich et al., 2009). Indeed it has been demonstrated that mice lacking the Duffy antigens exhibit a greater inflammatory response to CXC and CC chemokines compared to homozygotes or heterozygotes (Dawson et al., 2000). This is consistent with the role of Duffy as a "chemokine sink" created by chemokine-binding sites on DARC(+) erythrocytes. But, paradoxically, the absence of the Duffy antigens has also been associated with a low neutrophil count (Reich et al., 2009, Ramsuran et al., 2011). Given the observation that DARC also binds HIV and is capable of transfecting CD4 cells (Lachgar et al., 1998), it is probable that HIV-binding to erythrocytes represents an action of various molecules acting in synergy.

Blood Group Disease Associations

On the pathological front, however, certain blood groups have been associated with diseases. Individuals of blood group A, for example, are known to be more susceptible to coronary heart disease (CHD) independent of known risk factors than other ABO blood groups (Wazirali, 2005). This is further corroborated by the observation that group A individuals also have higher levels of low density lipoprotein (LDL) cholesterol (Greenwell, 1997). Although the molecular aspects of this observation still remains to be elucidated, the observation implies that this antigen has potential to either influence synthesis of, or inhibit the natural metabolism of these lipids, thus predisposing individuals to CHD. The same study, on the other hand, reported a lower risk for this condition among group O individuals.

Additionally, blood group A and B are known to be highly susceptible to thrombotic disorders in contrast to group O individuals who are more at risk for bleeding than thrombotic events(Clark and Wu, 2011, Schleef et al., 2005, Lourenço et al., 1996). This observation has been attributed to higher levels of Factor VIII and von Willebrand factor in A and B individuals while the levels may be as low as 30% in group O individuals. Blood groups A and B antigens have been demonstrated on these coagulation molecules and are thought to prolong their half-life, leading to higher concentration in non-O individuals (Jenkins, 2006).

Blood Group A has also been associated with malignancies such as cancer of the ovary, cervix, rectum, breast, stomach and leukemia (Greenwell, 1997). This is thought to be due to an abundance of high-affinity binding sites for epidermal growth factor (EGF) on blood group A red blood cells compared to blood groups O and B (Engelmann et al., 1992). Since blood groups may be expressed on tissues other than red cells(Telen, 1995), it is plausible that the binding of EGF to these binding sites may indeed promote cancer development. Conversely, some malignancies have been observed to positively or negatively modulate blood group antigen expression to varying degrees depending on the anatomical site (Greenwell, 1997). Thus, gastric carcinomas were found to be associated with increased expression of ABH and Le[a] antigens while colonic cancers were associated with expression of Le[b] and low expression of the other antigens. In other studies, enhanced fucosyltransferase activity was reported in endometrial cancer leading to unexpected expression of Le[a], Le[b] and H antigens (Ravn, 1992, Ravn et al., 1994).

The Duffy blood group is probably one of the most studied blood groups in relation to disease association. Individuals who are homozygotes or heterozygotes for the Duffy antigens, Fy[a] or Fy[b], are vulnerable to malaria parasites *Plasmodium vivax* and *Plasmodium knowlesi.* Individuals that lack both Duffy antigens, Fy(a-b-) and therefore lack the receptor for malaria parasites are constitutively resistant to these forms of malaria (Barnwell et al., 1989, Telen, 1995)**.**

Blood Groups in HIV and other viral Infections

Blood Groups in HIV Infection
Most research on HIV has focused on cells of the immune system to the exclusion of viral interactions with red blood cells. However, emerging evidence, suggests that red blood cells may be important in the pathogenesis of HIV as they enhance viral infectivity by binding free viruses (Beck et al., 2009) as well as viral immune complexes (Hess et al., 2002, Horakova et al., 2004, Beck et al., 2009), and through such binding transfect HIV-susceptible cells (Hess et al., 2002, Horakova et al., 2004).

HIV infection has been reported to occur in select blood groups in some regions of the world. A study by Sayal *et al.* in India reported a preponderance for infection in group O Rh(D)-positive men and least among Group B positive and D-negative ones. However, a close examination of the results reveals insufficient statistical analysis rendering the differences statistically insignificant. Similar studies by Nneli *et al* (Nneli et al., 2004) and John et al (John et al., 2011) suffered similar deficiencies. In these studies, group O positive individuals were thought to be highly susceptible, but again the studies lacked the statistical rigor to indicate the level of significance and have been contradicted by other investigators (Ukaejiofo and Nubila, 2006). A statistical interrogation of the data in each of the above case does not support the conclusion of statistical significance.

It would then appear that current scientific information does not support a potential role for ABO blood groups in HIV infection. In fact, evidence from other studies would suggest the contrary for group O individuals. Since HIV virions have been shown to acquire the blood group antigens of the infected individuals(Rachkewich et al., 1978, Neil et al., 2003, Arendrup

et al., 1991), such virions would be neutralized by naturally-occurring antibodies in group O individuals, thus offering protection in blood group-discordant couples (Neil et al., 2003). It is noted, however, that this protection will not be available if the source of infection was of a similar blood group. Moreover, given the apparently uniform risk of infection among ABO blood groups, it is doubtful if this neutralization is of any clinical consequence, especially with reference to HIV-1 infection. It remains to be demonstrated then whether HIV from blood group A or B is able to infect group O CD4 cells.

In a separate study, Abdulazeez and colleagues (Abdulazeez et al., 2008) working with HIV-1 and 2 reported a higher prevalence of HIV-2 (71.4%) compared to HIV-1 (7.1%) in the AB blood group and that Rh(D) positive (97.8%) was more susceptible than D negative (2.2%). However, these findings are yet to be confirmed by other investigators.

The lack of direct empirical evidence for ABO blood groups does not, however, obliterate the possibility of associations in other blood groups. Since secreted blood group substances can be adsorbed onto lymphocyte membranes (Rachkewich et al., 1978, Arendrup et al., 1991, Neil et al., 2003), the presence of these antigens could potentially alter cell behavior. Blood group antigens, being glycoproteins and glycolipids, are highly charged molecules that are bound to affect their molecular micro-environment, including protein conformation and receptor/CD4 localization and function (Greenwell, 1997, Lund et al., 2009). Glycosphingolipids and glycoproteins have in fact been demonstrated to facilitate fusion of HIV-1 with CD4 cells by independent investigators, thus acting as alternative co-receptors for the virus (Puri et al., 1998, Hammache et al., 1999). There exists a theoretical possibility therefore, that membrane-bound blood group substances on CD4-positive cells may thus affect the affinity of viral-binding proteins and viral infectivity, promoting or diminishing cell susceptibility to infection. Apparently, both the promotive and inhibitory effects of blood groups in HIV infection have been documented. The Duffy antigen receptor for chemokines (DARC) has been reported as a binding site for HIV-1 on red blood cells, which binding increases infectivity for permissive cells(Lachgar et al., 1998, He et al., 2008). Although some investigators have refuted the role of this antigen in HIV-1 infection(Winkler et al., 2009, Horne et al., 2009), the preponderance of evidence is heavily in favor of this role, including recent findings of sequence similarities between HIV-1 V3 loop and the *Plasmodium vivax* Duffy-binding protein, both of which bind to DARC.

Furthermore, the Duffy Null phenotype has been associated with increased susceptibility to HIV infection among African Americans, elevating the odds of acquiring HIV by as much as 40%, while those negative for the antigen were observed to exhibit slower progression to AIDS (He, 2008). Although these findings were later refuted by other investigators (Winkler, 2009, Horne et al., 2009), there is persuasive evidence suggesting the role of DARC in binding the virus and facilitating transfection of permissive cells(Ramsuran et al., 2011, Lachgar et al., 1998, Bolton and Garry, 2011). These reports were given further credibility by findings that the Duffy-binding protein of *Plasmodium vivax* bore sequence similarity with the V3 loop of HIV1(Bolton and Garry, 2011).

He et al (He et al., 2008) reported that the DARC+ phenotype was associated not only with higher rates of HIV-1 infection, but also that DARC+ red cells transfected HIV-susceptible

cells with X4 variants more efficiently than it did with X5 variants. Blocking of the DARC with monoclonal antibodies or other natural ligands significantly reduced viral binding, thus confirming the role of DARC in the interaction. However, the blocking of the DARC did not totally obliterate HIV binding to erythrocytes, thus suggesting possibilities of involvement of other binding molecules as hypothesized by Beck et al(Beck et al., 2009). Similar findings were also reported from a study involving commercial sex workers in South Africa, where investigators found that a Duffy-null phenotype with low neutrophil counts was predictive of HIV-1 sero-conversion (Ramsuran et al., 2011).

Another such molecule is the P^k RBC antigen, which has been reported to confer resistance to HIV infection in lymphocytes *in vivo* and *in vitro*. In this Canadian study, the level of expression of P^k antigen on peripheral mononuclear cells (PBMC) was positively correlated with anti-viral protection (Lund et al., 2009). The P^k antigen has a low frequency (one in a million)(Lund et al., 2009) among Caucasians and is thought to have contributed to reduction of HIV spread among such populations. The CCR5Δ32/Δ32 is another mutation that is prevalent in the same population at 1-3% (He et al., 2008) and is known to confer resistance to HIV infection. While it has been argued that the lack of P^k among Africans may not account for the difference in the epidemiology of HIV in sub-Saharan Africa (given its low frequency among Caucasians), one could nevertheless consider the combined effect of both genes in the same population and the number of contacts that could have been generated if such genes were not operational, compared to a population that lacks both.

In addition, Beck *et al* demonstrated that human red blood cells bind HIV virions independent of antibody or complement. The RBC selectively bound infectious HIV virions as opposed to other viral components and removed all infectivity from the suspension medium. The HIV-binding to erythrocytes was calcium-dependent but complement and CD4-independent. Moreover, RBC-bound virions were 100-fold more infectious to CD4 cells than cell-free particles(Beck et al., 2009), leading them to conclude that red blood cells could constitute a viral reservoir for trans-infection of CD4 cells. The Beck report(Beck et al., 2009) essentially confirmed earlier findings by Hess et al(Hess et al., 2002) of a pool of HIV viral RNA associated with RBC in HIV-1-infected patients. These included patients in whom plasma viraemia had been suppressed to undetectable levels for several months with anti-retroviral drugs. Their investigations revealed higher numbers of RNA copies per mL of whole blood in the RBC than white cell preparations of the same patients, suggesting that the bulk of the cell-bound virus circulates on RBC rather than white blood cells (WBC). RBC-bound HIV was also able to infect HeLaT4 cells. The number of erythrocyte-bound HIV copies significantly correlated with disease severity(Hess et al., 2002). The sum total of these findings is that RBC are important elements for consideration in HIV infection and could potentially influence epidemiology and clinical progression.

In order to deal with this reservoir of infection, there is a need to understand the nature of the RBC-HIV binding, especially now that there is evidence linking certain blood groups to susceptibility or resistance to HIV(Lund et al., 2009, Nneli et al., 2004, Neil et al., 2003, Puri et al., 1998).

Blood Groups in Other Viral Infections

The binding of viruses by red blood cells, including specific binding to blood group antigens is not unique to HIV, although the mechanisms involved may differ. Noroviruses have been observed to selectively bind to group A, H and difucosylated Lewis blood groups (Nilsson et al., 2009, Shirato-Horikoshi et al., 2007), and only volunteers who were secretors of these antigens were found to be susceptible to norovirus gastroenteritis(Le Pendu et al., 2006). Although this binding was not directly related to red blood cells, it nevertheless demonstrated viral affinity for a red blood cell antigen expressed on gastric mucosal cells and led to the conclusion that the group A, H and Le^b antigens act as viral receptors for the virus(Le Pendu et al., 2006, Tan and Jiang, 2005).

Anaemia

Anaemia is the most important prognostic indicator in AIDS patients (Mocroft et al., 1999, Harris et al., 2008, Olayemi et al., 2008). A large scale study involving over 4000 patients established an increasing hazard ratio of 1.42, 2.56 and 5.26 for mild, moderate and severe anaemia, respectively (Harris et al., 2008). Various mechanisms have been suggested, including bone marrow suppression by various cytokines, toxic depletion by the virus, and immune destruction following sensitization with viral proteins (Volberding et al., 2003, Weiss and Goodnough, 2005, Beck and Alving, 2011). However, the degree of anaemia has not been correlated with the amount of erythrocyte-bound HIV (E-HIV), a phenomenon that might account for the autoimmune haemolytic anaemia that commonly occurs in HIV-infected individuals. Of interest is the observation by Martins-Silva *et al* that HIV infection alters red cell and lymphocyte membrane fluidity, membrane protein activity and brings about changes in trans-membrane calcium transportation (Martins-Silva et al., 2006). These changes may disrupt erythrocyte membrane stability, thus promoting haemolysis and ultimately anaemia.

Blood group antigens have also been reported to occur on trans-membrane transport channels (Telen, 1995). These transport channels have an influence on membrane structure and integrity and HIV binding to these transport channels may explain some of the mechanisms that contribute to membrane instability with consequent haemolysis and cytopaenias in HIV patients. Binding of R5 viruses to CCR5, for example, has been shown to elicit phospholipase C production (Fantuzzi et al., 2008). Furthermore, others have reported that phospholipase C is translocated to the periphery of activated natural killer (NK) cells with possible role in cell-mediated cytotoxicity (Ramoni et al., 2001). However, the role of this enzyme in haemolytic processes in HIV patients remains a matter for further enquiry.

The haemolytic effects of phospholipase C could be further aggravated by the presence of lipolytic enzymes emanating from the various opportunistic infections. Many bacteria produce this lytic enzyme including *Mycobacterium tuberculosis*, a common opportunistic pathogen in HIV patients (Johansen et al., 1996, Côtes et al., 2008, Bakala N'Goma et al., 2010, Schué et al., 2010).

Conclusion

Finally, HIV continues to take a toll on the health and economies of the world especially the developing countries. To date a cure for this virus continues to be elusive. However, a virtually complete remission has been attained in one patient following a bone marrow transplant of a CCR5Δ32/Δ32 mutant cell line (Hütter, 2009). However, despite this milestone, donors with this mutation are still far too rare and limited to European populations. The P^k blood group has also been demonstrated to offer such protection (Lund et al., 2009). These findings indicate that there could be additional blood groups that can be explored in the fight against HIV. Very few studies have focused on the role of blood groups in preventing or enhancing HIV infection, and these few had the limited scope of only looking at the ABH and Rh(D) blood groups. Africa suffers from a lot of malignancies that require total body irradiation and bone marrow reconstitution, some of them linked to HIV infection. The occurrence of HIV-resistant blood groups may avail bone marrows that may well be an answer to HIV eradication in such cancer patients.

CONFLICT OF INTEREST

The authors have nothing to disclose and declare that there is no potential conflict of interest.

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CHAPTER THREE

BLOOD GROUP ANTIGENS C, Lu^b AND P$_1$ MAY HAVE A ROLE IN HIV INFECTION IN AFRICANS

[1,2]Modisa S Motswaledi, [1]Ishmael Kasvosve, [2]Oluwafemi O Oguntibeju

[1]Department of Medical Laboratory Sciences, Faculty of Health Sciences, University of Botswana, Gaborone, Botswana.
[2]Nutrition & Chronic Diseases Research Group, Oxidative Stress Research Centre, Department of Biomedical Sciences, Faculty of Health & Wellness Sciences, Cape Peninsula University of Technology, Cape Town South Africa

Address correspondence to: Department of Biomedical Sciences, Faculty of Health & Wellness Sciences, Cape Peninsula University of Technology, Bellville, 7535, Cape Town, South Africa.
Email: oguntibejuo@cput.ac.za, bejufemi@yahoo.co.uk
Tel: +27219538495, Fax: +27219538495

Modisa S Motswaledi, MT (ASCP), MSc, BSc, (modisa.motswaledi@mopipi.ub.bw)

Ishmael Kasvosve, PhD, MSc, BSc (ishmael.kasvosve@mopipi.ub.bw)

Oluwafemi O Oguntibeju, DTech, FIMLS, FACBS, FIBMS, CSci, Pr.Sci.Nat (oguntibejuo@cput.ac.za, bejufemi@yahoo.co.uk)

Abstract

BACKGROUND: Botswana is among the world's countries with the highest rates of HIV infection. It is not known whether or not this susceptibility to infection is due to genetic factors in the population. Accumulating evidence, however, points to the role of erythrocytes as potential mediators of infection. We therefore sought to establish the role, if any, of some erythrocyte antigens in HIV infection in a cross-section of the population.

METHODS: 348 HIV-negative samples were obtained from the National Blood Transfusion Service as residual samples, while 196 HIV-positive samples were obtained from the Botswana-Harvard HIV Reference Laboratory. Samples were grouped for twenty three antigens. Chi-square or Fischer Exact analyses were used to compare the frequencies of the antigens in the two groups. A stepwise, binary logistic regression was used to study the interaction of the various antigens in the light of HIV-status.

RESULTS: The Rh antigens C and E were associated with HIV-negative status, while blood group Jk^a, P_1 and Lu^b were associated with HIV-positive status. A stepwise binary logistic regression analysis yielded group C as the most significant protective blood group while Lu^b and P_1 were associated with significantly higher odds ratio in favour of HIV-infection. The lower-risk-associated group C was significantly lower in Africans compared to published data for Caucasians and might explain the difference in susceptibility to HIV-1.

CONCLUSION: The most influential antigen C, which also appears to be protective, is significantly lower in Africans than Caucasians. On the other hand, there are multiple antigens associated with increased risk that may override the protective role of C. A study of the distribution of these antigens in other populations may shed light on their roles in the HIV pandemic.

Background

A number of investigators have alluded to the role of blood groups in HIV epidemiology. Arendrup and colleagues, for example, reported that HIV from lymphocytes of blood group A individuals was neutralized by anti-A, implying that this mechanism could potentially reduce the likelihood of infection in ABO discordant couples (Arendrup et al., 1991). These findings were later corroborated by Neil and colleagues (Neil et al., 2003). Furthermore, other investigators have reported higher prevalence of HIV-2 in blood group AB (Abdulazeez et al., 2008), in blood group O (Sayal et al., 1996) as well as a protective function associated with blood group P^k (Lund et al., 2009, Lund et al., 2005).

Many studies have demonstrated the promiscuity of red blood cell antigens in binding to bacterial toxins, viruses and other molecules (Branch, 2010, Nilsson et al., 2009, Horakova et al., 2004, Lachgar et al., 1998). The Duffy antigen receptor for chemokines (DARC) is the receptor for *Plasmodium vivax (Barnwell et al., 1989, Hadley and Peiper, 1997)* and has stimulated debate on its role in HIV-infection as a candidate for HIV-binding and infection of susceptible cells (Lachgar et al., 1998, Horne et al., 2009, He et al., 2008, Ramsuran et al., 2011, Bolton and Garry, 2011). Other reports have suggested that certain erythrocyte lipids promote viral membrane fusion with CD4 cells and thus facilitate infection (Puri et al., 1998, Puri et al., 2004, Hammache et al., 1999). The predilection of HIV for any particular blood group could therefore explain the epidemiology among populations expressing that antigen to varying degrees.

Botswana is one of the countries in the world most hard-hit by the HIV pandemic with estimated national prevalence of 17.6%(Agency, 2008) and as high as 33.7% among pregnant women aged 15-49 years(Gomez et al., 2007). It is not known whether the susceptibility of this population was purely attributable to its social habits or whether there exists a genetic predisposition to HIV infection that exacerbated its vulnerability. If indeed erythrocyte surface molecules bind and increase the transfection of CD4+ cells, we hypothesize that this should reflect, in the prevalence of HIV associated with those erythrocyte surface molecules. Blood groups are probably the most abundant molecules on the red blood cell surface and are therefore logical candidates for this investigation. We thus sought to find out if the presence of any particular blood group antigen could be linked to increased HIV infection risk in Botswana.

Methods

Study Population

Three hundred and forty eight (348) HIV-negative blood samples were obtained from the National Blood Transfusion Service (NBTS) as residual samples. The National Blood Transfusion Service is the main center for donor recruitment as well as screening for suitability of blood donors and for ensuring the safety of donated blood and its components. The NBTS blood collections cover the southern part of the country and therefore represents a wide range of communities in the Southern part of Botswana. Moreover, Gaborone is a cosmopolitan city comprising of individuals from all over the country. Blood donors who tested positive for HIV were added to the HIV-positive pool. One hundred and ninety six (196) HIV-positive samples were obtained from the Botswana-Harvard HIV Reference Laboratory (BHHRL). This

laboratory is a referral laboratory in the management of HIV/AIDS patients and functions mainly to support the anti-retroviral treatment programme by providing CD4 counts and viral load testing services for prospective and ARV-enrolled patients. All samples were obtained as aliquots and assigned new study identification numbers. HIV-positive samples included both new cases and those from patients already on anti-retroviral therapy. The total number of samples tested for each blood group was determined by the availability of the specific antiserum. The HIV-positive samples from the HIV Reference Laboratory covered a wide range of communities in the southern eastern part of the country. As stated above, the city of Gaborone comprises of individuals from various parts of the country and therefore representative of the Botswana population.

Ethical clearance was obtained from the Ethics Committee of the University of Botswana's Office of Research and Development, Ministry of Health's Research Unit, BHHRL, Princess Marina Hospital Research Committee, and from the Research Ethics Committee of the Faculty of Health and Wellness Sciences, Cape Peninsula University of Technology. The study was thus approved by the ethics committees of Cape Peninsula University of Technology and Botswana's Ministry of Health. The collection of data was achieved between June 2013 and January 2015. Informed consent was not necessary since the research utilized residual samples that were rendered anonymous in accordance with the requirements of ISO 15189 (Standardisation, 2007).

Determination of Blood Groups

Patient and donor red cells were phenotyped using specific antisera (Fortress Diagnostics, Antrim, United Kingdom). Blood grouping was done following routine tube-grouping technique as outlined in the manufacturer's instructions for all blood groups, with the exception of anti-Jk^a and Jk^b which failed quality control unless subjected to the indirect antihuman globulin procedure (AHG). It was reasonable to subject them to AHG since they are also known to be non-agglutinating antibodies. The AHG procedure was used to determine the reactivity of the following antibodies: anti-Fy^a, anti-Fy^b anti-Jk^a, anti-Jk^b, anti-Kp^a, anti-Kp^b, anti-Lu^a, anti-Lu^b, Anti-S and anti-s. The AHG was also used to confirm negative results for anti-D. The specificity and reactivity of the antisera was confirmed by use of an antibody identification panel, DiaPanel®, Lot 16211.66.x-16311.66.x (BioRad®, Japan). The blood antigens tested for in this study included: A, B, "O", D, C, c, E, e, Fy^a, Fy^b, Jk^a, Jk^b, Kp^a, Kp^b, Le^a, Le^b, Lu^a, Lu^b, M, N, P_1, S, and s.

Statistical Analysis

Data analysis was conducted using IBM SPSS version 23 software. Frequencies were used to report blood group prevalence. Comparison of proportions of blood group antigens between HIV-infected and non-infected individuals were conducted using Pearson's chi-square test ($\chi 2$) or Fisher's exact test where appropriate. We explored the combined effect of various blood groups on HIV prevalence using a stepwise binary logistic regression analysis. Only blood group antigens with statistically significant association with HIV status in bivariate analyses

were included in the model and the results were reported as odds ratio with 95% confidence interval. Results were considered significant at p < 0.05.

Results

In total, 348 HIV-negative blood samples and 196 blood samples from HIV-infected patients were studied. The results comparing the prevalence of each antigen in the HIV group versus uninfected controls are summarized in **Table 3.1**. The frequency of C Rh antigen was lower in HIV-infected patients than in HIV-negative subjects, 17.1% (95% CI 10.7 – 23.5) versus 29.7% (95% CI 24.1 – 35.1), respectively p = 0.006. Similarly, the E Rh antigen was lower in HIV-infected patients than in HIV-negative subjects, 12.1% (95% CI 6.6 – 17.6) versus 21.2% (95% CI 16.2 – 26.2), respectively p = 0.024. Only these Rh-antigens raised the prospects of a protective function against HIV-1 infection.

In contrast, the HIV-infected group exhibited a significantly higher prevalence of blood groups Jk^a , Lu^b and P_1 compared to HIV-negative individuals, suggesting a potential infection risk associated with carriage of these antigens. Among the HIV-infected patients the prevalence of Jk^a was 85.3% ((95% CI 78.7 – 91.9) compared to 51.4% (95% CI 41.8 – 61.0) in non-infected individuals, p = 0.024. The prevalence of Lu^b was 86.7% (95% CI 80.3 – 93.1) among HIV patients versus 67.8% (95% CI 61.8 – 73.8) in non-HIV infected subjects, p = 0.0001. Among the HIV-infected patients, P_1 prevalence was 89.2% (95% CI 83.1 – 95.3) compared to 74.6% (95% CI 69.1 – 80.1), p = 0.003. All the other 19 red cell antigens we tested were not significantly different when stratified according to HIV status.

Table 3. 1 – Prevalence of blood group antigens in HIV-infected and uninfected subjects

Antigen	HIV-positive Subjects		HIV-negative Subjects		
	N	Antigen Frequency % (95% CI)	N	Antigen Frequency % (95% CI)	P-value
A	155	34.1 (26.5 – 41.7)	239	42.3 (35.9 – 48.7)	0.109
B	155	32.3 (24.8 – 39.8)	239	25.9 (20.2 – 31.6)	0.174
O	239	41.3 (47.3 – 65.9)	336	41.0 (35.6 – 46.4)	1.000
D	155	95.5 (92.2 – 98.8)	239	97.1 (94.9 – 99.3)	0.406
C	140	17.1 (10.7 – 23.5)	269	29.7 (24.1 – 35.3)	0.006
c	140	99.3 (97.9 – 100.7)	269	97.4 (95.5 – 99.3)	0.273
E	140	12.1 (6.6 – 17.6)	269	21.2 (16.2 – 26.2)	0.024
e	140	99.3 (97.9 – 100.7)	269	98.5 (97.0 – 100)	0.665
Fy^a	146	10.3 (5.3 – 15.3)	251	9.6 (5.9 – 13.3)	0.818
Fy^b	146	12.3 (6.9 – 17.7)	254	9.8 (6.1 – 13.5)	0.440
Jk^a	116	85.3 (78.7 – 91.9)	109	51.4 (41.8 – 61.0)	<0.0001
Jk^b	117	25.6 (17.5 – 33.7)	109	31.2 (22.3 – 40.1)	0.355
Kp^a	123	2.5 (-0.3 – 5.3)	264	1.1 (-0.2 – 2.4)	0.387
Kp^b	97	45.4 (35.3 – 55.5)	266	36.5 (30.6 – 42.4)	0.124
Le^a	82	30.5 (20.3 – 40.7)	257	25.3 (19.9 – 30.7)	0.354
Le^b	66	50.0 (37.7 – 62.3)	257	42.0 (35.8 – 48.2)	0.244
Lu^a	118	1.7 (-0.7 – 4.1)	266	3.8 (1.5 – 6.1)	0.358
Lu^b	113	86.7 (80.3 – 93.1)	240	67.8 (61.8 – 73.8)	0.0001
M	92	77.2 (68.5 – 85.9)	236	80.9 (75.8 – 86.0)	0.456
N	93	71.0 (61.6 – 80.4)	269	66.9 (61.2 – 72.6)	0.470
P1	105	89.2 (83.1 – 95.3)	248	74.6 (69.1 – 80.1)	0.003
S	125	33.6 (25.2 – 42.0)	225	31.6 (25.4 – 37.8)	0.695
s	111	95.6 (91.7 – 99.5)	226	89.4 (85.3 – 93.5)	0.060

We also investigated the possible combined influence of the protective and risk-associated blood group antigens on HIV status. For this purpose, data was subjected to a stepwise logistic regression model in which analysis was repeated after the systematic removal of the least significant among the covariates. In this model, only those blood groups that were significantly associated with HIV infection in bivariate analyses were tested. The results of this analysis are shown in **Table 3.2**.

Table 3. 2 – Logistic regression model showing the effect of blood group antigens on the risk of HIV infection

Antigen	Odds Ratio (95% Confidence Interval)	P-value
E	0.859 (0.169 - 0.437)	0.855
P_1	0.428 (0.108 – 0.169)	0.226
C	0.146 (0.047 – 0.455)	0.001
Jk^a	1.001 (0.156 – 6.408)	1.0
Lu^b	1.731 (0.314 – 9.541	0.529

After the stepwise elimination process, only the C, Lu^b and P1 maintained statistical significance as risk factors in the model. The results are shown in **Table 3.3**. Carriage of P_1 antigen increased the odds of HIV infection by 2.2 (95% CI 1.1 – 4.5), p = 0.026 and similarly, carriage of Lu^b increased the odds ratio for HIV infection by 2.9 (95% CI 1.5 – 5.5), p = 0.001. Conversely, presence of C Rh antigen resulted in 40% decrease in HIV risk of infection.

Table 3. 3 – Blood group antigens associated with HIV infection in multivariate logistic regression analysis

Antigen	Odds Ratio (95% Confidence Interval))	p-value
C	0.6 (0.3 – 1.0)	0.047
P_1	2.2 (1.1 – 4.5)	0.026
Lu^b	2.9 (1.5 – 5.5)	0.001

We further sought to determine if the frequency of protective and risk-associated antigens was any different from other populations of African ethnicity or Caucasian counterparts. The frequency of blood group C in this study, though not statistically different from that published for Blacks (p=0.8389), was much lower than that for Caucasians (p<0.0001)(Makroo et al., 2013) The frequencies for the risk-associated P_1 and Lu^b were also lower in this study than in Caucasians (p=0.0446 and p<0.0001, respectively (Hellberg, 2007, Badjie and Stubbs, 2012,

Hellberg et al., 2013). However, these other studies did not report on the HIV status of the populations from which the data was derived.

Discussion

Blood groups have been implicated as factors in the epidemiology of HIV(Arendrup et al., 1991, Bolton and Garry, 2011, Lachgar et al., 1998, Onsten et al., 2013, Puri et al., 1998, Ramsuran et al., 2011). We studied the distribution of blood groups in Botswana and identified those associated with HIV infection. We therefore determined the frequencies for ABO, Rh, Kidd, Kell, Duffy, Lewis, Lutheran, MNSs, and P_1 blood group systems in both HIV-infected patients and non-HIV-infected individuals. The frequencies of Fy^b, Jk^a, Jk^b, Kp^b, Le^b, N, P_1, Lu^b and s in Botswana were statistically lower than the published literature for Blacks (Badjie and Stubbs, 2012, Makroo et al., 2013, Cooling, 2011). This is not a surprising finding since blood group variations do occur within a population (Badjie and Stubbs, 2012) and may sometimes be aggravated by cultural marriage practices that promote peculiar phenotypes (Mavanga et al., 2013). Recent publications have also demonstrated the peculiarities of the Botswana population that necessitate the use of Botswana-specific reference values (Mine et al., 2011, Maphephu and Kasvosve, 2011, Segolodi et al., 2014). Other than these, the rest of the antigen frequencies were consistent with published literature as noted.

The C and E antigens in the Rhesus blood group system were found to be rarer in HIV-infected subjects while that of Jk^a, Lu^b and P_1, were more prevalent in this group. In comparison to individuals of African ethnicity, the Botswana population does not appear to have any higher frequency of risk-associated blood groups nor does it have any lower frequency of those associated risk mitigation. However, the prevalence of the risk-mitigating antigen C was lower in blacks (and in the Botswana population studied) than in Caucasians or Asians. Given the strength of the influence of this antigen in the model used, and given the relative rarity of its frequency in the Botswana population, we conclude that the C antigen likely plays an important role in mitigating against HIV-1 infection and could have played a role in the HIV pandemic in Botswana.

We hypothesized that a higher prevalence of an antigen in the HIV+ group than the control group possibly indicates that its presence promotes HIV infection, and vice versa. We therefore investigated the potential mitigation of the "protective" phenotypes against the "risky" ones. The odds ratios were therefore computed for risk-associated blood groups in the presence or absence of Rh C or E expression. The presence of C or E did not consistently result in obliteration of the risk-significance in the risk-associated phenotypes. This analysis was also hampered by the simultaneous carriage or alternate carriage of suspect antigens in different individuals, making it difficult to isolate the effect of each antigen.

To further elucidate how the odds ratio is impacted by interaction of all significant blood groups, the data was subjected to a logistic regression analysis. The Jk^a and E antigens, although highly significant in their association to HIV infection in the isolated Chi-square analysis, failed to maintain significance in the logistic regression model. On the other hand, the C, P_1 and Lu^b remained significant with C significantly reducing the odds of HIV infection while P_1 and Lu^b increased the odds of infection.

Our results for blood group O are not in concordance with the reports by other investigators (Nneli et al., 2004, Sayal et al., 1996) who documented an increased HIV susceptibility for this group. Neither did we find any increased risk in group B individuals as reported in Brazil (Onsten et al., 2013). There was also no difference in HIV prevalence between D-positive and D-negative subjects as reported in Nigeria (Abdulazeez et al., 2008). However, we take cognizance of the possible population differences and the effect of other factors such as viral strains in those populations that may account for the disparities observed. But for the Botswana population, the ABO groups do not appear to have any role in enhancing or diminishing HIV risk of infection.

Our findings indicate that P_1 antigen, a glycosphingolipid, is associated with a higher risk of HIV infection and thus corroborates the role of glycosphingolipids as facilitators of viral fusion to CD4+ cells (Hammache et al., 1999, Puri et al., 2004). In their experiments, Puri and colleagues demonstrated that HIV infection of CD4+ cells could be averted by addition of a glycosphingolipid inhibitor. Conversely, the presence of the glycosphingolipid increased susceptibility of CD4+ cells to HIV infection. The P_1 antigen is also known to bind several microorganisms including enteropathogenic *Escherichia coli* and parvovirus (Branch, 2010) and has a wide tissue distribution (Hellberg et al., 2013), providing multiple opportunities for viral binding. It is conceivable from its broad spectrum of binding that P_1 could also bind and act as a receptor for HIV. We conclude that our findings corroborate the role of P_1 as a potential aggravator of HIV infection.

We did not find any risks associated with the Duffy blood group system or its Duffy-null phenotype alluded to elsewhere (Thobakgale and Ndung'u, 2014, Ramsuran et al., 2011, He et al., 2008, Lachgar et al., 1998). Our results in this respect appear to support an insignificant role of Duffy blood group expression consistent with other investigators(Winkler et al., 2009, Horne et al., 2009). However, we did confirm that the Duffy-null phenotype was associated with leukopenia(Reich et al., 2009), though not neutropenia, in Botswana.

The Lutheran blood group is a member of the immunoglobulin superfamily and has been shown to function as a receptor for laminin (Parsons et al., 1995, Nemer et al.) with potential role for signal transduction and is widely distributed in various tissues (Parsons et al., 1995, Eyler and Telen, 2006) No data is available on the role of the Lutheran blood groups in HIV infection. However, its tissue-wide distribution would facilitate infection at various anatomic sites, if such a role could be confirmed.

Conclusion

We have studied the role of 23 blood group antigens in relation to susceptibility to HIV infection, this being hitherto the most comprehensive study on the role of blood groups in HIV infection. The strongest association in this study was with the Rh antigen C, which also appears to be protective, and is significantly lower in Africans than Caucasians. On the other hand, there are multiple antigens associated with increased risk that may override the protective role of C. A study of the distribution of these antigens in other populations may shed light on their roles in the HIV pandemic.

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CHAPTER FOUR
POTENTIAL ROLE OF LU/BCAM IN HIV-RELATED ATHEROSCLEROSIS

Modisa Sekhamo Motswaledi[1, 2](corresponding author), Ishmael Kasvosve[1], Oluwafemi Omoniyi Oguntibeju[2]

[1] Department of Medical Laboratory Sciences, Faculty of Health Sciences, University of Botswana, modisa.motswaledi@mopipi.ub.bw, ishmael.kasvosve@mopipi.ub.bw, Gaborone, Botswana, [2] Department of Biomedical Sciences, Faculty of Health & Wellness Sciences, Cape Peninsula University of Technology, Cape Town, South Africa. OguntibejuO@cput.ac.za (Address correspondence to: Department of Biomedical Sciences, Faculty of Health & Wellness Sciences, Cape Peninsula University of Technology, Bellville, 7535, Cape Town, South Africa. Tel: +27219538495, Fax: **+27219538495**

Abstract:

Atheromatous lesions are formed by macrophage and LDL-cholesterol invading the vascular intima. Here we show that increasing cholesterol levels are associated with peripheral monocyte depletion and this imbalance is aggravated by carriage of Lu/BCAM leukocyte adhesion molecule. This is true only in HIV infection and probably explains the risk of atherosclerosis observed in HIV infection.

Key words: Cholesterol, Non-DHL cholesterol, Lu/BCAM, atherosclerosis

Introduction

The pathology of atherosclerosis is tightly linked to three main factors; leukocytes, plasma lipids and vascular injury or chronic inflammation (Frostegard et al., 1990, Oh et al., 2009b). Leukocytes such as neutrophils, activated monocytes or monocyte-derived macrophages and platelets accumulate in the vascular sub-endothelial matrix by use of adhesion molecules. The Lutheran blood group, also known as basal cell adhesion molecule (Lu/BCAM), is one such molecule and is expressed on both monocytes and endothelial cells (Pedraza et al., 2000). It is the natural ligand for laminin, an extracellular basement membrane protein that facilitates both adhesion and extravasation of monocytes (Pedraza et al., 2000). In addition, monocytes and neutrophils also constitutively express α4β1 integrin (Chaar et al., 2010), another ligand for Lu/BCAM. These molecules enable these cells to adhere to the endothelium and have been implicated in the pathology of crescentic, sickle cell disease (Chaar et al., 2010) as well as in several cancers suggesting more efficient tumour tethering and greater tumour size (Chang et al., 2017, De Grandis et al., 2013).

In HIV-infected individuals, the viral Tat protein accumulates in the extracellular matrix of blood vessels and facilitates integrin-mediated adhesion (Chiodelli et al., 2012) with the consequence that HIV-infected monocytes have enhanced extravasation (Dhawan et al., 1992). HIV infection is also characterised by chronic inflammation resulting from the activation of T-cells, macrophages, neutrophils and NK cells– all of which release pro-inflammatory cytokines that create a state of persistent inflammation (Appay and Sauce, 2008). This chronic inflammation involves neutrophils which produce reactive oxygen species thought to contribute to the oxidation of LDL-C (Hartwig et al., 2015). Cholesterol enrichment of neutrophil membranes results in more stabilized rolling and adhesion on endothelial cells (Oh et al., 2009a), where they may recruit monocytes (Hartwig et al., 2015) to promote atherogenesis. In particular, non-HDL cholesterol has been documented as one of the more important promoters of atherosclerosis in HIV-infected patients (Badiou et al., 2008). The atheromatous lesion forms by gradual accumulation of oxidized LDL-C and infiltration by macrophages, smooth muscle cells and platelets (Østerud and Bjørklid, 2003). This may ultimately narrow the vascular lumen, causing tissue hypoxia distal to the atheroma. Moreover, the release of RANTES (CCL5) by activated platelets in the atheroma leads to further recruitment of monocytes and neutrophils (von Hundelshausen et al., 2001). A high monocyte count has thus been reported to be a long term predictor of plaque-formation (Johnsen et al., 2005), while neutrophil enzymes such as elastase and myeloperoxidase in the atheroma may destabilize it (Ionita et al., 2010), leading to its fragmentation and subsequent generation of emboli that may cause vascular occlusion at distant sites.

In our previous study, the Lutheran blood group, Lu[b], was associated with a three-fold risk for HIV infection (Motswaledi et al., 2016), and it was suspected that this could be due to its adhesiveness that promotes trans-endothelial migration and spread of infected monocytes to distant sites. The current study therefore sought to determine the effect, if any, of carriage of the Lu[b] antigen as an adhesion molecule on circulating monocytes and neutrophils as well as how these parameters related to cholesterol measurements. The results indicate that HIV does affect the relationship between monocytes and cholesterol, and that this relationship is accentuated by expression of Lu[b] antigen.

Materials and Methods

One hundred and one (101) blood samples comprising 59 females and 42 males received at Julia Molefe Clinic in Gaborone were enrolled on the study between December 2016 and January 2017. The patients had all tested positive for HIV and were being prepared for enrolment into the antiretroviral therapy programme. A full blood count (FBC) was done using the Sysmex ® XT1800i haematology analyser (Sysmex Corporation, Kobe, Japan). The samples were phenotyped for the Lutheran antigens (Lu^a and Lu^b) using specific antisera (Fortress Diagnostics, Antrim, United Kingdom) according to the manufacturer's instructions. The reactivity of the antisera was confirmed by use of antigen-positive and negative cells selected from an antibody identification panel (DiaPanel®, Lot 45241.88.1, Bio-Rad®, Cressier FR, Switzerland). Total and HDL-cholesterol measurements were also made using the AU480 Chemistry analyser (Brea, CA). LDL cholesterol was calculated using the Friedewald equation. We also revisited a previous dataset of 261 normal subjects as a control group to examine the same relationships in healthy, HIV-uninfected subjects(Mine et al., 2011).

Ethical clearance for the study was obtained from the Office of Research and Development (University of Botswana), Botswana's Ministry of Health and Wellness's Health Research Development Committee and Faculty of Health and Wellness' Research Ethics Committee (Cape Peninsula University of Technology)[Appendices A-C]. Informed consent was not necessary since de-identified, residual samples were used (ISO, 2007).

The results were analysed using the IBM SPSS version 24 statistical software. Statistical analyses included comparison of means according to carriage of the Lutheran antigens, as well as correlation of data to explore relationship between variables. Results were considered significant only if $P<0.05$.

Results:

An inverse correlation was observed between total cholesterol and absolute monocyte and neutrophil counts (See Table 4.1). In HIV-infected subjects, monocyte depletion was associated with relatively high LDL-cholesterol levels ($r=-0.435$, $p=0.003$). The correlation was stronger ($r=-0.479$, $p=0.010$) in Lu^b-positive but absent ($r=-0.212$, $p=0.414$) in those negative for the antigen. Among controls (n=261), LDL cholesterol did not correlate with monocyte counts at all ($r=0.031$, $p=0.619$). Lu^b expression was associated with lower absolute monocyte counts (0.46 ± 0.19 versus $0.54\pm0.22 \times 10^9$/L, $p=0.040$).

Table 4.1: Correlation of cholesterol with phagocyte counts in Lu^b-positive and negative subjects

Test on	Mean±SD (x10^9/L)	Pearson correlation coefficient (r) with TC	P-value
Lu^b –positive and negative subjects (n=101)			
Absolute neutrophil count	2.7±2.0	-0.207	0.038
Absolute monocyte count	0.48±0.20	-0.253	0.011
Lu^b-positive subjects only (n=76)			
Absolute neutrophil count	2.65±2.2	-0.310	0.006
Absolute Monocyte count	0.46±0.20	-0.330	0.004
Lu^b-negative subjects only (n=25)			
Absolute neutrophil count	2.8±1.3	0.153	0.465
Absolute Monocyte count	0.54±0.22	-0.047	0.822

To further elucidate the total cholesterol (TC) component responsible for these observations, correlations were done involving phagocyte counts versus HDL-C and non-HDL-C. Non-HDL-C was consistently associated with a lower monocyte count suggesting that the leukocyte-depleting effects of cholesterol are largely due to non-HDL-C and that depletion is enhanced in Lu^b-positive subjects. Neutrophils were less affected by non-HDL-C than monocytes (See Table 4.2).

Table 4. 2: Correlation of phagocyte counts with non-HDL cholesterol in Lu^b-positive and negative subjects

Test on	Mean±SD	Non-HDL Correlation coefficient (r)	P-value
Lu^b –positive and negative subjects (n=59)			
Absolute neutrophil count (x10^9/L)	2.70±2.00	-0.226	0.136
Absolute monocyte count (x10^9/L)	0.48±0.20	-0.401	0.006
In Lu^b-positive subjects (n=28)			
Absolute neutrophil count (x10^9/L)	2.65±2.20	-0.369	0.053
Absolute Monocyte count (x10^9/L)	0.46±0.20	-0.422	0.018
In Lu^b-negative subjects (n=31)			
Absolute neutrophil count (x10^9/L)	2.85±1.30	0.143	0.584
Absolute Monocyte count (x10^9/L)	0.54±0.21	-0.259	0.315

Discussion

We studied the relationship between cholesterol and phagocytes (monocytes and neutrophils) in HIV-1-infected and uninfected individuals in an attempt to determine the influence, if any, of the Lu/BCAM on phagocyte counts.

We hypothesized that carriage of the Lu[b] antigen, an adhesion molecule, would enhance margination of leukocytes leading to a peripheral deficiency of the affected leukocytes. Indeed, we observed a significantly lower absolute monocyte count in Lu[b]-positive individuals compared to Lu[b]-negatives.

We also observed a negative correlation between cholesterol and phagocytes (neutrophils and monocytes), which corroborates the findings by other investigators that membrane cholesterol, which is in equilibrium with plasma cholesterol (Buchwald et al., 2000), increases leukocyte adhesion and extravasation (Oh et al., 2009b, Frostegard et al., 1990). Moreover, phagocyte counts were negatively correlated with non-HDL-C and LDL-C, which lipids are known to promote adhesion and atherogenesis (Frostegard et al., 1990, Østerud and Bjørklid, 2003). The monocyte depletive effect of cholesterol was accentuated in Lu[b]-positive subjects but totally absent in the Lu[b]-negative subjects. We conclude that higher levels of cholesterol are associated with lower peripheral monocyte and neutrophil counts and that this depletion is accentuated in individuals expressing the Lu[b] antigen. This depletion is an effect of HIV infection since it was not observed in the uninfected population, and probably involves the particular role of the HIV-Tat protein's enhancement of vascular adhesion and trans-endothelial migration of monocytes reported by other investigators (Lafrenie et al., 1996). HIV-infected monocytes are known to migrate more efficiently across vessel wall while maintaining their infectivity (Dhawan et al., 1992). We conclude that the Lu[b] blood group contributes to the depletion of circulating phagocytes, possibly by enhancing their margination and extravasation (Oh et al., 2009b).

Working from the established fact that cholesterol, especially LDL-C, promotes adhesion of phagocytes (Oh et al., 2009a, Frostegard et al., 1990), with possible reduction of peripheral counts, we noted that in HIV infection, even normal cholesterol levels are associated with diminishing circulating phagocytic cells. This could be due to the chronic inflammatory status occasioned by HIV infection. It appears, therefore, that HIV-infected individuals are at higher risk of developing cholesterol-related monocytopenia and neutropenia, which, in the presence of increased non-HDL cholesterol, points to the peculiar vulnerability of HIV-infected patients to cardiovascular diseases. Although these observations were generally expected considering that cholesterol promotes tethering of phagocytes to the endothelium (Oh et al., 2009b, Dhawan et al., 1992), it was interesting that only in HIV infection was the phagocyte-depleting effect of cholesterol significant.

When cholesterol components were examined, non-HDL-C negatively affected phagocyte counts consistently, especially the monocyte counts, which finding corroborated previous reports by (Badiou et al., 2008) linking non-HDL-C to atherosclerosis in HIV infection.

Conclusion

Our findings suggest that the risk of cardiovascular disease in HIV-1-infected subjects may in part be due to an interplay between cholesterol, phagocytes and Lu^b-mediated adhesion, all of which tend to recruit phagocytes out of circulation. While a genetic predisposition for atherogenesis has always been alluded to, our results suggest the Lu^b adhesion molecule to be a potentially important genetic link. The results of this study also imply a need to manage cholesterol in HIV-infected patients to achieve lower levels than the general public, especially for those who are Lu^b-positive. Moreover, the vulnerability occasioned by the Lu^b-positive status may provide opportunity for personalized care in Lu^b-positive, HIV-infected individuals.

A limitation of our study was that we did not directly measure phagocyte migration but rather, implied phagocyte migration from diminished peripheral cell counts. Future studies need to focus on direct migration measurements of phenotyped phagocytes.

Competing interests:

The authors declare that they have no financial or personal relationship(s) that may have inappropriately influenced them in writing this article.

Author Contributions

MSM: Conceived the idea, carried out the experiments, analysed results and drafted paper. OO and IK provided experimental guidance, participated in data analysis, critiqued the content and gave final approval of the version to be published.

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CHAPTER FIVE

RHCE*C PHENOTYPE MAY EXPLAIN THE GEOGRAPHICAL DISTRIBUTION OF HIV INFECTIONS

Short Title: RHCE*C and HIV susceptibility

AUTHORS:

Motswaledi, Modisa Sekhamo[1], MS, MT(ASCP)

Kasvosve, Ishmael[2], PhD

Oguntibeju, Oluwafemi Omoniyi[3], PhD

AFFILIATIONS:

[1] Senior Lecturer, Department of Medical Laboratory Sciences, University of Botswana, Gaborone, Botswana, Motswaledims@ub.ac.bw

[2] Professor, Department of Medical Laboratory Sciences, University of Botswana, Gaborone, Botswana.

[3] Professor, Biomedical Science, Cape Peninsula University of Technology, Cape Town, South Africa, OguntibejuO@cput.ac.za.

<u>**CORRESPONDING AUTHOR DETAILS**</u>

Name: Oluwafemi Omoniyi Oguntibeju

> Department of Biomedical Sciences, Faculty of Health & Wellness Sciences, Cape Peninsula University of Technology, PO Box 1906, Cape Town, South Africa. 7535

Phone: +277-11400428

Email: OguntibejuO@cput.ac.za

Fax: +272-1 953 8490

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Abstract

BACKGROUND: Sub-Saharan Africa and the Caribbean countries bear a disproportionate burden of the HIV pandemic. This suggests a genetic predisposition arising from a common ancestry. We previously reported a 40% HIV risk-reduction associated with RHCE*C blood group. The current study sought to elucidate potential mechanisms for this risk reduction.

MATERIALS AND METHODS: Lymphocyte sub-populations and viral load measurements were achieved by routine diagnostic laboratory methods in 102 untreated, HIV-1 infected patients. Results were compared across categories of RHCE*C blood group.

RESULTS: Carriage of the RHCE*C antigen was associated with a higher proportion of T-cells ($82\pm8\%$ versus $75\pm10\%$, p=0.001), especially single-positive ($CD8^+$) cytotoxic T-cells (64 ± 14 versus $54\pm13\%$, P=0.004). High absolute CD8 counts were more prevalent among C-positive than C-negative subjects (90% versus 65%, p=0.023). Among C-positive subjects, the increase in CD8 count paralleled viral load compared to C-negatives ($r^2=0.630$, p<0.0001 versus $r^2=0.148$, p<0.001, respectively). Moreover, examination of data involving 20 countries or regions indicated that high HIV prevalence occurred only in those countries with RHCE*C frequencies lower than 30% ($\chi^2=20.0$, p<0.0001).

DISCUSSION: Results suggest that RHCE*C enhances CD8 counts in HIV infection, which cells are known to play a vital role in immunity against HIV. Invariably, the only geographical locations with high HIV prevalence rates are those where the frequency of this antigen is low. Results provide a possible genetic explanation for the worldwide distribution of the HIV pandemic, with a special emphasis on the vulnerability of individuals of African descent, where the prevalence of RHCE*C is consistently low across many countries.

Keywords: HIV-prevalence; RHCE*C / RHCE*C; CD8 count; CD4 count; viral load; African.

Introduction

HIV prevalence in Botswana is among the highest in the world (Weiser et al., 2006). Many studies have revealed risk factors that have contributed to the spread of the virus, some of which include poverty, multiple sexual partners, alcohol and drug abuse as well as improper use of condoms (Keetile, 2014). Excessive alcohol consumption was reported as the greatest risk since it promoted risky behaviours in general (Weiser et al., 2006). In some studies, the risk of infection in women, especially in Botswana and Swaziland, was reported to be due in part to cultural norms that predispose women to sexual abuse or assign them to lower economic status (Shannon et al., 2012).

The high prevalence of HIV in Africa as well as those communities comprising of people of African descent, such as the Caribbean strongly suggests a common genetic link. However, few such links have been alluded to. In at least two studies, the Duffy antigen receptor for chemokines, was reported to promote HIV infection (He et al., 2008, Lachgar et al., 1998) among Africans. However, these reports were strongly refuted by other investigators (Winkler et al., 2009). In yet another study, the P^k blood group was reported to protect against HIV infection (Lund et al., 2009). However, this antigen is rare among Africans (Cooling, 2014). While it is generally accepted that host genetic factors play a role in immunity against the virus (Chatterjee, 2010), no specific hereditary factors have been advanced to explain the pandemic in Africa.

Although the function of many blood groups remains unknown, some reports have demonstrated that erythrocytes bind HIV and that such HIV becomes more efficiently transferred to CD4+(Beck et al., 2009, Beck et al., 2013, Garcia et al., 2012) cells. Erythrocyte antigens are therefore logical targets for erythrocyte-virus interactions. In our previous work, we reported a 40% risk reduction for HIV-1 infection in individuals of the RHCE*C blood group (also denoted as *RHCE*C), while blood groups P_1 and Lu^b were associated with double and triple risks, respectively (Motswaledi et al., 2016). Of epidemiological importance was the observation that this protective blood group was very rare among Africans, while the risk-associated blood groups were much more common, raising the question of whether this antigenic profile could have contributed to the peculiar susceptibility of this population to infection with HIV-1.

RHCE*C is a component of the Rh blood group system. Antigens in this system are inherited in strong linkage disequilibrium on chromosome 1. The RHD gene codes for the D antigen while the RHCE gene caries a polymorphism that leads to the production of a range of RH phenotypes that include CE, Ce, cE or ce (Ripoche et al., 2004). The Rh gene products are organized on the erythrocyte membrane as a complex of proteins that include the Rh-associated glycoproteins (RhAG), LW, CD77, Duffy and CD47, which serve as ammonia transporters (Ripoche et al., 2004, Anstee and Tanner, 1993, Pourazar, 2007).

In the current study, we performed viral load, CD4 and CD8 counts and compared them among patients expressing or negative for RHCE*C. From these results and the review of available literature, we sought to find out if the observed risk reduction for HIV infection could be corroborated by clinical laboratory data.

Materials and methods

One hundred and twenty (120) HIV-1-infected (48 males and 72 females) treatment-naïve (self-reported) individuals were enrolled in the study. Since, no drug tests were done to verify their treatment-naïve status, 19 subjects with undetectable viral load were excluded on the presumption that they could be on treatment.

We used anonymized EDTA-anticoagulated samples from the Botswana-Harvard HIV Reference Laboratory in Gaborone. Ethical clearance was obtained from the University of Botswana's Office of Research and Development, Human Research and Development Committee of the Ministry of Health and Wellness, Gaborone District Health Management Team and the Research Ethics Committee of the Faculty of Health and Wellness Sciences at Cape Peninsula University of Technology. Individual consent was not needed since the study used anonymized, residual samples (ISO, 2007).

The viral load was measured using the Cobas® Taqman 48 analyser (Pleasanton, CA., USA). CD3, CD4, CD8 and CD45 counts obtained using the FacsCalibur ® flow cytometer (Becton-Dickinson, San Jose, CA, USA). The samples were further phenotyped for the C antigen using specific anti-C and anti-c antibodies (Fortress Diagnostics, Antrim, UK). The reactivity of the antisera was confirmed with C-positive and negative cells selected from an antibody identification panel, Bio-Rad DiaPanel®, Lot 45241.88.1, (Cressier FR, Switzerland).

Results of laboratory tests were interpreted in line with the reference ranges previously determined for the Botswana population (Mine et al., 2011).

The results were analyzed using the IBM SPSS version 24 statistical software. Correlations were used to study the effect of blood RHCE*C on the relationship between CD8%, log viral load, CD8% and CD4%. Results were considered significant only if $P<0.05$.

Results
Results from experimental data

The mean viral load did not differ between individuals positive (n=21) or negative (n=81) for C (p=0.398). In untreated, HIV-1-infected subjects, the CD4 count was significantly lower than that of the general uninfected population. On the contrary, the absolute CD8 count was significantly higher in the infected than uninfected subjects. The results are shown in Table 5.1.

Table 5.1: Comparison of CD4 and CD8 in untreated, HIV-infected individuals and normal controls.

Lymphocyte population	Mean in HIV-infected Subjects (n=101)	Mean in normal population (n=261)	P-value
CD4$^+$	307±208	859*	<0.0001
CD8$^+$	927±492	540*	<0.0001

*One sample (T-test) mean used from a previous study(Mine et al., 2011).

In individuals expressing the RHCE*C antigen, 19/21 had a high absolute CD8 count compared to 52/80 who were RHCE*C-negative. A high CD8 count was therefore more associated with C-positive individuals than C-negative (90% versus 65%, p=0.023). The mean CD8 count between the two categories of RHCE*C, though higher in the C-positive group, did not reach statistical significance. However, the proportion of T-lymphocytes (CD3$^+$) and cytotoxic (CD8$^+$) T-cells was higher in the C-positive population as reflected in Table 5.2.

Table 5.2: Comparison of mean Lymphocyte sub-populations in untreated HIV-infected patients according to RhC phenotype. The RhC phenotype was associated with higher CD8 counts.

Lymphocyte Sub-population	C-Positive (Mean±SD) n = 21	C-Negative (Mean±SD) n = 80	*P-value*
CD3+ (%)	82±8	75.0±10	0.001
CD8+ (%)	64±14	54±13	0.004
CD8 Absolute count (/µL)	1058±444	893±500	0.171

Among C-positive subjects, the increase in CD8 counts paralleled viral load more strongly than in C-negatives (r^2=0.630, p<0.0001 versus r^2=0.148, p<0.001, respectively). These results are shown in Fig 5.1.
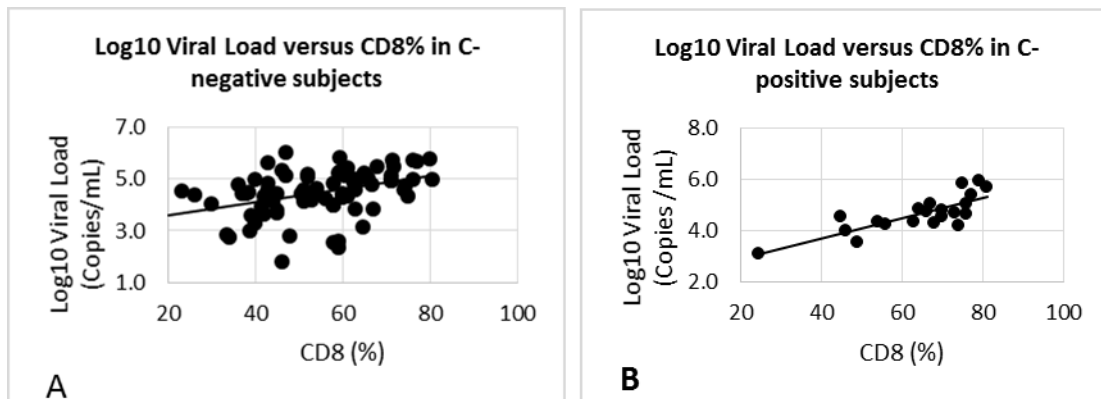
Figure 5.1: Relationship between viral load and CD8% in C-positive subjects shows strong positive correlation (A: r^2=0.148, p<0.001, versus B: r^2=0.630, p<0.0001)

Results from country HIV prevalence data

A literature search was also undertaken to investigate HIV prevalence in countries where the frequencies of the RHCE*C are known. Data was obtained for 20 countries or communities as shown in Table 5.3. In brief, a high HIV prevalence in any country or community was associated with an RHCE*C frequency less than 30% (Pearson χ^2=20.0, p<0.0001).

Table 5.3: Comparison of RHCE*C with HIV prevalence across geographical regions

Country/Community	RHCE*C Frequency	HIV Prevalence
Botswana	29.70	21.90
South Africa (Blacks)	18.80	18.90
Nigeria	28.2	2.9*
Cote d'Ivoire	21.97	2.70
Caribbean	24.50	1.60**
Uganda	3.62	6.5***
Thailand	51.50	1.10
Ethiopia (Blacks)	41.18	1.10
South Africa (Asians)	99.53	1.00
Mali	58.8	1.00
Brazil	63	0.6
Mauritania	42.69	0.5
Argentina	62	0.4
Laos	60.30	0.30
Southern India	88.00	0.30
Northern India	84.76	0.26
Sudan	58.40	0.20
Netherlands	68.00	0.20
Iran	75.90	0.10
China	78.22	0.04

*5.8% in 2001. Some states at some point exceeded 8%, and current rates may only reflect the effect of treatment. Nevertheless, Nigeria carries the world's heaviest HIV burden (Gwaram, 2013).

**Prevalence rates range from 1-5% for different islands, depending on the reporting dates (Coggins, 2006, UNAIDS, 2016a). RHCE*C information for Curacao used here.

***Prevalence is down from 24.8% in 1994 (Kilian et al., 1999)

Discussion
RHCE*C, CD8 Parameters and protection against HIV infection

This study sought to establish the mechanism of protection provided by carriage of RHCE*C observed in a previous study. In this study, expression of this antigen was associated with a higher proportion of cytotoxic (CD8+) T-cells (CTL). In C-positive subjects, the CD8 absolute count varied in direct proportion to the viral load, suggesting a coordinated response to viremia. This correlation was much weaker in the C-negative group. Moreover, the proportion of individuals with high CD8 counts was greater in the C-positive group. All these observations suggest a role for this antigen in cytotoxic T-cell kinetics and function, and may therefore

explain the protective role of this blood group observed in our previous study (Motswaledi et al., 2016). Our findings are also consistent with recent findings in South Africa where the odds of HIV infection were reported to be nil for individuals who are homozygous for RHCE*CC (Davison et al., 2018). This prompted us to revisit a previous dataset involving 544 subjects. In this dataset only 6 individuals were homozygous for the C antigen and none of them were infected.

CTLs are important in the control of viremia in acute HIV-1 infections (Goonetilleke et al., 2009). They also correlate negatively with infection rates(Oxenius et al., 2004) and with good prognosis (Rinaldo et al., 1995). Moreover, they have been linked to slower progression of disease in non-progressors by producing HIV-1-specific CTL responses (Betts et al., 2001, Alimonti et al., 2006) and increased perforin production in HIV-infected non-progressors (Migueles et al., 2002).

The enhancement of CTLs points to a mechanism involving major histocompatibility class I (MHC-I) response, in which a cell-mediated rather than humoral response is invoked, and this kind of response is critical for viral infections such as HIV. This phenomenon appears to be enhanced when the RHCE*C antigen is expressed on erythrocytes. Erythrocytes and erythrocyte-bound platelets harbor HIV virions (Beck et al., 2013). In this connection, platelets have been shown to engage in direct MHC-I antigen presentation to CTLs (Chapman et al., 2012), a phenomenon that has also been documented in murine megakaryocytes (Zufferey et al., 2015). The RHCE*C phenotype appears to enhance this process, possibly by engaging its associated CD47 as the activator of CTLs (Seiffert et al., 2001).

Antigen presentation is normally accompanied by co-stimulatory responses that enhance clonal expansion of the specific CTL (Watts, 2010). We propose therefore, that the virus-platelet-erythrocyte-T-cell interaction may explain the connection between RHCE*C and its protective role against HIV. Our results further suggest that the presence of C, rather than the other Rh antigens associated with CD47, may be responsible for the enhanced CTL response.

CD47 is the one erythrocyte antigen most commonly associated with T-cell activation events (Reinhold et al., 1997, Ticchioni et al., 2001, Seiffert et al., 2001, Pettersen et al., 1999). This glycoprotein is widely expressed on somatic cells including erythrocytes(Ripoche et al., 2004), a characteristic which makes the direct interaction of HIV-bearing erythrocytes with T-cells and phagocytes probable.

CD47 also interacts with its ligands on monocytes and T-lymphocytes in signal-transducing events. In this regard, it binds to signal regulatory protein-α (SIRPα) in a high-affinity interaction that results in T-cell activation(Seiffert et al., 2001). Binding of CD47 to SIRPβ2 enhances adhesion of T-cells to antigen-presenting cells and therefore enhances cell-mediated immunity(Piccio et al., 2005). In activated T cells, such as occur following HIV infection, CD47 promotes Fas-mediated apoptosis(Manna et al., 2005). All these events would work to eliminate HIV-infected CD4+ cells and therefore minimize the chances for an infection to be established.

Evidence from empirical HIV prevalence data

To further investigate its potential role in the epidemiology of HIV worldwide, we compared the frequency of the RHCE*C antigen across populations with varying degrees of HIV prevalence. Invariably, countries with an antigen frequency lower than 30% were consistently associated with highest prevalence rates in their regions. In Southern Africa, antigen frequency data was available only for Botswana (Motswaledi et al., 2016) and South Africa (Tax et al., 2002). Cote d'Ivoire and Nigeria (Gwaram, 2013) likewise had the lowest antigen frequencies (Bogui et al., 2014) and highest HIV prevalence in West Africa (UNAIDS, 2016a). In contrast, other African countries with higher antigen frequencies had low HIV-prevalence rates comparable to or slightly above other non-African countries, such as Argentina (Cotorruelo et al., 2008), Brazil (Guelsin et al., 2011), Thailand (Nathalang et al., 2001), Laos (Keokhamphoui et al., 2012), India (Makroo et al., 2013), Netherlands (Tax et al., 2002), Iran (Shokouhi Shoormasti et al., 2011) and China (Ma et al., 2018), where the RHCE*C frequency is higher than 40%. These African countries included Mali (Ba et al., 2015), Mauritania (Hamed et al., 2013), Ethiopia (Tax et al., 2002), Sudan (Elfadni et al., 2014) and South Africans of Asian origin (Tax et al., 2002).

Of further note and interest was the Caribbean statistics. The Caribbean Islands represent the second highest HIV-prevalence outside Africa (UNAIDS, 2016b). Apparently, a significant proportion of this population originates from Africa and the RHCE*C antigenic profile performed in Curacao (Tax et al., 2002) is similar to that of the African countries where high HIV rates are experienced. We propose that the RHCE*C antigen is important for HIV immunology and probably explains the genetic basis for the geographical distribution of the epidemic.

Conclusion

The results of this study indicate that high HIV rates occur only in geographical locations of low RHCE*C frequencies. The lack of this antigen in Botswana and countries with similar frequencies may have contributed to the rapid spread of HIV-1 in these populations. Results also suggest an immunological role of an Rh system antigen, unreported hitherto.

Acknowledgements

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CHAPTER SIX

HIV-1 RNA IS DETECTABLE IN P_1 ERYTHROCYTE LYSATE

Modisa Sekhamo Motswaledi, MS[1,2], Ishmael Kasvosve, PhD[1], Oluwafemi Omoniyi Oguntibeju, PhD[2]

[1] Department of Medical Laboratory Sciences, University of Botswana Faculty of Health Sciences, Block 246 Room A215. Phone +267-355-2572, Fax: +267-307-4538). Email: modisa.motswaledi@mopipi.ub.bw (Co-corresponding author), ishmael.kasvosve@mopipi.ub.bw, [2] Department of Biomedical Sciences, Faculty of Health & Wellness Sciences, Cape Peninsula University of Technology, PO Box 1906, 7535, Bellville, South Africa. Fax: +2721 953 8490. Email: OguntibejuO@cput.ac.za (corresponding author).

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Abstract

BACKGROUND: Many studies have implicated erythrocytes in HIV-1 pathogenesis and as viral reservoirs. We previously reported a high risk for HIV infection associated with P_1 blood group.in Africans. P_1 belongs to a group of glycolipids known to facilitate viral membrane fusion. The aim of this study was to investigate the possibility of HIV RNA inside P1 erythrocytes.

METHODS: Viral load measurements were made in 120 untreated, HIV-1 infected patients. Qualitative PCR (n=27) was done on purified erythrocytes to detect erythrocyte-associated HIV-1 RNA. Results were then compared across categories of P_1.

RESULTS: Viral RNA was detected in both lysate and membranous fraction of purified erythrocytes, including those with undetectable plasma viral load. Plasma viral load did not differ between individuals positive or negative for P_1. However, purified P_1-positive erythrocyte lysate contained more viral RNA (Mann-Whitney U-test, P= 0.032). Erythrocyte-associated RNA was independent of plasma VL.

CONCLUSIONS: The higher level of intra-erythrocytic RNA in P_1 subjects is a novel finding consistent with viral fusion facilitated by glycosphingolipids, possibly accounting for the observed risk of infection associated with P_1. The study also suggests a new viral reservoir hitherto undescribed.

Key words: HIV-1 RNA; P_1 blood group; viral load; glycosphingolipids, erythrocytes.

Introduction

The P-blood group system is comprised to three antigens – P^k, P and P1. The antigens are glycosphingolipids (GLS) built by addition of sugars to a galactosyl ceramide, the basic sphingolipid backbone(Hellberg, 2007). The P antigen is derived by addition of sugars to the P^k antigen. The P_1 antigen synthesis follows a separate pathway resulting in a more complex structure carrying more glycosyl residues(Spitalnik and Spitalnik, 1995). In blood transfusion, the P_1-blood may occasionally cause transfusion reactions(Spitalnik and Spitalnik, 1995), although most of the cases are generally of the cold-reacting IgM class and therefore clinically insignificant. This blood group occurs among individuals of African descent with high frequency but relatively rare among Caucasians(Hellberg, 2007).

Seemingly contradictory reports have been published regarding the role of the P^k antigen with respect to predisposition to HIV infection. In some reports, GLS were reported to promote HIV membrane fusion to susceptible CD4 cells (Hammache et al., 1999, Puri et al., 1998a). In particular, the P^k blood group was thought to enhance infection with X4 viruses while P enhanced R5 strains(Puri et al., 1998a). In other reports, lipid rafts comprising of GSL and cholesterol were considered to be sufficient for viral fusion without the need for co-receptors(Yang et al., 2015, Rawat et al., 2006).

In a contrary report, the P^k blood group was reported to be protective against HIV infection in vitro(Lund et al., 2009). This perspective was further strengthened by the observation that patients with Fabry Disease, in which an intracellular accumulation of the P^k antigen occurred, were resistant to HIV infection (Lund et al., 2005, Ramkumar et al., 2009). A possible explanation of this apparent contradiction was that optimal amounts of P^k are required for viral fusion. However, excess quantities of GSL protects the cell from infection by competing for the virus and thus preventing it from effectively fusing to the cell membrane(Lund et al., 2009).

To date, no reports specifically linking the P_1 antigen to the risk of HIV infection have been found. However, a number of other reports have demonstrated that HIV directly interacts with RBC, some via glycosphingolipids, and this interaction may be important in the epidemiology and pathogenesis of the virus (Beck et al., 2009). For example, in 2002, Hess and colleagues reported some HIV-1 to be associated with erythrocyte membranous ghosts in about 98% of HIV-1-infected patients (Hess et al., 2002). The erythrocyte-associated HIV was detectable in one third of the patients in whom plasma viral load was undetectable. The erythrocyte-associated HIV in some of the patients exceeded that associated with leukocytes and was associated with advanced clinical stages of the disease. Subsequent reports by Fierer and colleagues (2007), however, strongly disputed the presence of erythrocyte-associated HIV in patients with long term viral suppression while acknowledging it in those who have not achieved viral suppression .

The binding of the virus to the erythrocytes has been attributed to a number of mechanisms, including the use of complement receptors normally used for clearance of antigen/antibody (immune) complexes(Horakova et al., 2004). Although some of the virus was bound to erythrocytes via the normal mechanisms of immune-complex clearance using complement receptor 1 (CR1), these investigators also noted that the virus also bound to erythrocytes via a

non-complement receptor pathway, suggesting that molecules other than complement may be operational in this association(Horakova et al., 2004).

In 2012, Garcia and colleagues (2011) reported the presence of anti-gp160/120 antibodies on erythrocyte surfaces. In addition, they documented that such antibodies on the red cells were responsible for capturing free plasma viral particles and may account for reduction of plasma viral load. This followed their earlier report in which viral p24 was detected on erythrocytes even in patients with undetectable plasma viral load (Garcia et al., 2011).

In addition to erythrocytes, P_1 antigen is also expressed on lymphocytes (Pourazar, 2007) and therefore presents a theoretical possibility of heightened risk of HIV-infection for subjects expressing the P_1 antigen.

In the current study, we investigated the possibility of the virus being present inside erythrocytes solely on the basis of the fusogenic property of the P_1GSL. . If found inside the erythrocytes, we also sought to find out if this was dependent on plasma viral load.

Methods

The study enrolled120 residual blood samples from HIV-1-infected individuals, (48 males and 72 females) prior to initiation of highly active anti-retroviral therapy at Julia Molefe Clinic in Gaborone, between May and June 2016.

. The study was approved by the University of Botswana's Office of Research and Development, Human Research and Development Committee of the Ministry of Health and Wellness, Gaborone District Health Management Team and the Research Ethics Committee, of the faculty of health and Wellness Sciences, Cape Peninsula University of Technology (Appendix A-D). Individual consent was not needed since the study used anonymized, residual samples(ISO, 2007).

The viral load was determined by use of the Cobas® Taqman 48 analyser (Pleasanton, CA., USA), while the RNA qualitative test for HIV-1 was performed using the COBAS® Ampliprep/COBAS® Taqman HIV-1 qualitative test, version 2.0 (Pleasanton, CA., USA). The samples were further phenotyped for using anti-P_1 antibody (Fortress Diagnostics, Antrim, UK) according to the manufacturer's instructions. The reactivity of the antiserum was confirmed by use of P_1-positive and P_1-negative erythrocytes selected from an antibody identification panel, Bio-Rad DiaPanel®, Lot 45241.88.1, (Cressier FR, Switzerland). CD4, CD3, CD8 and CD45 counts were enumerated using the FacsCalibur ® flow cytometer (Becton-Dickinson, San Jose, CA. USA).

Viral RNA detection was performed on purified erythrocytes. To achieve this, 1 ml of the packed red cells was diluted with an equal volume of normal saline and then overlaid on 14ml of ficoll, followed by centrifugation at 400xG for 30 minutes. The suspended leukocyte layer was removed by aspiration together with all the saline and ficoll above the erythrocytes. The remaining cells were again diluted in 1 ml normal saline and subjected to a second round of ficoll gradient centrifugation. The cells were then washed three times with 14 ml of normal saline. The supernatant of the last wash was tested for viral RNA or DNA using the COBAS®

AmpliPrep/COBAS® Taqman® (Pleasanton, CA., USA) and none was detectable, indicating a successful removal of unbound viral nucleic acids.

A sample was reconstituted for CD45 testing by adding equal volumes of the purified packed red cells and normal saline to bring the haematocrit to 41.3±1.8%. The CD45 determination was repeated to assess white cell depletion and a reduction of more than 97% after the first and over 99% after the second purification was achieved.

Subsequently, the purified cells were lysed with 1 ml of distilled water and allowed to lyse on a rotary mixer for 5 minutes before being centrifuged at 1000xG for 3 minutes to pellet erythrocytic ghosts and any virus that might have been attached to them. The top 500μL of the RBC lysate was removed and tested for viral nucleic acids as described above, to demonstrate the presence, if any, of intra-erythrocytic viral RNA in the lysate.

The remainder of the sample was mixed by vortexing and viral RNA was also determined, which would represent the membrane-bound RNA plus that from the residual hemolysate. The number of PCR cycles taken to show a positive reaction in the membranous fraction or lysate was used as a proxy for viral concentrations in the two fractions against an internal standard. The median difference in cycles to reach a positive reaction were compared according to P_1-carriage for statistical significance (number of cycles for lysate minus the number of cycles for stroma). For the purposes of comparison in this study, a high viral load in HIV-infected patients was defined as any value above $Log_{10}VL=4.97$, the viral set-point previously determined for this population. The results were analysed using the IBM SPSS version 24 statistical software.

Results

The viral load and lymphocyte populations did not differ between individuals positive or negative for P_1. However, the median number of additional cycles required to reach a positive reaction for erythrocyte lysate-associated viral RNA was smaller for P_1-positive individuals than for the P_1-negative (Mann-Whitney U-Rank-Sum test, P=0.032), indicating a higher intracellular content of viral RNA in P_1-positive erythrocytes. The data is presented in Table 6.1.

Table 6. 1 - Comparison of RNA-PCR results in untreated HIV-1-infected patients.

Blood Group (n=10)	No of PCR Cycles	
	Median	IQR
P_1-positive	0.9	0.3
P_1-negative	4.5	3
P-value	0.032	

Erythrocyte-associated RNA (E-RNA) was estimated in 27 patients and was detectable in 22 (81%) and undetectable in 5. Of these 5, only one had undetectable plasma viral load (pVL). On the other hand, E-RNA was demonstrable in 6 patients who had undetectable pVL. Of the 22 with demonstrable E-RNA, 10/22 (45%) demonstrated the virus on both the stroma and

lysate. Most of the E-RNA was associated with the stroma than the lysate. Only one sample yielded more RNA from the lysate than the stroma.

We also compared the prevalence of HIV across countries for which P1 frequencies were known. The results are shown in Table 6.2

Table 6.2: Comparison of P_1 frequency with HIV prevalence in various countries showing that high P_1 frequency is associated with high HIV prevalence

Country	P_1 frequency (%)	HIV Prevalence (%)	
Botswana	74.6	21.9	
Uganda	89	6.5	
Laos	18.97	0.3	
Turkey	0.5	*	
Malaysia	40	0.4	
Thailand	30	1.1	

*Data for Turkey not available, but official reports suggest low prevalence (Ay and Karabey, 2006)

Discussion

The presence of HIV virions on erythrocytes and erythrocyte-bound platelets has been documented by various reports (Beck et al., 2013, Hess et al., 2002, Garcia et al., 2011). However, some investigators have refuted the presence of erythrocyte-bound HIV in virally suppressed patients(Fierer et al., 2007). The current study confirms the presence of viral RNA from purified erythrocytes in patients with undetectable plasma viral load. The detection of intra-erythrocytic viral RNA is another novel finding hitherto unreported.

Although the P_1 positive and P_1-negative groups did not differ with respect to plasma viral load, the PCR analysis of the erythrocyte lysate revealed the presence of more intra-erythrocytic viral RNA in P_1-positive than negative samples. This observation is consistent with the role of glycosphingolipids in enhancing viral fusion (Hammache et al., 1999, Puri et al., 2004, Puri et al., 1998b, Puri et al., 1998a) and probably explains the increased risk of HIV infection observed in P_1-positive individuals (Motswaledi et al., 2016). P_1 has a wide tissue distribution (Hellberg et al., 2013), including its expression on HIV-susceptible cells such as monocytes and lymphocytes (Dunstan, 1986, Pourazar, 2007). Our results corroborate the findings of Yang et al. (2015) who reported that lipid rafts, which are rich in sphingolipids are "necessary and sufficient for membrane targeting and fusion" in human immunodeficiency virus infection

Erythrocyte-associated HIV (E-RNA) was demonstrable in the majority of patients with or without detectable plasma viral load (pVL), consistent with other reports (Beck et al., 2009, Hess et al., 2002, Garcia et al., 2012, Garcia et al., 2011). However, E-RNA could not be demonstrated in some samples with detectable or even high pVL, suggesting that the association of the virus with erythrocytes is not simply a function of plasma viral load. The

detection of viral RNA in erythrocyte lysate suggests alternative pathways for viral entry into cells independent of CD4+ as suggested by other investigators (Garcia et al., 2012). However, this analysis was limited by the relatively small sample size for intra-erythrocytic viral RNA.

Our results suggest that the risk of infection associated with P_1 potentially emanates from its ability to promote viral fusion. Furthermore, the presence of viral RNA in erythrocyte lysate suggests an additional viral reservoir that may need to be addressed for successful eradication of HIV infection. This blood group has a high frequency among individuals of African descent and may have contributed to the high infection rates in this population.

We also looked at HIV prevalence among countries for which P1 frequencies were known. Although data was only available from five countries, the only country with a high prevalence rate was also the only country with a high P1 frequency. The presence of P_1 can therefore not be excluded as a contributing factor to HIV prevalence in those countries.

Conclusion

We report for the first time, the occurrence of HIV RNA in the lysate of erythrocytes. This phenomenon is independent of plasma viral load and potentially explains a GSL-based, receptor-independent entry of the virus into those cells possessing adequate levels of membrane GSL. Furthermore, The low frequency of P1 in countries with low HIV prevalence might corroborate our findings that P1 carriage increases vulnerability to HIV infection.

Authors' disclosure

The authors declare that there is no conflict of interest in this study.

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GENERAL DISCUSSION AND CONCLUSION

Aims and Objectives

The aim of this project was to study the relationship between blood groups and HIV-infection. HIV infection in Botswana has not been linked to particular blood groups. In fact, the distribution of blood groups other than ABO in Botswana is not known. Furthermore, very few studies elsewhere have addressed this matter and the few that have been done only looked at ABO and Rh(D) blood groups. This is hitherto the most exhaustive study of the role of various blood groups in HIV pathology and epidemiology. The study therefore aimed to:

1. Determine the frequencies of blood groups in the Botswana population with respect to the following antigens among uninfected controls and HIV-infected individuals: A, B, "O", D, C, c, E, e, Fy^a, Fy^b, Jk^a, Jk^b, Kp^a, Kp^b, Le^a, Le^b, Lu^a, Lu^b, M, N, P_1, S, and s.
2. Compare the occurrence of blood groups in HIV-infected versus uninfected individuals.
3. Determine, where possible, the underlying mechanisms.

The frequency of the selected blood groups in Botswana were determined and found to be compatible with documented frequencies for Africans in general. The occurrence of HIV infection in these blood groups was also compared and the antigens Rh2, Lu^b and P_1 were found to be associated with infection as detailed below.

ABO Blood Groups

Many studies have reported a relationship between the risk of HIV-infection and some blood groups (Abdulazeez et al., 2008, Anstee, 2010, Branch, 2010, Neil et al., 2003). In one study, naturally-occurring ABO antibodies were reported to neutralize HIV from lymphocytes of group A blood donors (Arendrup et al., 1991). According to this report, it would be expected that ABO antibodies should offer some level of protection against HIV across ABO blood groups (Neil et al., 2003, Neil et al., 2005). However, there is no consensus on the role of ABO blood groups with respect to the risk of HIV infection (Nneli et al., 2004, Abdulazeez et al., 2008).

We compared the prevalence of 23 blood group antigens in HIV-infected and uninfected individuals in Botswana. Our study did not find any relationship between risk of HIV-infection and any ABO blood group. In particular, none was found for blood group O, which produces both anti-A and anti-B, and should be expected to be least susceptible to HIV-infection if these conferred any level of protection. It is noted, however, that blood group O is the most prevalent among the subjects of the current study, and therefore infections between group O individuals would not be affected by presence of naturally occurring anti-A or anti-B. Reports concerning susceptibility of ABO blood groups to HIV infection therefore vary extensively. Considering the inadequate statistical analysis employed in some of the studies that reported such a

relationship (Ukaejiofo and Nubila, 2006, Sayal et al., 1996, Nneli et al., 2004), it is probably safe to conclude that no data exists to support the role of ABO blood groups in affecting the outcome of HIV exposure.

Blood Group C (rh', Rh2 or RHCE*C)

Of all the antigens studied, the blood groups C (Rh2) and E (Rh3) in the Rh system were associated with a reduced risk. However, with rigorous statistical analysis, the carriage of blood group E did not sustain significance as a protective surface molecule. We attributed this initial significance to its simultaneous co-expression and co-localization with C (Ripoche et al., 2004). Blood group C was associated with a 40% risk reduction for HIV infection. The association of this blood group to potentially-enhanced cellular cytotoxicity is a novel finding in this current study which has not been reported elsewhere in the literature. Other than its association to urea transporter proteins, the function of group C antigens is unknown. We provide here a potential immune functional role of an antigen in the Rh blood group system.

Further analysis revealed that the frequency of a high absolute CD8 count among HIV-infected patients was higher among C-positive subjects, indicating a heightened cell-mediated cytotoxicity. This is the major mode by which the immune system deals with cells that present non-self-antigens such as virally-infected or cancer cells (Gulzar and Copeland, 2004, Migueles et al., 2002). The evidence gleaned from this study suggests that the protective effect of blood group C emanates from its ability to eliminate the infection via cell-mediated immunity.

This immune function, hitherto unreported, is likely associated with CD47, a glycoprotein that is spatially associated with Rh antigens on the erythrocyte membrane (Dahl et al., 2003) and functions to enhance antigen presentation (Piccio et al., 2005) and cytotoxicity (Seiffert et al., 2001, Ticchioni et al., 2001). It is likely that the C-antigen accelerates the effectiveness of CD47, since other Rh antigens that are known to be spatially associated with CD47 (E, e, c, Duffy) did not show any evidence to suggest a protective role.

It is noted, however, that Rh antigens are expressed primarily on erythrocytes (Anstee and Tanner, 1993), and that erythrocytes are not considered immunologically active cells. This notwithstanding, it is an established fact that erythrocytes bind HIV by various means (Beck et al., 2013, Garcia et al., 2012, Hess et al., 2002, Horakova et al., 2004). HIV also binds to other cellular components such as platelets (Beck et al., 2013), and these antigens are presented to cytotoxic T-cells via MHC class I restriction (Chapman et al., 2012, Zufferey et al., 2015) to provoke a cell-mediated immune response. Erythrocytes and platelets are the most numerous blood cells. The amount of HIV bound to these components by various mechanisms is not only substantial, but erythrocytes preferentially capture the infectious virions (Beck et al., 2009, Beck et al., 2013) and present these via MHC class I restriction (Chapman et al., 2012, Zufferey et al., 2015). Dendritic cells also present exogenous viral antigens by a special cytotoxic MHC-I pathway (Buseyne et al., 2001, Moris et al., 2004). However, since Rh antigens are not expressed on leukocytes, the anti-viral mode associated with Rh(C) is more attributable to erythrocyte associated virions, where they are captured and concentrated on the erythrocyte membrane (Beck et al., 2009). Moreover, since erythrocytes bind the most infective form of

the virus (Beck et al., 2013, Beck et al., 2009), and then subject it to cell-mediated cytotoxicity (Alter et al., 2002, Buseyne et al., 2001), it is plausible that a blood group that facilitates cell-mediated immunity would prevent establishment of an infection.

During blood grouping with anti-C, the strongest agglutination reactions (4+) were more common among uninfected than infected individuals. Since Rh antibodies react stronger with homozygous cells, which have higher antigen density than heterozygous ones, we further examined the data for homozygous carriage of Rh(C). Here, only six individuals out of 409 were homozygous for the C antigen. Interestingly, none of these six were infected with HIV. It would be interesting in a large sample study to see if individuals who are homozygous for blood Rh2 ever get infected with HIV. A recent study in South Africa reported a zero probability for infection in homozygous C individuals (Davison et al., 2018). Particularly interesting would be the Rh2 profile of non-progressors and elite controllers. Our data suggests that they would be mostly Rh2-positive and more likely to be homozygous for the antigen.

This observation suggests that the protection is related to the density of antigens on the erythrocytes. From a population genetics perspective, this is a blood group with a low frequency among individuals of African origin (27%), compared to Northern Indians (85%) or Caucasians (68%). These findings are important from an epidemiological perspective as the rarity of Rh2 among Africans may have contributed to the severity of the epidemic in the continent (Motswaledi et al., 2016). In fact a close examination of the world statistics demonstrates that HIV infections are high only in those regions of the world where Rh(C) frequency is low (Table 7.1).

Duffy Blood Group

Another blood group system implicated in HIV-infection is the Duffy blood group system. A fierce debate has previously raged between investigators whose data suggested an increased risk (Lachgar et al., 1998, He et al., 2008) while others suggested a protective role (Ramsuran et al., 2011) and others argued for no effect at all (Winkler et al., 2009). Our study did not find any relationship of the Duffy antigens to the risk of HIV infection. However, we note that the Duffy antigen co-localizes on the erythrocyte membrane with the C antigen (Ripoche et al., 2004, Anstee and Tanner, 1993), which was found to be protective in this study (Motswaledi et al., 2016).

Lutheran Blood Group

The Lutheran antigens are also known as basal cell adhesion molecules (BCAM) (Chang et al., 2017). Lu[b], but not Lu[a], was associated with a three-fold risk for HIV-infection (Motswaledi et al., 2016). This antigen has wide tissue distribution and is present on monocytes, erythrocytes and endothelial cells (Parsons et al., 1995). It is a natural ligand for laminin, an intercellular matrix protein that has been implicated in vascular occlusion occasioned by adhesion of sickled erythrocytes to vascular endothelia. The role of adhesion molecules in cardiovascular disease is an active area of research (Østerud and Bjørklid, 2003, Lafrenie et al., 1996, Pedraza et al., 2000, Weerasinghe et al., 1998). Monocytes not only adhere to laminin, but also migrate on it into the tissues (Pedraza et al., 2000). For this reason, Lutheran blood groups are an important

consideration in cardiovascular disease. The most significant finding in this study was a lower absolute monocyte count in individuals positive for Lu^b antigen.

The correlation between total cholesterol and phagocytic cells was also studied. As reported by others, cholesterol levels correlated negatively with phagocytes (Solomkin et al., 2007, Frostegard et al., 1990). This cholesterol-related phagocyte depletion was present only in HIV infection and probably reflects the other aspect of atherosclerosis – inflammation, resulting from chronic HIV infection. Considering that atherogenesis requires both macrophages (transformed monocytes), cholesterol and inflammation, we observe here a combination of these factors in a fashion that logically favours atherogenesis. Moreover, we note that this interaction is enhanced in Lu^b-positive subjects but absent in Lu^b-negatives. This evidence strongly suggests that Lu/BCAM likely plays a role in endothelial retention of monocytes that leads to atherogenesis, and that such adhesion may be a function of non-HDL cholesterol.

Moreover, since HIV-infected monocytes have enhanced epithelial transmigration, it is probable that the presence of the Lu/BCAM adhesion molecule enhances this process and results in the introduction of the virus to anatomical sites such as the lymph nodes, where HIV-susceptible cells are concentrated. This would explain the observed, increased risk associated with this blood group.

Since the negative correlation between cholesterol and phagocyte counts was not observed in uninfected individuals, we conclude that HIV or its effects, especially its inflammatory effects, are responsible for promoting leukocyte depletion in the presence of cholesterol. This, if confirmed by further investigations, could explain the increased risk of cardiovascular diseases in HIV infected patients.

The P_1 Blood Group.

A novel finding regarding the P1 blood group in this study was its association with risk of HIV infection and the presence of HIV RNA in the lysate of P_1 erythrocytes. The P_1 blood group is comprised of glycosphingolipids, which are known to facilitate viral fusion independent of co-receptor expression (Hammache et al., 1999) or enhance cofactor efficiency (Puri et al., 1998).

The current study corroborated the risk associated with the glycosphingolipid P_1. This blood group was associated with a two-fold odds ratio for HIV infection. Moreover, the study confirmed association of the virus with erythrocytes even in those patients with undetectable plasma viral load. The erythrocyte-associated virus appears to be independent of the viral load as it was detectable in those with undetectable plasma viral load and absent in some with high viral load. The detection of viral RNA in the lysate of erythrocytes suggests presence of the virus inside the erythrocytes, which was another novel finding of the study.

Combination of risk of HIV-1 Infection based on the ratio of the risk to protective blood group.

Table 7.1 presents frequencies of the protective RHCE*C and the risky P_1. In Africa, P_1 frequencies were only available for Uganda and Botswana. The ratio of the frequencies of the risky over the protective blood groups indicates Uganda as the most HIV-risk-prone population followed by Botswana. For comparison sake, the frequency ratio for China is used as the denominator. The least vulnerable, according to this model would be Turkey, Laos, Malaysia and China. Other countries have a ratio less than one, indicating a protective advantage. The Lu^b blood group was excluded from this analysis since it occurs in high frequency across most ethnic groups (Karamatic et al., 2003).

Table 7.1: Comparison of the risk of HIV-1 Infection based on the ratio of the risk to protective blood group.

Country/Community	RHCE*C Frequency	P$_1$ Frequency	P1/RHCE*C ratio	Risk Factor based on China	HIV Prevalence
Botswana	29.70	74.6	2.51	5.8	21.90
Uganda	3.62	89	24.59	57	6.5*
China	88.1	37.82	0.43	1	0.04
Laos	60.30	18.97	0.31	0.72	0.30
Malaysia	96	40	0.42	1	0.4
Northern India	82.02	71.92	0.88	2.0	0.26
Thailand	51.50	30	0.58	1.3	1.10
Turkey	43	0.5	0.01	0.02	**

*1994 prevalence data = 24.8% (Kilian et al., 1999)

**Official statistics not available, but HIV not considered a threat(Ay and Karabey, 2006).

Conclusion

Taking all data into account, human blood groups, especially Lu^b, and P$_1$, were observed to increase susceptibility to HIV infection with data consistent with their known biochemical properties. Particularly novel in this study is the finding of viral RNA in erythrocyte lysate since HIV has not been reported to enter erythrocytes. Moreover, Rh(C) was found to be protective against HIV and was for the first time linked to an immunological function, particularly involving CD8+ lymphocytes. Our data also shows that a combination of the risky blood groups and the protective activity may influence the severity of the HIV epidemic in a region, especially exemplified by analysis of reports on the prevalence of HIV and the distribution of the blood groups in Africa and other parts of the world. This, to our knowledge is the first comprehensive documentation of the role of blood group systems in HIV immunology.

The study was limited in that while it documented the association of RH2 with cell-mediated immunity, no specific CD functions were investigated. Future experiments will need to include cytotoxicity studies that include measurement of granzyme and interferon.

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CHAPTER 8

RECOMMENDATIONS

Blood Group C

1. This blood group was protective and showed an increased proportion of cytotoxic T cells. Moreover, the frequency of this antigen was consistently low in countries with high HIV rates of infection. However, HIV-specific CD8 T-cell responses were not measured in this study to confirm enhanced cytotoxicity. A study is hereby recommended to investigate HIV-1-specific T cell responses such as Interferon gamma response to stimulation with HIV-specific peptides. This would confirm the functional role of the Rh2 antigen in the immunological response to HIV.
2. Most countries in Africa do not have data relating to these antigens that relate to HIV susceptibility. A multi-national study is needed to corroborate our findings, similar to the South African study cited in this document.
3. Another pertinent study would be to determine the RhC frequency among HIV-infected non-progressors and elite controllers.
4. Other profitable studies could investigate how erythrocyte-bound virions are presented to T-cells and the role, if any, of CD47 in enhancing the presentation of such antigens.
5. The use of homozygous Rh2 hematopoietic stem cells may benefit those who are Rh2 homozygotes. These would not reject the Rh2 cells but would increase the antigen expression and consequent resistance to HIV. Moreover, a more permanent vaccine may be possible by introducing hematopoietic stem cells to developing foetuses who are likely to take adopt the immune hematopoietic cells and acquire HIV-resistance.

Lutheran Blood group (Lu/BCAM, LU2 or Lu^b)

1. The results concerning this blood group showed that the Lutheran blood group, in the presence of high cholesterol levels, promotes depletion of circulating monocytes. This phenomenon constitutes an atherogenic environment. Recommended studies include the use of a larger sample size to study the relationship between high lipids, inflammation, LU2 antigen carriage, and carotid intima/medial thickness. This could help in establishing the genetic link of the Lutheran blood group to cardiovascular disease in HIV-infected individuals.
2. Other studies could also focus on the effect of the blood group on monocyte tethering, migration, as well as the combined atherogenic effect of HIV infection and Lu^b.
3. Furthermore, measures of monocyte activation such as soluble CD163 (sCD163) could be used to study monocyte activation between Lu^b-positive and negative individuals.

P_1 Blood Group

1. The P_1 blood group was associated with a high risk for HIV infection. This was attributed to its ability to facilitate viral fusion to erythrocytes. However, this study used

only a small sample size to demonstrate viral RNA in erythrocytes. Large scale studies are needed to explore the *in vitro* fusogenic effect of the blood group.

2. If corroborated by such studies, the findings would pave the way for further studies to determine if such intra-erythrocytic RNA has potential to infect splenic macrophages when they phagocytose senescent erythrocytes, which could eventually lead to the development of strategies for dealing with this viral reservoir. In particular, the results hold promise for using RNase-laden glycosphingolipid liposomes to capture and lyse circulating virions as a way to effect a rapid drop in the viral load.

APPENDICES

APPEDNDIX A – University of Botswana IRB Permit

UNIVERSITY OF BOTSWANA

Office of the Deputy Vice Chancellor (Academic Affairs)

Office of Research and Development

Corner of Notwane	Pvt Bag 00708	Tel: [267] 355 2900
and Mobuto Road,	Gaborone, Botswana	Fax: [267] 395 7573
Gaborone, Botswana		E-mail: research@mopipi.ub.bw

Ref: URB/IRB/1365

30th November 2012.

The Permanent Secretary
Ministry of Health
Private Bag 0038
Gaborone, Botswana

RE: APPLICATION FOR A RESEARCH PERMIT: MODISA S. MOTSWALEDI

Since it is a requirement that everyone undertaking research in Botswana should obtain a Research Permit from the relevant arm of Government, The Office of Research and Development at the University of Botswana has been tasked with the responsibility of overseeing research at UB including facilitating the issuance of Research permits for all UB Researchers inclusive of students and staff.

I am writing this letter in support of an application for a research permit by Mr Modisa Motswaledi an academic staff member in the Faculty of Health Sciences. Mr Motswaledi has proposed to conduct a study titled **"The role of blood groups in preventing or enhancing HIV infection in Botswana."**The main aim of this project is to study the relationship between human blood groups and susceptibility to HIV-infection. It will also compare the viral binding ability of specific blood groups and how such binding impacts haematological and biochemical prognostic indicators in HIV-infected patients. The findings of this study may be used in future to improve the care of people who are at risk of, or are infected with HIV.

The Office of Research and Development is satisfied with the process for data collection, analysis and the intended utilisation of findings from this research. We will appreciate your kind and timely consideration of this application.

We thank you for your usual cooperation and assistance

Sincerely,

Dr M.B.M. Sekhwela
For Director, Office of Research & Development
Encls: Completed Application for Research Permit
Research Proposal
Informed Consent document
Data collection tools
Comments from the UB IRB

www.ub.bw

APPENDIX B – Ministry of Health Permit

Telephone: (267) 363200
FAX (267) 353100
TELEGRAMS: RABONGAKA
TELEX: 2818 CARE BD

MINISTRY OF HEALTH
PRIVATE BAG 0038
GABORONE

REPUBLIC OF BOTSWANA

REF NO: PPME-13/18/1 Vol VII (698)

05 December 2012

Mr Modisa Sekhamo Motswaledi
Pvt Bag 00708
University of Botswana

Dear Sir/Madam

PERMIT:THE ROLE OF BLOOD GROUPS IN PREVENTING OR ENHANCING HIV INFECTION IN BOTSWANA

Reference is made to your application submitted to the Health Research Unit (HRU) for permission to conduct above mentioned study. The HRU reviewed and approved the study.

Permission is therefore granted to conduct the above study. This approval is valid for a period of 1 year effective 05 December 2012.

You are requested to submit at least one hardcopy and an electronic copy of the report to the Health Research and Development Division, Ministry of Health within 3 months of completion of the study. Copies should also be submitted to all other relevant authorities.

Yours sincerely

P. Khulumani
FOR PERMANENT SECRETARY

MINISTRY OF HEALTH
RESEARCH DIVISION
2012 -12- 0 5
P/BAG 0038
GABORONE
REPUBLIC OF BOTSWANA

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APPENDIX C – CPUT ETHICS PERMIT

Cape Peninsula
University of Technology

HEALTH AND WELLNESS SCIENCES RESEARCH ETHICS COMMITTEE (HW-REC)
Registration Number NHREC: REC- 230408-014

P.O. Box 1906 • Bellville 7535 South Africa
Symphony Road Bellville 7535
•Tel: +27 21 959 6352 • Fax +27 21 953 8490
Email: danielso@cput.ac.za

26 April 2013
CPUT/HW-REC 2013/H17

Faculty of Health and Wellness Sciences - Biomedical Sciences Department

Dear Modisa Sekhamo Motswaledi

APPLICATION TO THE HW-REC FOR ETHICAL CLEARANCE

Approval was granted on 24 April 2013 by the Health and Wellness Sciences-REC to Modisa Sekhamo Motswaledi for your Ethical Clearance application (pending corrections that have been received and reviewed if applicable). This approval is for research activities related to a DTech: Biomedical Technology at this institution.

TITLE: The role of blood groups in preventing or enhancing HIV infection in Botswana.

Internal Supervisor: **Prof O Oguntibeju**
External Co-supervisor: **Prof I Kasvosve**

Comment:
Approval will not extend beyond 26 April 2014. An extension should be applied for 6 weeks before this expiry date should data collection and use/analysis of data, information and/or samples for this study continue beyond this date.

Note:
The investigator(s) should understand the conditions under which they are authorized to carry out this study and they should be compliant to these conditions. **It is required that the investigator(s) complete an annual progress report that should be submitted to the HW-REC in December of that particular year, for the HW-REC to be kept informed of the progress and of any problems you may encounter.**

Kind Regards

Zuléika Nortjé
CHAIRPERSON – ETHICS RESEARCH COMMITTEE
FACULTY OF HEALTH AND WELLNESS SCIENCES

APPENDIX D – National Blood Transfusion Permit

TELEPHONE: (+267) 3974 482

FAX   : (+267)3974 494

National Health Laboratory
Private Bag 0057
GABORONE
Botswana

REPUBLIC OF BOTSWANA

TEL: 3686700

30th January 2013

Ref: NBTS 007/01/13

Mr Modisa S. Motswaledi
University of Botswana
Department of Medical Laboratory Sciences

Dear Sir

RE: PERMISSION TO USE RESIDUAL BLOOD DONOR SAMPLES

Permission is granted to you to use residual blood samples in National Blood Transfusion Centre (EDTA samples) however you will be collecting them on the seventh day when they are due to be discarded.

Thank you

Yours faithfully

Mr K. Mosenki /for Ag. Medical Director

gmm

BOTSWANA HARVARD AIDS INSTITUTE PARTNERSHIP
FOR HIV RESEARCH AND EDUCATION

29th January 2013

Mr Modisa Motswaledi
Department of Medical Laboratory Science
Faculty of Health Sciences
University of Botswana
P/Bag UB 00712
Gaborone

Dear Mr Motswaledi,

Re: The Role of Blood Groups in Preventing or Enhancing HIV Infection in Botswana.

Thank you for your detailed proposal to undertake a study on "The Role of Blood Groups in Preventing or Enhancing HIV Infection in Botswana". The BHP recognizes the importance of studying various factors that are important in HIV Infection and how those can inform new strategies for prevention of HIV transmission in Botswana.

This letter is in full support of the proposed research study being conducted at the Botswana Harvard HIV Reference Laboratory.

This research will be conducted under the agreement for collaborative research on HIV/AIDS in Botswana between the Botswana Harvard Partnership and The University of Botswana.

I take this opportunity to wish you success on your study and look forward to finalizing the specific details relating to conducting the study.

Yours Sincerely,

Dr M. J. Makhema
CEO Botswana Harvard Aids Institute Partnership

ADDENDUM: RESEARCH OUTPUT

Published articles

1. MOTSWALEDI, M. S., KASVOSVE, I. & OGUNTIBEJU, O. O. 2013. The Role of Red Blood Cells in Enhancing or Preventing HIV Infection and Other Diseases. BioMed Res Intern.Volume 2013, Article ID 758682, 7 pages. Available at: http://dx.doi.org/10.1155/2013/758682.

2. MOTSWALEDI, M. S., KASVOSVE, I. & OGUNTIBEJU, O. O. 2016. Blood Group Antigens C, Lub and P1 may have a role in HIV infection in Africans. PLoS ONE, 11, e0149883.

3. Motswaledi, MS, Kasvosve, I & Oguntibeju OO (2019). Rh2 Phenotype Affects Cytotoxic T- Cell Counts. *Jordan Journal of Biological Sciences* 12(3). In Press

Articles submitted to journals

1. MOTSWALEDI, M. S., KASVOSVE, I. & OGUNTIBEJU, O. O. 2018. Potential role of Lu/BCAM in HIV-related atherosclerosis. Submitted to *African Journal of Laboratory Medicine*

2. MOTSWALEDI, M. S., KASVOSVE, I. & OGUNTIBEJU, O. O. 2018. HIV-RNA is detectable in P_1 erythrocyte lysate. Submitted to *Transfusion and Apheresis Science*

Conference Presentations

1. MOTSWALEDI, M. S., KASVOSVE, I. & OGUNTIBEJU, O. O. 2013. Blood Groups in HIV infection. Botswana Institute for Clinical Laboratory Professionals. 2nd Scientific Conference 2016.

2. MOTSWALEDI, M. S., KASVOSVE, I. & OGUNTIBEJU, O. O. 2017: Potential Mechanisms for HIV risk of infection in subjects carrying blood groups C, Lub and P_1. Botswana Institute for Clinical Laboratory Professionals – 3rd Scientific Conference, 2017.

3. MOTSWALEDI, M.S., KASVOSVE, I. & OGUNTIBEJU, O. O.2018. Lack of Rh2 as a risk factor for HIV among Africans. 4th Biennial Conference of the African Society for Laboratory Medicine. ASLM Abstract Control ID: 3054843, Abuja Nigeria.