UNIVERSITY FOR DEVELOPMENT STUDIES, TAMALE

IMMORTALIZATION OF B CELLS FROM *PLASMODIUM FALCIPARUM* EXPOSED INDIVIDUALS FOR THE PRODUCTION OF PARASITE-SPECIFIC ANTIBODIES

BY

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DECLARATION

Student

I hereby declare that this thesis is the result of my own original work conducted at the Noguchi Memorial Institute for Medical Research and that no part of it has been presented for another degree in this University or elsewhere.

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I hereby declare that the preparation and presentation of the dissertation/thesis was supervised in accordance with the guidelines on supervision of dissertation/thesis laid down by the University for Development Studies.

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ABSTRACT

The continuous production of antibodies with desired properties has become very important in fields, such as biomedical research and in medicine. Methods for making such useful antibodies include the hybridoma technology and Epstein-Barr virus (EBV) transformation of antibody-secreting cells (ASCs) to make them live longer in *ex vivo* culture. Transformed cells are then capable of multiplying rapidly over longer periods and producing large amounts of the desired antibodies. The aim of this study is to perform an EBV transformation of ASCs in peripheral blood mononuclear cells (PBMCs) to establish immortalized ASCs and to subsequently screen culture supernatants for *Plasmodium falciparum*-specific antibodies.

PBMCs were isolated from malaria exposed individuals by density gradient centrifugation using Ficoll-hypaque. Isolated PBMCs were co-cultured with EBV using the EBV-based Human Blood B Booster Kit from *Dendritics*™. Transformed B cells clones after day 21 were expanded and screened for total human and *Plasmodium*-specific antibody production using ELISA. Signs of transformation (cell aggregates, increased size and rate of growth) were seen within 3 days of commencement of the immortalization process, and after 21 days some of the polyclonal population of B cells were frozen and stored in liquid Nitrogen. Upon thawing, recovered cells were shown to be viable and underwent clonal expansion in culture. Screening of culture supernatants with chicken anti-human antibody capture ELISA showed good production of human antibodies. The quantity of human IgG produced by immortalized ASCs that had been in culture for 11 days was greater compared to yield from the same culture by day 25. Western Blot analysis confirmed human IgG bands of 160 kDa. After about 20 rounds of screening, however, *Plasmodium falciparum-specific* antibodies could not be isolated and this reflects a very low frequency of parasite-specific B cell clones in these exposed individuals.

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DEDICATION

TO

The Suurbaar family

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ADCI	Antibody-Dependent Cellular Inhibition
ICD	Induced Cytidine Deaminase
APC	Antigen Presenting Cell
ASC	Antigen Secreting Cell
BAFF	B Cell Survival Factor
BCR	B Cell Receptor
CR2	Complement Receptor Type 2
DCs	Dendritic Cells
DMSO	Dimethyl Sulfoxide
EBNAs	EBV Nuclear Antigens
EBV	Epstein-Barr Virus
ELISA	Enzyme-Linked Immunosorbent Assay
GAEC	Ghana Atomic Energy Commission
HLA	Human Leukocyte Antigen
iB cells	EBV Immortalized B cells
IgG	Immunoglobulin

IM	Infectious Mononucleosis
IRB	Institutional Review Board
kitHuBBB®	Human Blood B Booster Kit
LCLs	Lymphoblastoid Cell Lines
LMPs	Latent Membrane Proteins
mAbs	Monoclonal Antibodies
MACS	Magnetic Activated Cell Sorting
NMIMR	Noguchi Memorial Institute of Medical Research
OD	Optical Density
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
Pf	*Plasmodium falciparum*
*Pf*RBC	*Plasmodium falciparum* Red Blood Cells
*Pf*Spz	P*lasmodium falciparum* Sporozoite
RAG	Recombination-Activating Genes
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SHM	Somatic Hypermutation

TCR	T cells Receptors
TMB	Tetramethylbenzidine
V1	Volunteer 1
V2	Volunteer 2
VH	Heavy Chain Variable
VL	Light Chain Variable

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Malaria is caused by eukaryotic single-celled parasites of the genus *Plasmodium.* The species infecting humans are *P. vivax, P. malariae, P. ovale* and *P. falciparum. P. knowlesi,* a simian malaria parasite, is also known to infect humans (Lee *et al.*, 2010; White, 2008). Of all these parasites, *P. falciparum* is the most lethal and responsible for approximately 40 % of all malaria cases globally and nearly all malaria related deaths (Wellems *et al.*, 2009).

In malaria endemic areas, protection against clinical malaria is acquired gradually after repeated infections. This immunity is referred to as clinical immunity. Several factors of the innate and adaptive immune systems play complementary roles in combating malaria (Artavanis-Tsakonas and Riley, 2002; Cohen *et al.*, 1989). Adaptive immunity has two components: antibodies and T cells, both of which protect against infection and severe diseases.

Antibodies are proteins that are produced by activated B cells known as plasma cells. They circulate in the blood and bind specifically to foreign antigens such as those of infectious micro-organisms. This interaction can result in direct inactivation of the microorganism or activation of a variety of immune mechanisms that will destroy the pathogen. Antibodies are important component of the adaptive immune response against the *Plasmodium* parasite (Perlmann and Troye-Blomberg, 2004)

Pathogen-neutralizing antibodies are usually produced as a polyclonal mix *in vivo* as they are elicited by a wide diversity of B cells with varying antigen specificities. Antibodies produced by a single clone of B cells are however described as being monoclonal since all antibodies will have a single antigen binding specificity. Neutralizing monoclonal antibodies (mAbs) can also be made *ex vivo* through the isolation of a single B cell clone with known pathogen antigen specificity (Chappel and Holder, 1993).

1.2 Problem Statement and Justification

Antibodies are important for the development of disease diagnostic tools and for immunological assay design as they can recognize and bind strongly with antigens. Fully human mAbs are also gradually becoming important pharmaceutical agents for the treatment/management of both infectious and non-infectious diseases (Goding, 1996).

Technologies for the production of antibodies were described years ago, and have now proven indispensable for immunological research (Boerbeak, 2004). These technologies have however not been fully deployed and utilized in sub-Saharan Africa. Establishment of protocols for the production of such antibodies is therefore very important especially in sub-Saharan Africa where disease specific antibodies can be very useful for the development of diagnostic research tools.

A number of strategies have been reported for the establishment of perpetual life span of B cells to generate human antibodies. These strategies include hybridoma generation through fusing of human B cells with a myloma cell line, immortalizing B cells with Epstein Barr Virus and selection of positive anti-fragments from phage display libraries,

production of human antibodies from vaccinated transgenic mice carrying human immunoglobulin loci (Jin *et al.*, 2009; Karpas *et al.*, 2001; Sui *et al.*, 2009).

Epstein Barr Virus (EBV) which is a member of the herpes virus family can be used to immortalize human B cells to produce specific antibodies. The EBV method has been described in some publications since 1977 (Rosen *et al.*, 1977; Steinitz, 2014). However, the standard EBV method has limitations, including the low efficiency of immortalization, the low cloning efficiency of EBV-immortalized B cells (EBV-cells) and slow growth rate and, in some cases, low antibody production. The technical procedures used to establish EBV transformed B cell lines have not changed substantially over the last 25 years. Free EBV particles are produced by maintaining an EBV infected marmoset cell line, which is overgrown and subsequently lysed. Human lymphocyte cultures are inoculated with free virus that gains entry into B lymphocytes via their CD2I (CR2) cell surface molecules (receptor for complement C3 fragments). As the virus becomes integrated into the B cell, cytotoxic T lymphocytes can be generated, which subsequently kill the infected B cells, leading to transformation failure (Louie and King, 1991; Ventura *et al.*, 1988). A range of techniques have been developed to avoid this, these include removal of T lymphocytes following immune suppression of T cells using cyclosporine A (Reidy and Wheeler, 1992; Tremblay and Khandjian, 1998).

Dendritics™ has developed a Human Blood B Booster Kit (KitHuBBB®) for the B cell Immortalization. The kit HuBBB® is packaged so as to make it very easy to handle and contains all the reagents needed for the immortalization process (*Human Blood Booster Kit*, 2010). The resulting cells can be screened for detection of human IgG and subsequently antigen-specific cells and cloned to generate *Plasmodium falciparum* specific

mAbs. EBV-immortalized B cells strategy may also be applicable to other infectious or autoimmune diseases.

The aims of this study are thus to develop a protocol for the continuous production of immortalized human B cells, detect antibody production, and subsequently purify antibodies from culture supernatant by affinity chromatography using protein G.

Antibody products from this study can be used for important downstream processes such as immunological research, development of diagnostic tools and potentially as therapeutics. The protocol when established can also be applied to making monoclonal antibodies for the treatment of other acute infectious and non-infectious diseases.

1.3 Objectives of the Study

- To immortalize B cells in freshly isolated human Peripheral Blood Mononuclear Cells (PBMCs) from malaria exposed individuals.
- To identify B cell clones that actively secretes human antibodies.
- To identify B cell clones that secretes antibodies with specificity for selected malaria vaccine candidate antigens.
- To purify and characterize expressed antibodies from culture supernatants.

1.4 Significance of the Study

Purified human antibodies can be employed as reagents in immunoassays, radioimmunoassay (RIA) and enzyme-linked immunosorbent assays (ELISA). They can also be used for the identification and characterization (epitope mapping) of pathogen

antigens recognized by semi-immune individuals and also important component of pharmaceutical compositions.

Developing a protocol using a novel method that allows efficient B cells immortalization will help in the identification of essential antibodies with useful downstream applications.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1.0 The Immune System

The immune system is typically defined as the body's way of fighting foreign material (antigens). Immunity process can be divided into two parts, innate and adaptive immune responses. The innate immune system comprises physical, chemical and microbiological barriers and includes elements of the immune system such as neutrophils, monocytes, macrophages, complement factors, cytokines and acute phase proteins, which provide instant host defenses. While the innate response is rapid, and is unable to memorize the same said pathogen should the body be exposed to it in the future the adaptive response takes some days or weeks to develop. The latter is characterized by specificity and memory that offers long lasting defense against specific antigens (Bouharoun-Tayoun *et al.*, 1995).

Lymphocytes are the main effectors in the adaptive immune system. They play a central role in the regulation of both cell-mediated and antibody-mediated responses against different antigens. It has been estimated that only 2% of all lymphocytes are circulating in the peripheral blood. Thus, the vast majority of lymphocytes are in the lymphoid organs (tonsils, spleen, lymph nodes and Peyer's patches). This is important since it is here they exhibit their functional competence via encountering antigens in association with antigen presenting cells (APCs), such as monocyte derived macrophages, dendritic cells and Langerhans cells. The activated cells in lymphoid organs interconnect with the tissues via the lymphatic and the blood vessel systems (Chaplin, 2010).

Two classes of lymphocytes are documented, the B and T lymphocytes. The former are precursors of plasma cells that secrete antibodies while the latter are in charge of regulating the immune responses. Precursors of both B and T cells develop in the bone marrow. B lymphocytes mature in the bone marrow, whereas the precursor T lymphocytes migrate to the thymus where they undergo the process of maturation (Chaplin, 2010; Whiteside, 2010).

2.1.2 T Lymphocytes

In the thymus, T cells distinguish between self and non-self-antigens through surface expression of T cells Receptors (TCR) (Thibault and Bados, 1995). TCR comprised of alpha- Beta T cells or Gamma-Delta T cells subunits and are expressed in association with several monomorphic proteins collectively called Cluster Differentiation (CD), constituting the TCR/CD3 complex. The TCR generation is a complex process that creates a repertoire in the order of more than 10^{14} through combinatorial joining of Variable (V), Diversity (D) and Joining (J) (Beta and Delta chains) or V and J (Alpha and Gamma chains) segments out of about 200 germline exons (Chaplin, 2010).

Naive T cells are those that have not yet encountered foreign Antigens (Ag) and have not yet been activated. Antigenic peptides are presented to the naive T lymphocyte in secondary lymphoid organs by dendritic cells (Salgame *et al.*, 1991). Dendritic cells (DC) are the most efficient Antigen Presenting Cells (APC) since they also provide signals for effective T cell activation. DC acquire Antigen (Ag) in non-lymphoid tissues throughout the body and migrate into secondary lymphoid organs guided by inflammatory stimuli and cytokines (Banchereau and Steinman, 1998).

There are two T cells of immune importance, they are Cytotoxic CD8+ T cells and CD4+ T helper cells, Cytotoxic CD8+ T cells are very effective in direct lysis of infected or malignant cells bearing the Ag, while CD4+ T helper cells produce cytokines that can be directly toxic to the target cells or can stimulate other T cell effector functions and B cell antibody production, as well as mobilize powerful inflammatory machineries. Most effector T cells will disappear after the antigenic agent is removed, although others will remain and form memory T cells. Unlike naive T cells that live for few months or effector cells that disappear at the end of the immune response, memory T cells may survive for years in lymphoid organs and peripheral tissues. The easily activated memory T cells can perform immediate effector functions in peripheral tissues or undergo activation and clonal expansion in lymphoid organs to mount a secondary immune response if the same Ag appears again (Sun and Bevan, 2003; Veillette *et al.*, 1988).

2.1.3 B Lymphocytes

B cells constitute 15% to 20% of peripheral blood lymphocytes. They differentiate from haematopoietic stem cells in the bone marrow where their B cells receptors (BCR) are assembled from genetic building blocks in recombination-activating genes (RAG1)/RAG2-mediated process similar to that used for the production of functional T cell receptors (TCR) (Lantelme *et al.*, 2000; Schatz *et al.*, 1989).

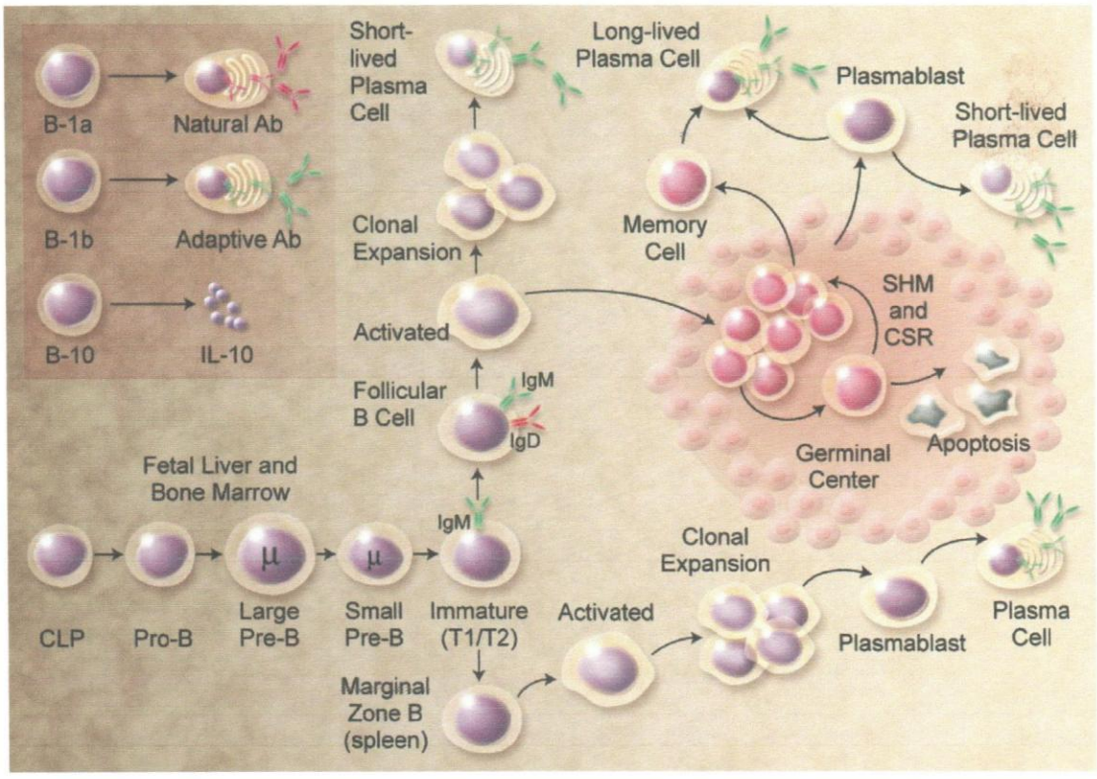

Figure 2.1: B cell development. The figure shows the broad outline of B cell developmental stages in humans. CLP indicates common lymphoid progenitor; SHM, Somatic Hypermutation (LeBien and Tedder, 2008).

Early B cells development is characterized by ordered rearrangement of Ig H and L chain loci, and Ig proteins themselves play an active role in regulating B cells development. Pivotal to understanding how early B cells development is regulated was the discovery of surrogate L chains (SLCs) (Melchers, 2005; Sakaguchi and Melchers, 1986). A decade after the discovery of RAG1/2, Honjo and his colleagues demonstrated Somatic Hypermutation (SHM) are mediated by an enzyme designated activation-induced cytidine deaminase (AID) (Honjo *et al.*, 2005). Expectedly, B cells AID expression is induced in GCs where CSR and SHM occur. There are 2 theories on how AID functions to promote antibody diversification. One suggests that AID carries out an RNA editing function not

being the source of hypermutator activity, but cooperating with another protein to mediate SHM. A more prevailing view suggests that AID participates more directly to effect mutation of Ig H genes at the DNA level (Honjo *et al.*, 2005; Lam *et al.*, 1997; Milne and Paige, 2006; Okazaki *et al.*, 2007).

The marginal zone B cells and GC B cells subsets (shown in Figure 2.1) all contribute to the circulating natural antibody pool, thymic-independent IgM antibody responses, and adaptive immunity by terminal differentiation into plasma cells, the effector cells of humoral immunity. Antigen activation of mature B cells leads initially to GC development, the transient generation of plasmablasts that secrete antibody while still dividing, and short-lived extra follicular plasma cells that secrete antigen-specific germ line–encoded antibodies (Figure 2.1). GC-derived memory B cells generated during the second week of primary antibody responses express mutated BCRs with enhanced affinities, the product of SHM. Memory B cells persist after antigen challenge, rapidly expand during secondary responses, and can terminally differentiate into antibody-secreting plasma cells. In a manner similar to the early stages of B cells development in fetal liver and adult marrow, plasma cell development is tightly regulated by a panoply of transcription factors, most notably Bcl-6 and BLIMP-1(McHeyzer-Williams and McHeyzer-Williams, 2005; Radbruch *et al.*, 2006; Shapiro-Shelef and Calame, 2005).

Primary and secondary immune responses generate separate pools of long-lived plasma cells in the spleen, which migrate to the marrow where they occupy essential survival niches and can persist for the life of the animal without the need for self-replenishment or turnover (McHeyzer-Williams and McHeyzer-Williams, 2005; Shapiro-Shelef and Calame, 2005).

The marrow plasma cell pool does not require ongoing contributions from the memory B cells pool for its maintenance, but when depleted, plasma cells are replenished from the pool of memory B cells (DiLillo *et al.*, 2008). Thereby, persisting antigen, cytokines, or Toll-like receptor signals may drive the memory B cells pool to chronically differentiate into long-lived plasma cells for long-lived antibody production (Klein *et al.*, 1998; Slifca *et al.*, 1998). It has been shown, in vitro, that human memory B cells proliferate and differentiate in response to polyclonal stimuli (Melzner *et al.*, 2006).

2.2 Antibodies

Antibodies are immunoglobulins capable of specific combination with antigens that caused its production in an organism. They are produced in response to the invasion of foreign molecules in the body (Spaulding *et al.*, 2006).

Antibodies exist as one or more copies of a Y-shaped unit as shown in figure 2.2, composed of four polypeptide chains. Each Y contains two identical copies of a heavy chain, and two identical copies of a light chain, named as such by their relative molecular weights. Antibodies can be divided into five classes: IgG, IgM, IgA, IgD and IgE, based on the number of Y units and the type of heavy chain. Heavy chains of IgG, IgM, IgA, IgD, and IgE, are known as gamma, mu, alpha, delta, and epsilon, respectively. The light chains of any antibody can be categorized as either a kappa (k) or lambda (l) type based on small polypeptide structural differences However, the heavy chain decides the class or subclass of each antibody (Boniesch, 2005 and Boneisch, 2001).

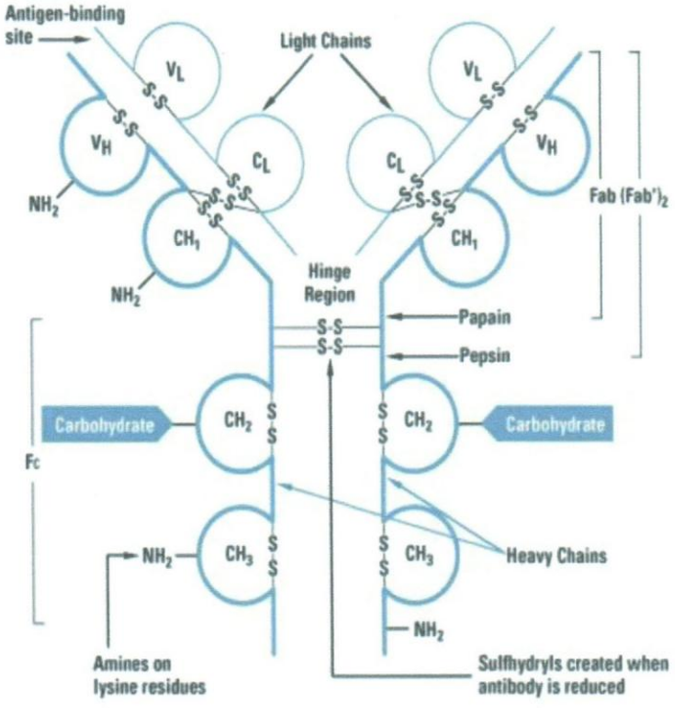

Figure 2.2. Diagram showing the structure of an immunoglobulin molecule. It comprises two identical heavy (H) chains and two identical light (L) chains. Inter and intra chain disulfide bonds contribute to the structure and stability of the molecule (Boneich *et al.*, 2006).

2.3 Antibody - Antigen Interactions

The specific association of antigens and antibodies is dependent on hydrogen bonds, hydrophobic interactions, electrostatic forces, and van der Waals forces. These are all bonds of a weak, non-covalent nature, yet some of the associations between antigen and antibody can be quite strong. Like antibodies, antigens can be multivalent, either through multiple copies of the same epitope, or through the presence of multiple epitopes that are recognized by multiple antibodies. Interactions involving multivalence can produce more

stabilized complexes, however multivalence can also result in steric difficulties, thus reducing the possibility of binding (Hershowitz, 1985).

Affinity describes the strength of interaction between antibody and antigen at single antigenic sites. Within each antigenic site, the variable region of the antibody "arm" interacts through weak non-covalent forces with antigen; the more interactions, the stronger the affinity (Hershowitz, 1985; Steward and Steensguard, 1983).

Avidity is perhaps a more informative measure of the overall stability or strength of the antibody-antigen complexes. It is controlled by three major factors: antibody epitope affinity; the valence of both the antigen and antibody; and the structural arrangement of the interacting parts. Ultimately these factors define the specificity of the antibody, that is, the likelihood that the particular antibody is binding to a precise antigen epitope. Cross-reactivity refers to an antibody or population of antibodies binding to epitopes. This can be caused either by low avidity or specificity of the antibody or by multiple distinct antigens having identical or very similar epitopes. Cross reactivity is sometimes desirable when general binding to a related group of antigens or when attempting cross-species labeling, when the antigen epitope sequence is not highly conserved in evolution (Steward and Steensguard, 1983).

Immunochemical techniques capitalize upon the specificity, at the molecular level, of each immunoglobulin for its antigen, even in the presence of high levels of contaminating molecules. The multivalancy of most antigens and antibodies enables them to interact to form a precipitate. Examples of experimental applications that use antibodies are Western

Blot, Immunohistochemistry and Immunocytochemistry, Enzyme-Linked Immunosorbent Assay (ELISA), Immunoprecipitation, and Flow Cytometry (Boneisch, 2006)

2.4 Polyclonal and Monoclonal Antibodies

Polyclonal antibodies are heterogeneous mixture of antibodies directed against various epitopes of the same antigen or multiple antigens. The antibodies are generated by different B cells clones and as a consequence are immunochemically dissimilar (Hornick and Kurash., 1979).

Polyclonal antibodies may thus recognize a variety of epitopes on antigen, which can be an especially useful feature in some experimental procedures. Because these polyclonal mixtures of antibodies react with multiple epitopes on the surface of their specific antigen, they will be more tolerant of minor changes in the antigen, e.g., polymorphism, heterogeneity of glycosylation, or slight denaturation, than will monoclonal (homogenous) antibodies. Depending upon the antigen that is used to create the antibody, one may use polyclonal antibodies to identify proteins of high homology to the antigen or to screen for the target protein in tissue samples from species other than that of the antigen (Carson *et al.*, 2010; Ramakrishna *et al.*, 2010).

In contrast Monoclonal antibodies are a homogeneous population of immunoglobulin directed against a single epitope. The antibodies are generated by a single B cells clone and are therefore immunochemically similar (Hornick and Kurash., 1979).

A homogeneous population of antibodies can be raised by immortalizing of B lymphocytes and this will produce many copies of antibodies. This remarkable technology has been

instrumental in the development of antibodies for diagnostic applications, because monoclonal antibodies react with one epitope on the antigen.(Ramakrishna *et al.*, 2010).

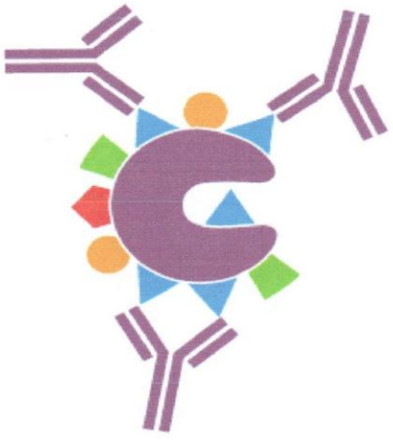

Figure 2.3. A given clone of monoclonal antibodies reacts with a specific epitope on an antigen.

2.6 Production of Human Antibodies

2.6.1 The Hybridoma Technology

The production of Antibodies by hybridoma technology was discovered in 1975 by Georges Kohler of West Germany and Cesar Milstein of Argentina, Kohler and Milstein developed a technique which relies on immortalizing B lymphocytes by fusion to myeloma cells. Fused cells will then possess the ability to grow over long periods of time and produce the specific antibody of the lymphocyte. Supernatants from hybridoma cell cultures are then screened for desired antibody specificities and positive cultures are cloned to obtain a pure cell line producing the monoclonal antibodies of interest (Kohler and Milstein, 1975)

Human antibodies can be produced by the hybridoma technology by using transgenic mice. These are mouse lines that have had human antibody genes inserted and their own antibody genes knocked out. In this way human mAbs can be made by the hybridoma technology. Genetically engineered mice expressing human antibody repertoires were first reported for producing human mAbs using the hybridoma technology in 1994.Transgenic mice producing human mAbs with various heavy-chain isotypes have also been produced to tailor effector functions. At contemporary, more than 50 human mAb produced in transgenic mice are in clinical trials and six have been accepted for marketing (Nelson *et al.*, 2010). Although immune responses in transgenic mice are sometimes less robust than those observed in wild type mouse strains that are used to generate mouse mAbs, the expression of human Ig in transgenic mice prevents human anti-mouse antibody responses and maintains the advantages of mouse hybridoma technology for the production of antibodies for potential clinical uses (Tiller *et al.*, 2008; Lonberg, 2005).

2.6.2 The Phage Display Technology

Phage display technology was first developed in 1985 and has been used to produce large numbers of peptides and proteins on the bacteriophage. It is the next method being successfully used to select antigen-specific variable region genes and to express functional antibody fragments with unique specificity (Scofield, and Clementel, 2007; Hoogen, 2000 and 2002). In principle, for the isolation of human antibodies, the library of diverse human immunoglobulin-heavy chain variable (VH) gene and light chain variable (VL) gene sections are set by reverse transcription of mRNA from B cells and PCR amplification. The gene encoding single chain variable fragment can be created by arbitrarily joining VH

and VL gene sections using PCR. The large antibody repertoires can be produced using the process of combinatorial infection and in vivo recombination, to display single chain variable fragment on the surface of the phage (Scofield *et al.*, 2007).

After bio-panning the phages bound to a range of antigens, antigen-specific antibody fragments with good affinities can be known (Scofield *et al.*, 2007; Williamson *et al.*, 1991 and Marks, 2004). One phage display-derived mAb has been approved by the Food drugs agency and at least 35 human mAbs generated by phage display technology have entered into clinical development (Marks, 2004).

2.6.3 Epstein Barr Virus Technology

EBV infects human B lymphocytes and transforms them into immortal cell lines. The complicated intracellular processes which eventually result in the transformation of a part of the B cell population following primary infection *in vitro* have been described (Alfieri *et al.*, 1991; Mellinghoff *et al.*, 1991; Middleton *et al.*, 1991; Thorley-Lawson, 1988). This immortalization approach has been very useful in human monoclonal antibody technology, since it allows relatively efficient immortalization of B cells, and a number of stable antibody-producing cell lines have been established by use of this method (James & Bell, 1987). In addition, the technique is not always applicable since only a minor part of the B cell pool is susceptible to this immortalization procedure, thus not only reducing the probability of the rare specific B lymphocytes being found, but also possibly restricting the repertoire that can be obtained with this technique. Since virtually every available immortalization technique has some restriction on the B cell populations which it can

efficiently immortalize, EBV-immortalization may actually complement the other technologies which are available for the production of antigens specific antibodies.

EBV-immortalized B cells secrete antibodies in large amounts so that screening to detect specific antibodies can be performed with culture supernatants not only using binding assays but also functional assays. In addition, EBV-immortalized B cells also retain expression of surface Ig and consequently can be selected for their antigen binding (Wang, 2011).

2.7.1 Epstein Barr Virus

Epstein-Barr virus (EBV) is the cause of heterophile-positive infectious mononucleosis (IM), which is characterized by fever, sore throat, inflammation, and atypical lymphocytosis. EBV is also associated with several human tumors, including nasopharyngeal carcinoma, Burkitt's lymphoma, Hodgkin's disease, and (in patients with immune deficiencies) B cells lymphoma (William & Crewford., 2006)

EBV enters B cells through CD21 marker on the surface of B cells. EBV infection of epithelial cells results in viral replication and production of virions. During latent infection of B cells, only the EBV nuclear antigens (EBNAs), latent membrane proteins (LMPs), and small EBV RNAs are expressed *in vitro*. EBV-transformed B cells secrete immunoglobulin; only a small fraction of cells produce virus (Fafi, 2005)

2.7.2 The Mechanism of EBV Immortalization of B cells.

The virus is known to infect mostly B cells in a mixed population of B, T and natural killer cells. The presence of complement receptor type 2, commonly known as CR2 (CD21) on B cells creates a route for virus entry into the cell. Viral envelope glycoprotein gp350 binds to CR2 and triggers endocytosis (Fingeroth *et al.*, 1984). In addition, a second glycoprotein gp42 binds to a human leukocyte antigen (HLA) class II molecule as co-receptor (Wang and Hutt-Fletcher, 1998). Through these interactions, the fusion machinery is triggered and the viral membrane fuses with the endosomal membrane to release viral genetic materials into the cell. EBV infection in other cell types, mainly epithelial cells, is less efficient and occurs through poorly defined separate pathways (Young and Rickinson, 2004; Borza and Hutt-Fletcher., 2002; Nemerow *et al.*, 1987 and Fingroth *et al.*, 1984)

This difference in cell that supports the entry of EBV can be attributed, to some extent, to the type of cell from which viral arrangements are made. For B cells immortalization, EBV creates latent infection mainly surviving as covalently closed circular episome with 5-800 copies per cell. This latent infection is characterized by expression of a number of viral genes (Hurley *et al.*, 1988 and Neitzel, 1987).

The expressed viral gene products comprises six different EBV encoded nuclear antigenic proteins namely EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, EBNA Latent Protein and three latent infection membrane proteins, LMP1, LMP2A and LMP2B (Hurley *et al.*, 1988). In addition, LCLs also show abundant expression of two non-polyadenylated RNAs, EBER1 and EBER2 with unclear function. This pattern of latent EBV infection which is activated only in B cells infection is referred to as latency III. The role of EBV latent genes

has been confirmed by recombinant EBV genetic analyses with *in vitro* B lymphocyte transformation assays. Studies using recombinant EBV revealed indispensable requirement of EBNA2 and LMP1 for immortalization process with an EBNA-1, EBNA-LP, EBNA-3A, and EBNA-3C playing a role in the immortalization process (Kempkers *et al.*, 1995). EBNA2 acts as a transcriptional activator by interacting with sequence specific DNA binding protein to regulate EBV latency gene expression in B cells and to modify cellular gene expression which results in stimulation of G0 to G1 cell cycle progression resulting in B cells immortalization (Kempkers *et al.*, 1995). Similarly, LMP1 is a critical determinant of EBV-mediated transformation as it is required for both, the outgrowth of LCLs *in vitro* as well as continued proliferation of an established LCLs. (Kaiser *et al.*, 1999; Kempkers *et al.*, 1995, Sinclair *et al.*, 1994 and Cohen *et al.*, 1989).

2.8 Human Blood B Booster Kit (kitHuBBB®)

Historically, Marmoset lymphoblastoid cell line (LCL) B95-8, which was established by infecting marmoset B lymphocytes with EBV isolated from a human patient with infectious mononucleosis, has been a constant source for producing transforming virus, but this has resulted very low efficiency (Neitzel, 1986)

There is however the development of Blood B Booster kit by *Dendritics*, a biomedicals manufacturing company in Lyon, France, which is based on the existing approaches and the generation of new reagents. In the existing approaches, the classical EBV infection yield had been $1/10^6$ of B lymphocyte immortalized and the combination of CD40 and EBV increased the efficiency by1% of B lymphocyte immortalized (Lanzavecchia, 2004;Rosen *et al.*, 1977 and Steintz *et al.*, 1977;).

Dendritics has developed a very powerful medium additive called the Booster reagent which contains more than 80 components and which increases 100 fold the CD40 - induced B cells proliferation. Under the best conditions, the kitHuBBB® kit allows the immortalization of up to 20% of the starting B cells population. and contains all the reagents needed to obtain from 5.10^4 to 2.10^5 independent B cells clones from 10^7 peripheral blood mononuclear cells using ten 96 wells culture plates (*Human Blood Booster Kit*, 2010).

2.9 ELISA Technique for Detecting Antibodies

The ELISA technique allows the detection and quantification of very small quantities of antigens such as proteins, hormones, or antibody in a fluid sample example plasma serum, utilizing enzyme-labeled antigens and antibodies to detect the biological molecules. The antibodies bind to a specific antigen which is then detected by a secondary, enzyme-coupled antibody. A chromogenic substrate is added to form a coloured solution that is measured as optical density (Thermo Fisher Scientific, 2013). This technique will allow screen antibody of interest.

The success of an ELISA assay is dependent upon the underlying level of immunoreactivity of the capture and detection antibodies to the target analyte. Two separate antibodies are involved: the first to recognize and bind the target analyte, the second to detect the bound target. One of the antibodies is applied to the well of a microtitre plate: this is known as the capture antibody. The capture antibody binds to the plate via passive adsorption and this step is often performed at -4°C overnight. A blocking solution (typically milk protein

(casein), bovine serum albumin or fish gelatin) is applied. These proteins adhere to any vacant sites on the plastic surface of the well that are not occupied by capture antibodies thereby minimizing the effect of non-specific binding by other reagents to the plate surface during subsequent incubation steps. Excess blocking agent is removed and the plate is rinsed before addition of the test sample (wash steps are incorporated between all incubation steps to minimize the background signal due to non-specific binding). If the test sample contains the target analyte, this is bound by the capture antibody that is anchored to the plate. After the incubation step with the test sample, the plate is washed before the addition of the enzyme-linked detection antibody. The detection antibody is conjugated with an enzyme which is commonly either horseradish peroxidase, alkaline phosphatase or β-D-galactosidase. These enzymes are proteins that catalyze the hydrolysis of a chromogenic substrate, such as 3, 3', 5, 5'-tetramethylbenzidine or 2, 6-dichlorophenolindophenol, which undergoes a colorimetric change that is measurable using a spectrophotometric plate reader at specified wavelengths. With the catalysis of the traditional chromogenic substrates the reaction is terminated by the addition of a stop solution prior to measuring the absorbance of each of the wells of the microtitre plate (Lequin, 2005; Voller *et al.*, 1976).

ELISAs may utilize an indirect detection approach. In this instance, as illustrated in Figure 2.4, the secondary antibody used will recognize and bind to the appropriate species-specific sub-class of the antibodies (or immunoglobulins). It is the secondary detection antibody that is conjugated to the enzyme, as opposed to the primary antibody (Koenig, 1981).

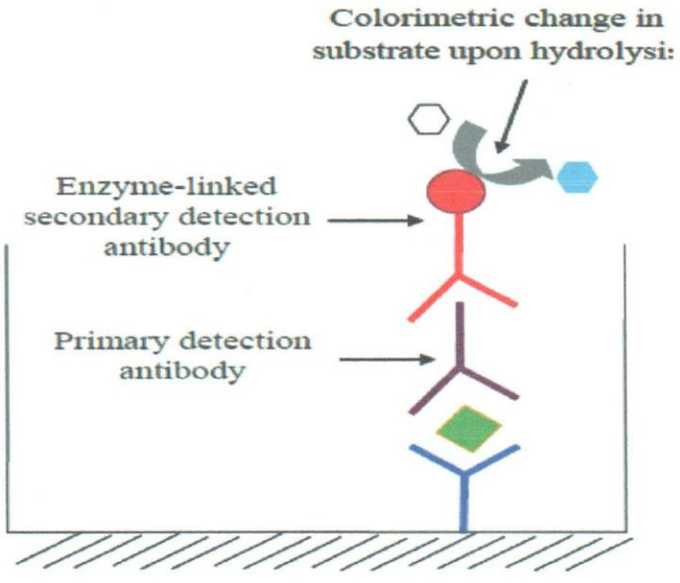

Figure 2.4: A Typical Indirect-Detection ELISA

2.10 Principle of SDS-PAGE analysis

Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) involves the separation of proteins (Human IgG) based on their size. By heating the sample for denaturing , proteins become unfolded and coated with SDS detergent molecules, acquiring a high net negative charge that is proportional to the length of the polypeptide chain (Porsch-Özcürümez *et al.*, 2004; Schmitt *et al.*, 2005). When loaded onto a gel matrix and placed in an electric field, the negatively charged protein molecules migrate towards the positively charged electrode and are separated by a molecular sieving effect. After visualization by a protein-specific staining technique, the size of a protein can be estimated by comparison of its migration distance with that of a standard of known molecular weight. It is also possible to blot the separated proteins onto a positively charged membrane and to

probe with protein-specific antibodies in a procedure termed western blotting (Boschetti, 2002). Blotting is the transfer of resolved proteins from the gel to the surface of a suitable membrane. The separated proteins are transferred out of the gel either by the capillary action of the buffer or in an electric field (Goldbaum *et al.*, 1993; Porsch-Özcürümez *et al.*, 2004; Schmitt *et al.*, 2005; Williamson *et al.*, 1991). The transferred proteins are bound to the surface of the nitrocellulose membrane and are accessible for reaction with immunochemical reagents. After electro-transfer, the Nitrocellulose membrane is placed in a Petridish and the non-specific sites which is the unabsorbed sites on nitrocellulose membranes will be blocked by agitating the membrane for 1 hour in serum diluent buffer. After incubation, the membrane is washed with wash buffer (Ibraghimov and Rokhlin, 1985; Spickett, 2006). After that a Labeled antibody (anti-human IgG HRP-conjugate) is added to the membrane. Excess antibody enzyme conjugate is removed by washing the membranes. Substrate solution is added and agitate to observe gray coloured bands.

2.11 Application of Antibodies

Antibodies have proved to be extremely valuable for basic immunological and molecular research. They are used in human therapy, commercial protein purification, suppressing immune response, diagnosis of diseases, cancer therapy, diagnosis of allergy, hormone test and purification of complex mixtures (Edward, 1981).

In diagnostic application antibodies are by far the most advanced, especially for tests that are performed on body fluids such as blood and urine sample. (Tyagi *et al.*, 2011; Zola, 2010; Ling, 1983; Edward, 1981; Kohler and Milstein, 1975).

In protein purification, Antibodies affinity columns are readily prepared by coupling Antibodies to a cyanogen bromide-activated chromatography matrix, e.g., Sepharose. Since the Antibodies have unique specificity for the desired protein, the level of contamination by unwanted protein species usually is very low (Tyagi *et al.*, 2011; Zola, 2010).

2.12 Malaria

Malaria is caused by a protozoan parasite that belongs to the group haemosporidians, Phylum *Apicomplexa* and family *Plasmodium*, and consists of several parasite species found in the blood of mammals, birds, reptiles. There are 10 sub-genera of the Genus *Plasmodium. Plasmodium (Plasmodium)* and *Plasmodium* (*Laverania*) are the sub-genera that infect humans and other primates, while the heterogeneous sub-genus *Plasmodium (Vinckeia)* infects other mammals (Janeway *et al.*, 2001). Four species of the genus *Plasmodium* naturally infect humans, namely: *Plasmodium falciparum (Pf)*, *Plasmodium vivax (Pv)*, *Plasmodium malariae (Pm)* and *Plasmodium ovale (Po)* (Hoffman, 1992). *Pf* is the most fatal of the four. The simian parasite *Plasmodium knowlesi*, known to cause disease in monkeys in Asia, has also been reported to cause fatal human infections in Malaysia and often misdiagnosed as *Plasmodium malariae* (Cox-Singh *et al.*, 2008).

Mostly the disease caused by parasites *Plasmodium falciparum* can be found mostly in the tropics. High severity of clinical symptoms that results in disease complications is caused by *P. falciparum*. These include severe anaemia and cerebral malaria. Other complications are respiratory distress, hypoglycaemia and generalized convulsion (Schien, 2011; McCance *et al.*, 2010).

According to the latest estimates, released in December 2013, there were about 207 million cases of malaria in 2012 (with an uncertainty range of 135 million to 287 million) and an estimated 627 000 deaths (with an uncertainty range of 473 000 to 789 000) (WHO,2013). In Ghana, about 3.5 million people get infected with malaria every year. In children, 20,000 die of malaria every year and 25% of the deaths occur in children under the age of five (UNICEF, 2007).

The major ways by which malaria contributes to deaths in children are: acute malaria infections which manifest as seizures or cerebral malaria, repeated plasmodium specie infections which contribute to the progress of severe malaria. Low birth-weight also occurs as a result of malaria during pregnancy (Adams *et al.*, 2004).

During *Plasmodium* infection, the parasite undergoes a series of developmental stages in the infected host which includes the pre-erythrocytic, and asexual blood stages. The parasite has a complex life cycle with various stages with each stage's blood stream being associated with stage specific antigens which are distinct from each other (Barrett and Stanberry, 2009). Malaria is transmitted through the bite of a *Plasmodium*-infected female *Anopheles* mosquito which transfers sporozoites in the salivary gland into the host's blood stream during a blood meal. In the host, sporozoites moves through the blood stream to the liver where they infect parenchymal cells, undergo development within seven days and rupture to release about Thirty thousand (30000) merozoites per hepatocyte into the blood stream (Greenwood *et al.*, 2008). About 20 minutes after invading red blood cells, merozoite forms trophozoites which develop into schizonts containing merozoites. Infected erythrocytes rupture to release merozoites within 48 hours in *P. falciparum, P. vivax* and *P. ovale* and 72 hours in *P. malariae* (Bray and Garnham, 1982). While some merozoites

invade more red blood cells, others develop into male and female gametocytes which are picked up by the mosquito during a blood meal. The life cycle continues in the mosquito with the male and female gametocytes developing into the microgametocytes and macrogametocytes which fuse to form the zygote in the mosquito gut. The zygote undergoes development between 15 to 30 hours to form ookinetes in the midgut of the mosquito. Ookinetes invade the stomach wall and develop to form the oocyst which raptures between 10 to 14 days to release sporozoites and the sporozoites migrate to the salivary glands of the mosquito, ready to infect the host during the next blood meal to continue the cycle (Florens *et al.*, 2002; Greenwood *et al.*, 2008).

2.13 Malaria Disease

Malaria is prevalent in tropical and subtropical regions because rainfall, warm temperatures, and stagnant waters provide habitats ideal for mosquito larvae (Cox, 2010). Malaria is seen as an acute febrile illness with symptoms such as headache, chills, vomiting and periodic fevers Clinical disease is a result of the blood stage infection. The results of the reactions of cytokine production by stimulated macrophages and other cells including other soluble factors gives rise to the clinical symptoms associated with the disease during the asexual/blood stage (CDC, 2010). Although the disease is agonizing, most patients, especially adults, survive uncomplicated malaria even without treatment. However, in 1-2% of cases the disease develops into severe, potentially fatal malaria. This is seen as further complications such as cerebral malaria, severe anemia or metabolic acidosis (Ascenzi, 1999 and Dobson, 1999). Other complications such as organ failure are also seen in countries with low transmission. Parasitaemia can also occur without the development

of any disease symptoms, known as asymptomatic malaria (Mackintosh *et al.* 2004; Rowe *et al.* 2009; Taylor and Molyneux 2002).

Health care providers may suspect malaria in anyone who has recurrent fever; however medical diagnosis of malaria involves examining blood smears taken from a finger prick under a microscope. The rapid test kit can also be used to diagnose malaria but with this method the quantity of parasite in the blood cannot be determined (Moody, 2002).

Chloroquine was the third most widely used drug in the world until the mid-1990s. Unfortunately, chloroquine-resistant malaria parasites have developed and have spread to most areas of the world nearly all *P. falciparum* malaria-endemic regions ("Understanding Malaria," 2007). Since the mid-2000s, the Artemisinin derivative Artesunate, which is superior to quinine is mostly used in malaria treatment for both children and adults, however emerging resistance to artemisinin has become a problem in some parts of Southeast Asia ("Understanding Malaria," 2007).

2.14 Immune Responses to Malaria

Plasmodium infection gives rise to host responses, which involve both the innate and adaptive arms of the immune system. Both antibody and T-cell mediated immune responses have been shown to be important for acquiring immunity to malaria (Perlmann and Troye-Blomberg, 2004). Several stage specific parasite antigens elicit immune responses in the human host. However, these responses generally reflect parasite presence or exposure. Clinical immunity to malaria is shown to develop slowly amongst naturally exposed populations (Macdonald, 1957; Richie *et al.*, 2009; Teirlinck *et al.*, 2011 ; WHO, 2011) but the mechanism of cellular responses in relation to exposure are not yet

established and being studied. Measured induced cellular responses to both P*lasmodium falciparum* Sporozoite (*Pf*Spz) and *Plasmodium falciparum* Red Blood Cells (*Pf*RBC) in semi-immune individuals showed that immune responses are present up to 14 months after even a single malaria episode (Roestenberg *et al.*, 2013). Both adaptive and innate lymphocyte subsets which include αβT cells, γδT cells and NK cells together contribute to the increased levels of IFN-γ response. A study by Chandele *et al.*, (2011) stated the importance of CD8 and CD4 T-cell response induced by blood-stage malaria antigens such as PfAMA-1 and Pf MSP-2 in malaria vaccine development targeting erythrocytic stage. On the other hand, a rodent study have shown that $CD8^+$ T cells are associated with the pathogenesis of cerebral malaria (Miyakoda *et al.*, 2013).

Humoral immunity is mainly linked with B cells and circulating antibodies (Benjamini *et al.*, 1996). Antibodies circulating in the blood bind with antigens on infectious agents and cause the inactivation of micro-organisms or elicit the production of different types of inflammatory mediators (complement, cytokines etc.) to destroy the micro-organism (MacCance *et al.*, 2010).

In malaria, protective immunity is said to be related with the different classes and subclasses of the antibodies. Antibody levels are generally found to increase with the extent of exposure to malaria (Gupta *et al.*, 1999). Different levels of antibodies are associated with different intensity of clinical malaria risk. A high level of IgG antibodies to CSP, LSA-1 and TRAP is associated with decreased risk of clinical malaria (John *et al.*, 2008)

2.15 Anti-Malarial Antibodies during Infection

The human body fights against infections and malaria is no exception, this is acquired through long term exposure to the parasite and is dependent on immunological memory. The T-cells known to be the key directors in immune recognition and regulation responses, mediate immunity by regulating macrophage and B cells activities. They may also act directly as cytotoxic cells on infected hepathocytes and through production of parasite-toxic cytokines (Donati, 2005).

The B cells upon malaria parasites infection for the first time, begins to produce antibodies, proteins that recognize molecules (antigens) on the parasite's surface and that act directly or cooperate with other parts of the immune system to kill malaria parasites. The production of these "naturally acquired" antibodies is initially slow so the individual can become ill when infected. However, because the immune system "remembers" how to make the antibodies, its response to subsequent infections is quicker. The levels of these antibodies also build up with each infection and become more effective at killing parasites.

Antibodies are produced by terminally differentiated B cells known as plasma cells and protect against malaria by a variety of mechanisms. They may mediate their effector roles against malaria parasites on their own or in association with effector cells. On their own, antibodies against merozoites surface-associated proteins may block RBC invasion (Wahlin *et al.*, 1984) and by hindering merozoites release from schizonts either by binding to surface exposed antigens (Green *et al.*, 1981). Moreover, antibodies may block cytoadherence avoiding infected RBC to being sequestered in the periphery, and allowing them to be removed by the spleen (David *et al.*, 1983). They may also inhibit spontaneous

binding of uninfected RBC to infected RBC (rosetting) (Carlson *et al.*, 1990) and consequently may guard against cerebral malaria. In collaboration with other effector immune cells, parasite antigen–specific antibodies play an important role via antibody-dependent cellular inhibition (ADCI), whereby binding of antibodies to phagocytes via Fc receptors lead to inhibition of parasite growth (Bouharoun-Tayoun *et al.*, 1990; Bouharoun-Tayoun *et al.*, 1995 and Tebo *et al.*, 2001). Alternatively, antibodies may initiate parasite clearance by opsonization, thus enhancing the activity of phagocytic cells or initiating complement-mediated damage (Giridaldi *et al.*, 2001 and Ramasamy *et al.*, 1997). It is widely believed that periodic reinfection is required to maintain acquired immunity to malaria and that antimalarial antibodies are short-lived in the absence of reinfection implying that B cells memory to malaria may be defective or suboptimal (Carvalho *et al.*, 2007). However, the development and persistence of B cells memory following malaria infection has long been a matter of debate. Some studies in animal models have shown that memory B cells do develop and are maintained normally after malaria infection (Langhorne *et al.*, 2008; Stephens *et al.*, 2005) whereas others have found that malaria infection interferes with the development of memory B cells and long-lived plasma cells (Carvalho *et al.*, 2007). In humans, several studies have demonstrated stable antibody responses to malaria antigens, however, short-lived antibody responses have also been observed, especially in young children (Drakeley *et al.*, 2005; Stephens *et al.*, 2005; Taylor *et al.*, 1996; Udhayakumar *et al.*, 2001).

2.16 Potential of Anti-malarial Antibodies for Therapy and Diagnostics

Malaria presents a diagnostic challenge to laboratories in most countries. Endemic malaria, population movements, and travelers all contribute to presenting the laboratory with diagnostic problems for which it may have little expertise available. Drug resistance and genetic variation has altered many accepted morphological appearances of malaria species, and new technology has given an opportunity to review available procedures (Cheng and Bell, 2006). Scientists, clinicians, and manufacturers need the realistic possibilities for developing accurate, sensitive, and cost-effective rapid diagnostic tests for malaria, capable of detecting 100 parasites/μl from all species and with a semi quantitative measurement for monitoring successful drug treatment (Cheng and Bell, 2006)

Most new technology for malaria diagnosis incorporates immunochromatographic capture procedures, with conjugated monoclonal antibodies providing the indicator of infection (Piper *et al.*, 1996).

Immunochromatography relies on the migration of liquid across the surface of a nitrocellulose membrane. Immunochromatographic tests are based on the capture of parasite antigen from peripheral blood using monoclonal antibodies prepared against a malaria antigen target and conjugated to either a liposome containing selenium dye or gold particles in a mobile phase. A second or third capture monoclonal antibody applied to a strip of nitrocellulose acts as the immobile phase. The migration of the antigen-antibody complex in the mobile phase along the strip enables the labeled antigen to be captured by the monoclonal antibody of the immobile phase, thus producing a visible colored line. Incorporation of a labeled goat anti-mouse antibody capture ensures that the system is

controlled for migration. Migration depends on several physical characteristics of the component reagents, primarily the porosity of the membrane controlling the flow rate and the components of the buffer solution used to transport the labeled antigen-antibody complex in the lysed blood sample (Iqbal *et al.*, 2001; Piper *et al.*, 1996).

In addition antibodies are multifunctional glycoproteins that are found in blood and tissue fluids, and can protect against malaria by binding and neutralizing malaria parasites and preparing them for destruction by immune cells. Important technical advances mean that it is now possible to synthesize antibodies against important *Plasmodium* antigens that could be used for therapeutic purposes. These reagents could be designed to act like a drug and kill parasites directly, or could be used in vaccine strategies to protect individuals from infection (Pleass and Holder, 2005)

CHAPTER THREE

3.0 METHODOLOGY

3.1 Ethical Clearance

Ethical and scientific clearance was obtained from the Institutional Review Board (IRB) and the Scientific and Technical Committee of the Noguchi Memorial Institute of Medical Research (NMIMR). Informed consent was obtained from all subjects before samples were taken. All other protocols in relation to good laboratory practices were strictly followed.

3.2 Samples Collection

Two volunteers were recruited under this study; Volunteer 1 (V1) had just recovered from a laboratory confirmed infection with *P. falciparum* using a thick blood smear at the Ghana Atomic Energy Commission (GAEC) clinic, Accra, Ghana. Volunteer 2 (V2) was an individual who frequently experiences malaria episodes but had no parasites at the time sample was taken. Twenty milliliters (20ml) of venous blood was taken from each volunteer into Acid Citrate Dextrose (ACD) tubes and the tubes transported on ice to the laboratory at NMIMR.

3.3 Isolation of Peripheral Blood Mononuclear Cells (PBMCs)

Blood from the ACD tubes for each volunteer was carefully transferred into labeled 50 ml falcon tubes. An equal volume of dilution medium (Appendix I) was added to the transferred blood and carefully layered on 20 ml of lymphoprep (Axis - Shield PoC AS,

Oslo , Norway) in 50 ml falcon tubes. Tubes were span at 2000 rpm at 25 °C for 7 minutes. The uppermost plasma layer was carefully pipetted off and the middle band was gently pipetted into a 50 ml falcon tube ensuring that no contaminating Red Blood Cells was transferred. Thirty (30) ml 5% Foetal Calf Serum (FCS) in RPMI – 1640 (Appendix I) was added and this was span at 2000 rpm for 7 minutes at 25 °C. The supernatant was quickly poured off and the tube gently tapped to loosen the pellet cells. Washing was repeated under this same condition and the pellet reconstituted in 10 ml with 5 % FCS. A one in five dilution with 0.4 % Trypan blue (Sigma) was prepared and the concentration and viability of cells estimated by the use of a Heamocytometer (Hawsley Counting Chamber, UK).

3.4 Immortalization of B cells

The immortalization process involved the use of Human Blood B Booster Kit (Dendritics, Lyon, France). Complete culture medium containing feeder cells was distributed in ten (10) 96 well plates and was incubated at 37 °C, 5 % CO_2 on day 0. On day 1, 100 µl/well of isolated PBMCs and EBV1 was distributed and the plates were incubated at 37 °C, 5 % CO_2. After day 5, the wells were observed for proliferating patches of "hairy" cells under an inverted microscope (Olympus BH 2) and 300 µl of fresh complete culture medium was added. On day 10, 200 µl/well of culture supernatant was discarded and 100 µl of complete media containing EBV was added. Culture plates were incubated at 37 °C, 5% CO_2. On day 21, fractions of immortalized cells from each volunteer were frozen down for storage in liquid nitrogen and a portion of cells from each well were transferred into 24 well plates for further cultivation. Cell concentration and viability were determined by staining with 0.4 % Trypan blue and counted by a haemocytometer.

3.5 Cryopreservation and Recovery of Immortalized B cells

Span cells were tapped to loosen the cells and were re-suspended in freezing mixture containing 90 % FCS and 10 % Dimethyl Sulfoxide (DMSO). The freeze mixture was added drop-wise onto the cells up to 4 ml. One milliliter aliquots of the cells in the freezing mix were pipetted into the labelled cryo-tubes and the tubes quickly transferred into pre-cooled strata cooler (4 °C) before transport into a -80 °C freezer overnight. The cells were subsequently transferred from -80 °C into liquid nitrogen at -196 °C for long term storage.

For recovery, cryopreserved cells were carried on ice from liquid nitrogen and placed in a water bath (37 °C). The thawed cells were transferred directly into labelled 50 ml falcon tubes and initially about 3 - 4 ml of wash medium was added drop-wise and it's then topped up to 40 ml of 5 % FCS. This was span at 2000 rpm at 25 °C for 7 minutes and the supernatant was quickly poured off and the tube was tapped to loosen the cells. The cell pellets were suspended in 1 ml of culture medium. 500 µl of the resulting cell suspension was distributed into 2 wells in a labelled 24 well plate for incubation at 37 ° C, 5 % CO_2.

3.6 Crude Malaria Antigen Preparation

Plasmodium falciparum, 3D7 laboratory strain was used to prepare crude parasite lysates. Parasites were grown in blood type 0 Rh+ erythrocytes, and incubated at 37 °C with 5 % CO_2. The culture was maintained by changing media daily and parasitaemia was checked daily by smears made on a glass slide, fixed in methanol (AppliChem) and stained with Giemsa (Merck,) for 10 minutes.

To prepare the crude lysate, a column containing iron filings was mounted between two strong magnets on a Magnetic Activated Cell Sorting (MACS) equipment. The MACS system enriches the schizont fraction of parasites by using the ferromagnetic property of haemozoin in the schizonts that makes them stick to the column containing iron fillings. The iron filings column with stuck schizonts was washed with plain Phosphate Buffered Saline (PBS) to remove all other culture medium components continually until the flow-through changed from red to colourless. The flow-through was discarded and the column removed from the magnetic field of the MACS system. The schizont-infected RBC's were eluted from the column by flushing with PBS and then tapping the sides of the column. Harvested parasites were washed twice by centrifugation at 2000 rpm for 7 minutes, the supernatant discarded and the pellet re-suspended in 5 ml plain PBS.

The parasite concentration was determined by using a haemocytometer. The harvested schizont-infected RBCs were subsequently lysed by three freeze-thaw cycles in liquid nitrogen (-196 °C) and water bath (37 °C). After this process the schizonts extract were centrifuged at a speed of 1200 rpm for 7 minutes, the supernatant was discarded and the pellets (crude malaria antigens) were re-suspended in PBS and then stored in a (-80 °C) freezer until use.

3.7 Screening of Culture Supernatants for Human Antibodies

ELISA was used for screening the supernatants for the human antibodies identification. The plates were coated with 1 µg/ml Anti-Human IgG in 1% Phosphate Buffered Saline (PBS) and was incubated overnight at 4 °C. The plates were washed four times with 200 µl of wash buffer (appendix II). Blocking was done by adding 200 µl/well of blocking

buffer (Appendix II) and incubated for 1hour 30 minutes at room temperature. Blocking solution was discarded and plates were washed 4 times as described above. 100 µl of immortalized cells supernatants were added and this was incubated for 1 hour at room temperature. Plates were again washed 4 times as described above and 100 µl of Goat anti-Human IgG (H+L), horseradish peroxidase conjugate (1/5000 in diluent buffer) was dispensed in the wells and was incubated for 1 hour at room temperature. Plates were again washed 4 times and 100 µl of the substrate which is 3, 3′, 5, 5′-Tetramethylbenzidine (TMB) Liquid was dispensed into the wells and was incubated to allow a colour to develop for ten minutes in the dark after which 100 µl of stopping solution which is 0. 2 M Sulphuric acid (0.2 M H_2SO4) was added to stop the reaction.

The absorbance was measured at 450 nm and the Optical Density (OD) values were obtained.

3.8 Screening for Malaria-specific Antibodies

ELISA was used for screening the supernatants for the malaria specific antibodies production. A day before ELISA assay the plates were coated with crude antigen extract of *Plasmodium Falciparum*.100 µl of 100,000 parasites per well in 1% Phosphate Buffered Saline (PBS) The plates were stored at 4 °C until use and all following steps were performed at room temperature. The plates were washed four times with 200 µl of wash buffer (Appendix II) and this was repeated 3 times. Blocking was done by adding 200 µl/well of blocking buffer (Appendix II) and incubated for 1hour 30 minutes. Blocking solution was discarded and plates were washed 4 times as described above and blotted on tissue to dry. About 100 µl of immortalized cells was added and this was incubated for 1

hour at room temperature. Plates were again washed 4 times as described above. Exactly100 µl of Goat anti-Human IgG (H+L), horseradish peroxidase conjugate (1/5000 in diluent buffer) was dispensed in the wells and was incubated for 1 hour. Plates were again washed 4 times. The substrate which is 3,3′,5,5′-Tetramethylbenzidine (TMB) Liquid of 100 µl was dispensed into the wells and was incubated to allow a colour to develop for 10 minutes in the dark after which 100 µl of stopping solution which is 0.2 M H_2SO4 was added to stop the reaction. The absorbance was measured at 450 nm and the Optical Density (OD) values were obtained.

3.9 Purification and Concentration of Human IgG

Immortalized B cells culture supernatant was prepared for purification by diluting with binding Buffer (0.1 M Phosphate, 0.15 M Sodium Chloride, pH 7.2 in 1X PBS). The top cap of spin columns pre-packed with a Protein G matrix (Pierce) was loosened and the bottom closure was snapped off, the columns were placed in a 15 ml collection tube and centrifuged for 1 minute. The flow through containing storage buffer (Appendix III) was discarded and the columns were equilibrated twice by adding 2 ml of binding Buffer (Appendix III) and span at 1000 x g for 1 minute. Exactly two (2) ml diluted samples were applied to columns and capped tightly to the top and the bottom and this was incubated for 10 minutes at room temperature. The columns in the 15 ml collection tube was span for 1 minutes at a speed of 1000 x g with the top cap loosen and the bottom cap removed. The flow through was discarded and eight rounds of the binding step was repeated. The columns were washed three times with 2 ml binding buffer (Appendix III) and was span for 1 minutes. After the unbound materials were removed by washing, 100 µl of neutralization

buffer (1 M Tris at pH 8 – 9) was put into three 15 ml collection tubes and the spin column was placed into one of the tubes. Exactly 1 ml of Elusion Buffer (0.1 M glycine, pH 2 – 3) was added to the column and was centrifuged for 1 minute. The spin column was transferred into another collection tube containing neutralization buffer saving the collected solution as the first elution fraction. This was repeated two times to obtain three fractions. After collecting the fractions, the concentration of all three eluates was determined by measuring the relative absorbance at 280 nm. The concentration of the eluates of the purification process was so small and this necessitated its concentration by the aid of spin cartridge. The eluates were put inside the spin cartridge and this was span for 7 minutes at a speed of 1,500 g to reduce the buffer volume in the spin cartridge. PBS was added and this was span again, the excess buffer collected in the collection tube between spins were discarded. The concentrated antibody from the top of the spin cartridge was collected into an Eppendorf tube and the volume was adjusted to 500 µl with PBS and the concentration of the Human IgG was determined by measuring the absorbance at 280 nm.

3.9 SDS-PAGE and Western blotting

Purified IgG for each volunteer were mixed with loading dye and heated for 5 minutes at 96°C, all sample prepared and molecular weight markers were loaded per lanes on SDS-PAGE and gel. Polyclonal purified Human IgG was used as positive control and the gels were run at150 V for 1 hour 15 minutes at room temperature. After the completion of the run, the gel was carefully removed from in between the glass plates and was stained with Coomassie Brilliant Blue R 250 solution (Appendix IV) for 30 minutes. After staining, the

gel was destained with destaining solution (Appendix IV) to remove the background stain. The gel was then photographed for permanent record.

Also Separated samples on different gel were electro-blotted onto a nitrocellulose membrane with transfer buffer (Appendix IV) at 40 V for 1 hour 30 minutes. For immune detection the transfer membranes were blocked with 5 % skimmed milk in 1X PBS for 1 hour at room temperature. Membranes were washed three times using PBS of 0.05 % Tween and then incubated with Horse Radish Peroxidase conjugated goat anti-human IgG (1:5000 dilution) in 5 % skimmed milk for 1 hour at room temperature. The membranes were washed in PBS and developed for 10 minutes in substrate solution containing hydrogen peroxide and 3,3 9 – diaminobenzidine tetrahydrochloride (DAB-Sigma) in PBS. The reaction was stopped by washing with distilled water and positive reactions were determined by the appearance of clearly defined bands. The relative molecular mass (Mr) of each recognized band was determined by comparison with standard molecular markers (Sigma).

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 Peripheral Blood Mononuclear Cells

Peripheral Blood Mononuclear Cells (PBMCs) from two volunteers were used for this study. A volume of 20 ml blood was taken and the yield and viability of PBMCs are presented in the Table 4.1 below.

Table 4.1: Number of PBMCs isolated and their percentage viability.

Volunteer's ID	Yield of PBMCs (Cells)	Viability (%)
V1	$7.48 * 10^7$	87
V2	$7.40 * 10^7$	97

4.2 Immortalization of B cells

For each volunteer, 500,000 PBMCs were co-cultured with EBV in a specialized growth medium that had feeder cells and a cocktail of B cell growth factors from the Blood B Booster kit. Two rounds of immortalization with EBV were performed, first on day 0 and again on day 10. Signs of transformation were seen from day 3 onwards and transformed cells were seen as large cell clumps with pseudopodia-like extensions from the individual cells. The cells were enlarged and slightly iridescent. Cell growth was qualitatively assessed by a change in colour of culture medium from pink to yellow after every 3 to 4 days (Figure 4.1).

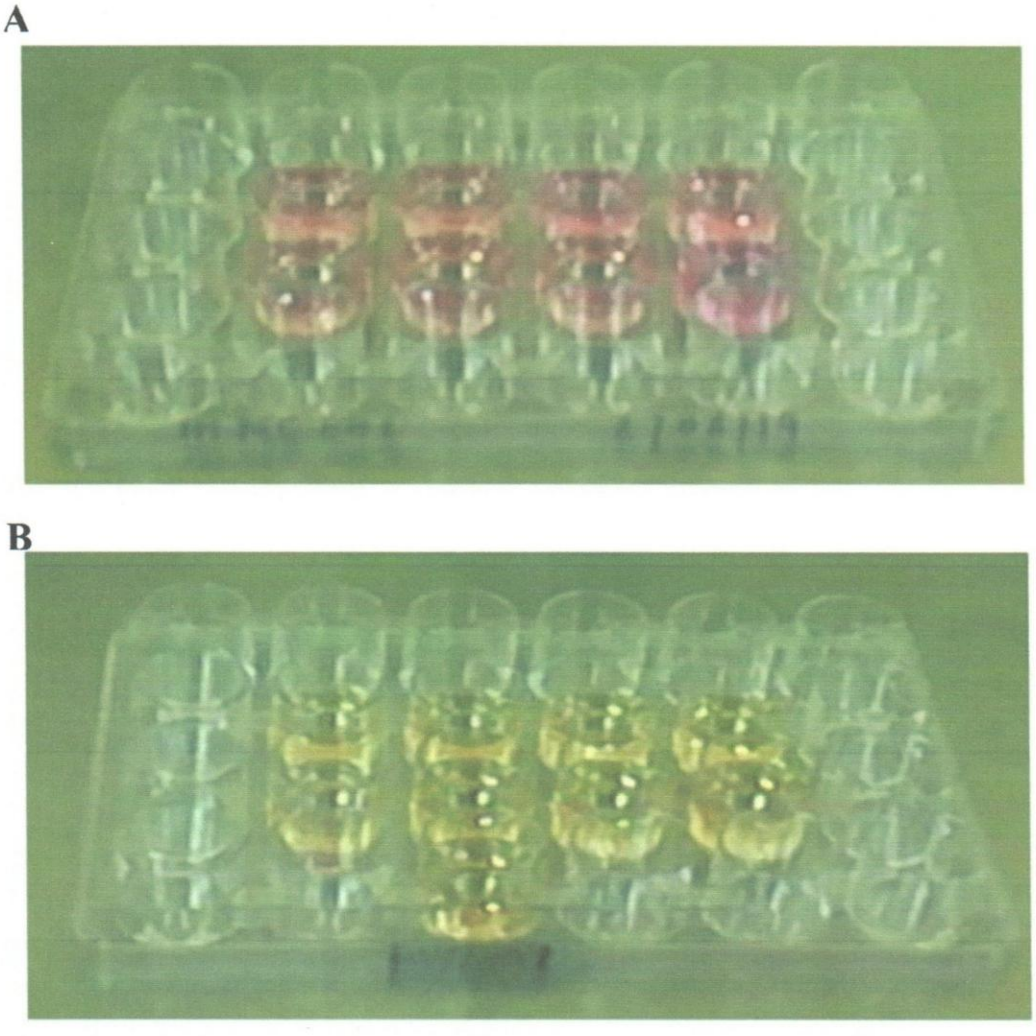

Figure 4.1. Culture plates of 24 wells. (A) Four days after Cells thawed showing Pink coloured wells indicating the original colour of the medium where there is no apparent cellular activity (B) Four days after Cells thawed showing change in medium colour from pink to yellow, indicating wells with metabolic activity.

Microscopically, aggregated immortalized cell images were observed and these are shown in Figure 4.2. Immortalized cells that were cryopreserved also showed good growth characteristics upon recovery. Immortalized cells were kept in culture for up to four (4) weeks.

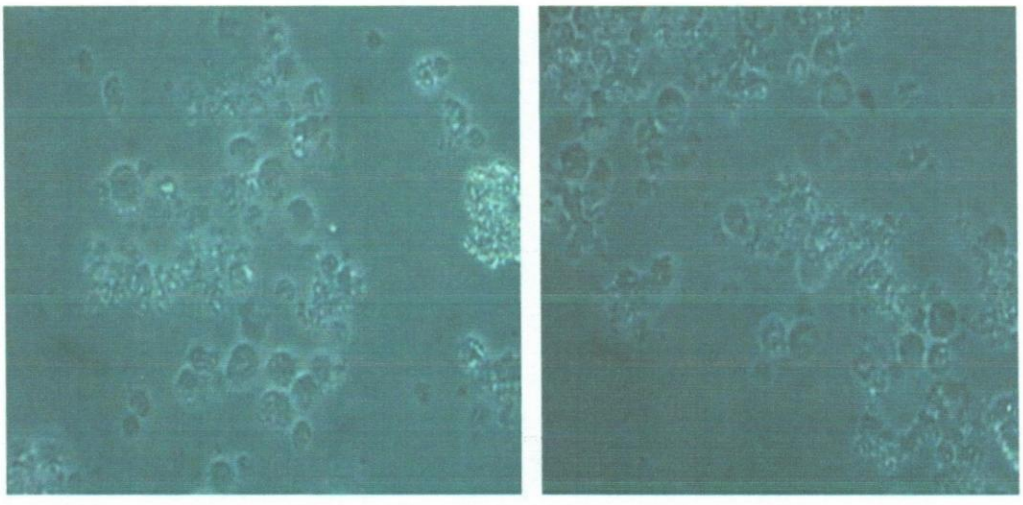

Figure 4.2. Representative images of EBV-immortalized B cells on day 15 after EBV immortalization. (Inverted Phase Contrast; Magnification 20 X)

4.3 Human antibody production

Human antibodies in culture supernatants were first identified using indirect ELISA. Immortalized B cells (iB cells) cultures were initially in 10 % normal human serum in RPMI-1640 (HR10) but this was changed to 10 % Foetal calf serum in RPMI-1640 (10 % FBS) to allow the quantitation of human antibodies made by iB cells . Figure 4.3 and 4.4 presents a representative of a number of ELISA analyses for the screening process. OD values of supernatants screened for human antibodies for V1 and V2 at different durations of culturing, namely 4, 11, 20 and 25 days of culturing the iB cells after thawing cells

from liquid Nitrogen. A standard of 40 ng/ml of commercially acquired purified human IgG was included on each ELISA plate as a control. A cut-off for antibody positivity was defined using the mean of medium blank plus three times the blank standard deviation, and ODs above this cut off value were considered to have significant levels of human IgG.

High OD values were observed for all supernatants screened on day four of cell culture and when observed under the inverted microscope iB cells showed increased number of cell clumps. On day eleven all supernatants screened had high levels of polyclonal human IgGs. On day twenty five the presented wells screened for Human IgG production for Volunteer one (V1) showed relatively low IgG levels as shown in Figures 4.3. For Volunteer two (V2) out of the presented wells screened, only Two (2) wells showed positivity in IgG production as shown in figure 4.4 below. OD values observed were above the cut-off point established except on day twenty five some wells were negative for both V1 and V2 as shown in Figures 4.3 and 4.4 below.

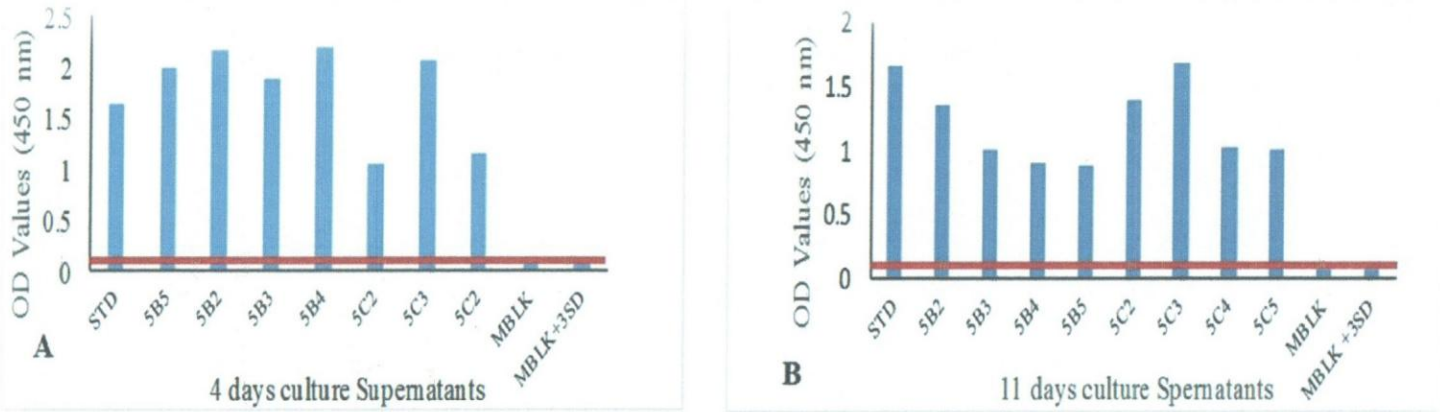

Figure 4.3: Representative OD Values of supernatants screened for human IgG production for V1. The solid red line represents a cut-off for positivity for human IgG established using the mean of medium blank (MBLK) plus three times standard deviations (STDEV), above which supernatants were described as being positive for IgG production.

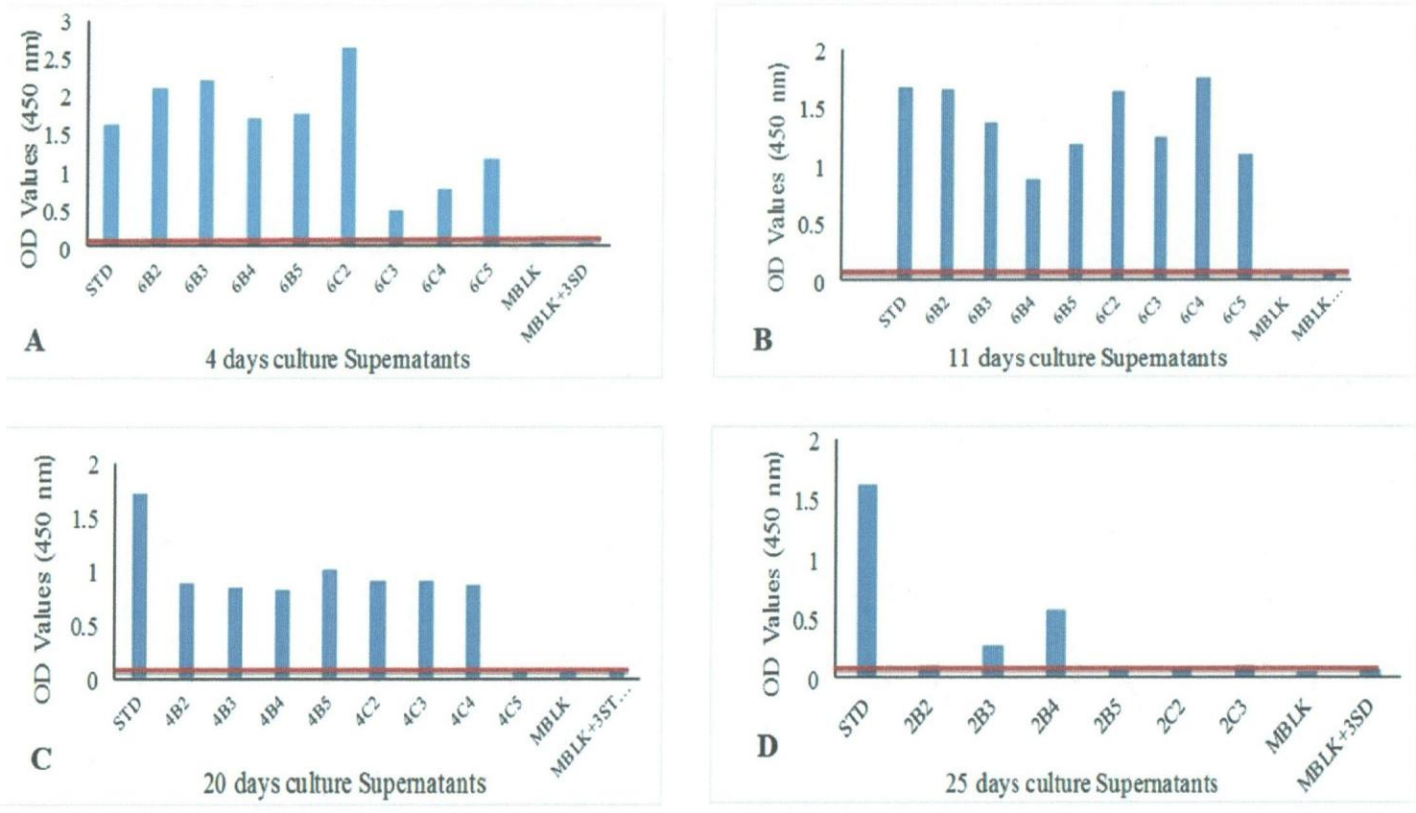

Figure 4.4: Representative OD Values of supernatant screened for human IgG production for V2. The solid red line represents a cut-off for positivity for human IgG established using the mean of medium blank (MBLK) plus three times standard deviations (STDEV), above which supernatants were described as being positive for IgG production.

4.4 Screening for Production of *Plasmodium falciparum*-specific Antibodies.

Screening for production of *plasmodium falciparum*-specific antibodies was done using indirect ELISA. About 20 rounds of screening were done on supernatants collected from 15 different cultures of immortalized cells for each volunteer. Figures 4.5 and 4.6 present a representative data on the OD values of the supernatants screened for malaria-specific antibodies. A standard of 1:16000 dilution of semi-immune serum was included on each plate as a control. OD values for all supernatants from the different time points were below the cut-off point established, which is medium blank plus three times the blank standard deviation.

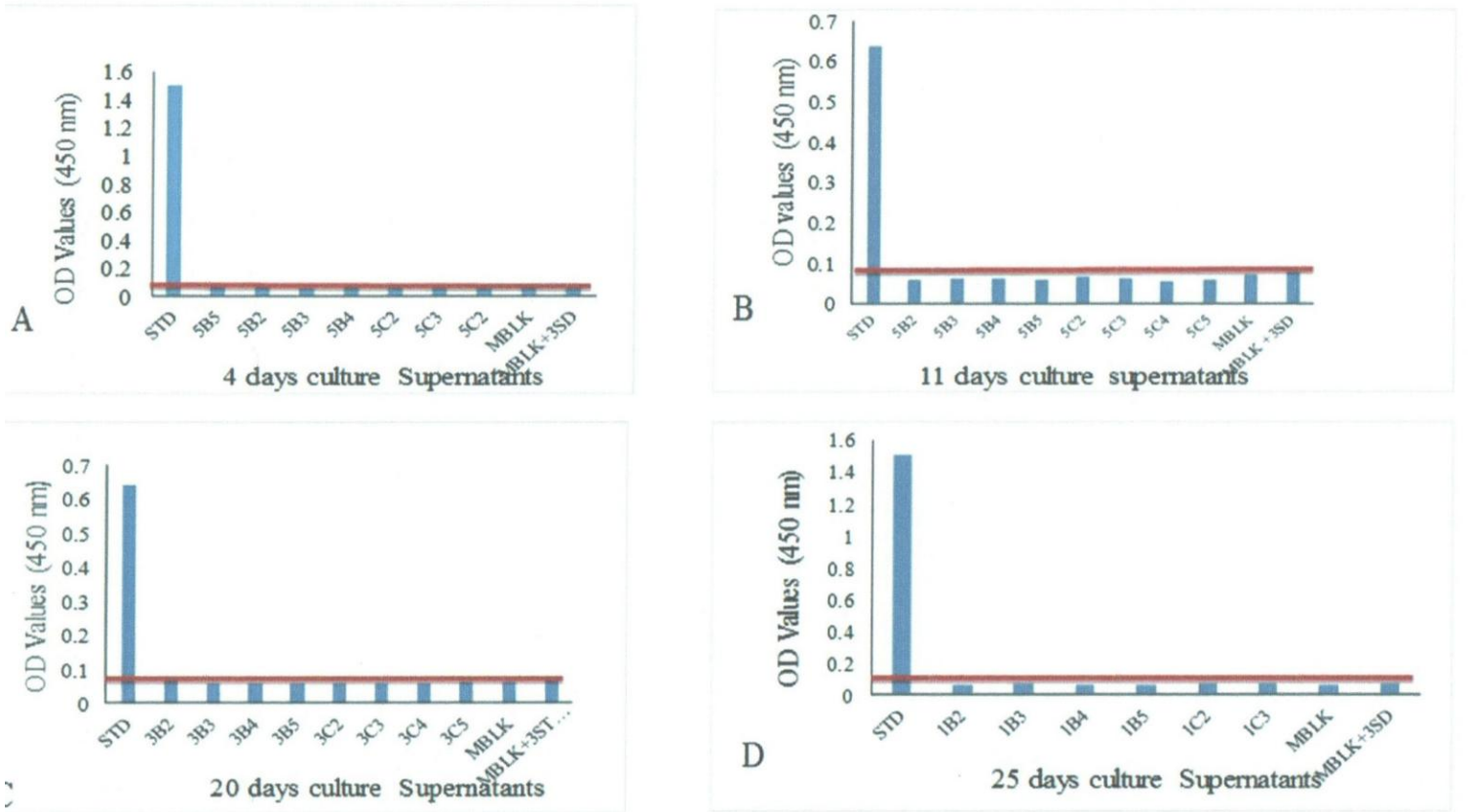

Figure 4.5: Representative OD Values of supernatant screened for Antibodies against Schizont Extract of *Plasmodium Falciparum* for V1. The solid red line represents a cut-off for positivity for human IgG established using the mean of medium blank (MBLK) plus three times standard deviations (STDEV), above which supernatants were described as being positive for IgG production.

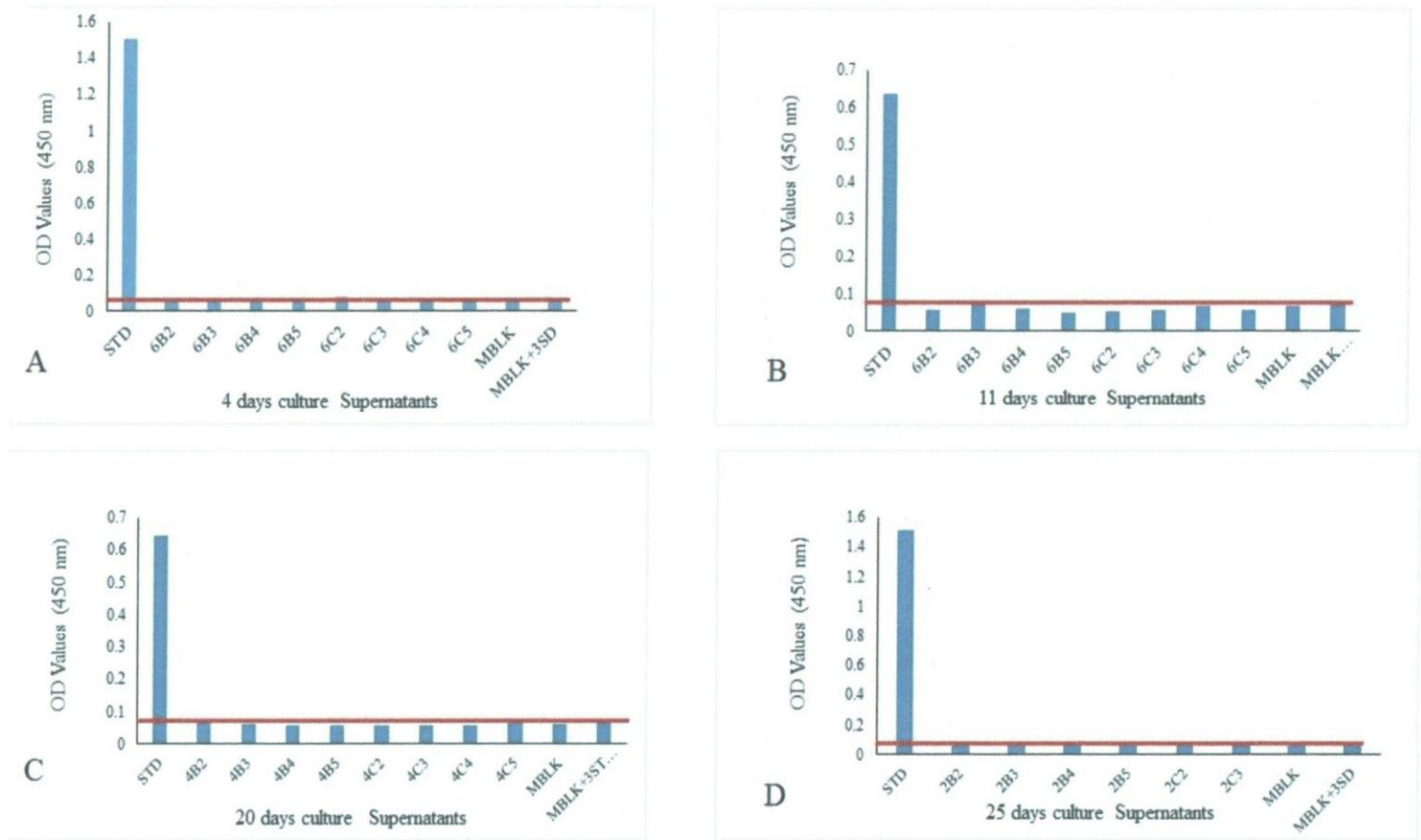

Figure 4.6: Representatives OD Values of supernatant screened for antibodies production against schizonts extract of *Plasmodium falciparum* for V2. The solid red line represents a cut-off for positivity for human IgG established using the mean of medium blank (MBLK) plus three times standard deviations (STDEV), above which supernatants were described as being positive for IgG production.

4.5 Human Antibody Purification and Concentration

After verifying the production of human antibodies by immortalized B cells , non-specific IgGs were purified and concentrated from supernatants of each volunteer. IgGs were purified and concentrated from13 ml supernatant of 11 day old cultures and from 20 ml of 25 day old culture supernatants for each volunteer. The yields of purified IgG from the two time points are presented in Table 2 below. The yields per ml of culture supernatant were much higher on day 11 (1.992 mg/ml for V1, 1.824 mg/ml for V2) compared to yields on day 25 (0.434 and 0.234 mg/ml for V1 and V2, respectively), suggesting a decrease in antibody secreting ability of the immortalized cells with time.

Table 4.2. Yield of purified IgGs from the two volunteers

	Day 11 culture supernatants		Day 25 culture supernatants	
Volunteers	**V1**	**V2**	**V1**	**V2**
Starting volume of supernatants (ml)	**13**	**13**	**20**	**20**
Concentrated volume (µl)	**500**	**500**	**500**	**500**
Concentration (mg/ml)	**1.992**	**1.824**	**0.434**	**0.234**
Amount of IgG (mg)	**0.996**	**0.912**	**0.212**	**0.117**

4.6 SDS PAGE and Western Blotting analysis

Purified human IgGs were further characterized by SDS –PAGE and Western Blotting. Upon staining of gels with Coomassie blue, bands were observed for IgGs from the two study volunteers and for the commercially purified IgG that was used as positive control (Figure 4.7 and Figure 4.8, below). Multiple bands were observed since Coomassie blue stains all protein present. Among the multiple bands, intact IgG was expected to be in a band which corresponds to 160 kDa (Molecular weight of human IgG) (Figures 4.7 A and B) There was a heavier band (> 260 kDa) and other smaller bands (< 80 kDa), and these could be modified/break down products of the purified IgGs.

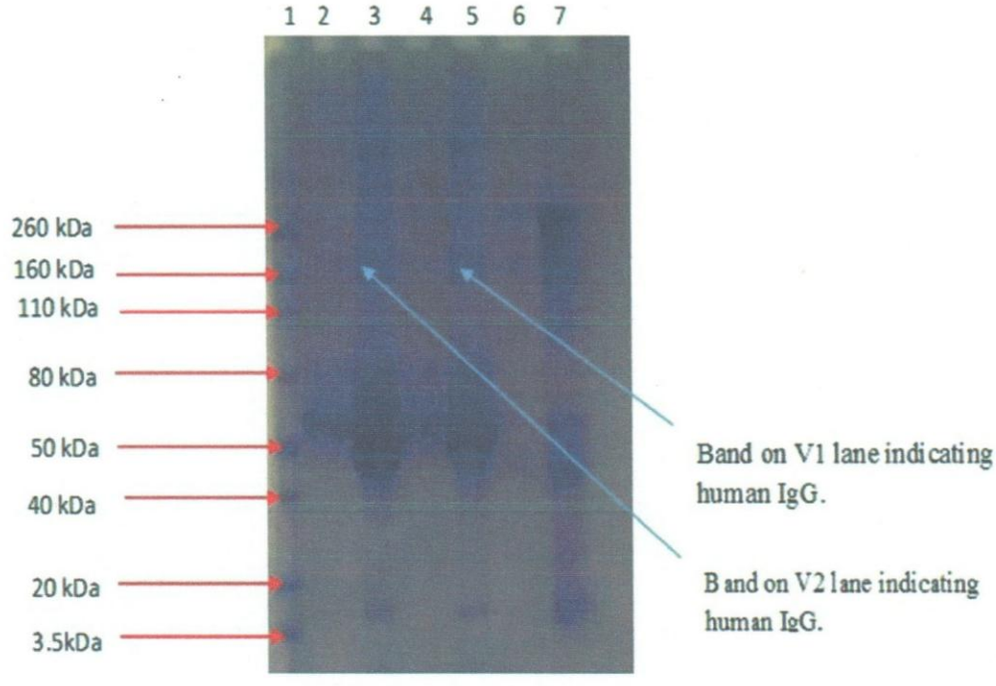

Figure 4.7: Coomassie stain for detection of Human antibodies. Day 11culture supernatants purified antibodies Lane 1 is molecular weight marker, lane 2 is 1.824 µg of V2, lane 3 is 18µg of V2, Lane 4 is 2.0 µg of V1, Lane 5 is 19.92 µg of V1 lane 7 purified IgG set as positive control

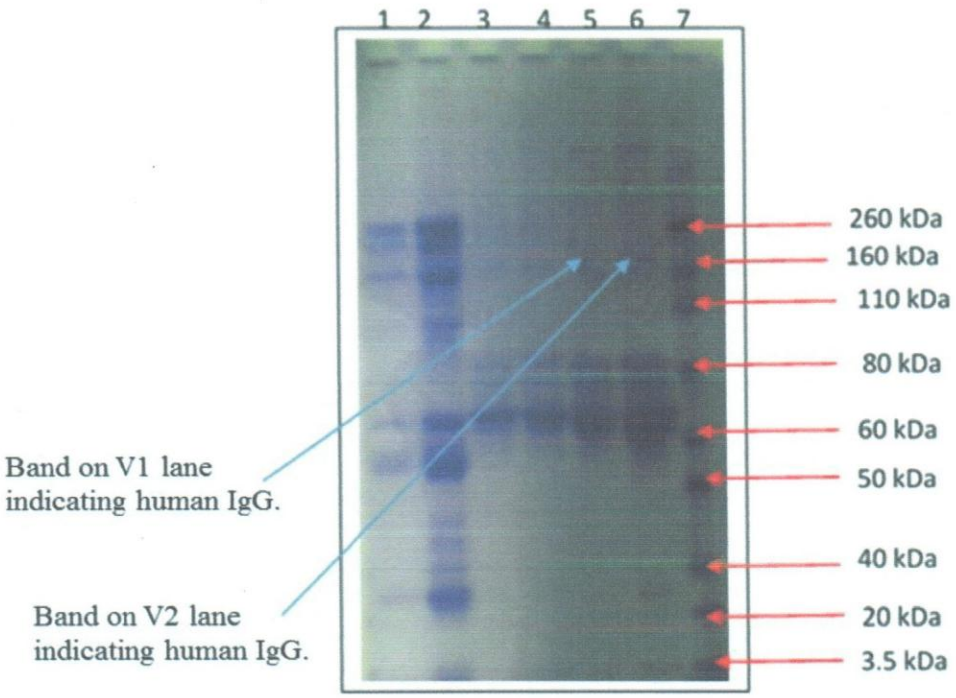

Figure 4.8: Coomassie stain for detection of Human antibodies. Day 25 culture supernatants purified antibodies, lane 2 purified IgG set as positive control, lane 3 is 0.234 µg of V1 lane 4 is 2.34 µg of V1. Lane 5 is 0.434 µg of V2, lane 6 is 4.34 µg of V2 Lane 7 is molecular weight marker,

Also in Western blot analysis was observed that the anti-human IgG-HRP conjugate reacted with human IgG resulted in bands that were observed for V1, V2 and positive control of purified IgG which corresponds to 160 kDa, molecular weight of human IgG presented in Figure 4.9 below. An additional band was also observed which may have resulted from breaking of intra-disulphide of some of the human IgG during heating of sample. The bands that showed corresponding to 160 kDa clearly suggest that the proteins present were indeed human IgG.

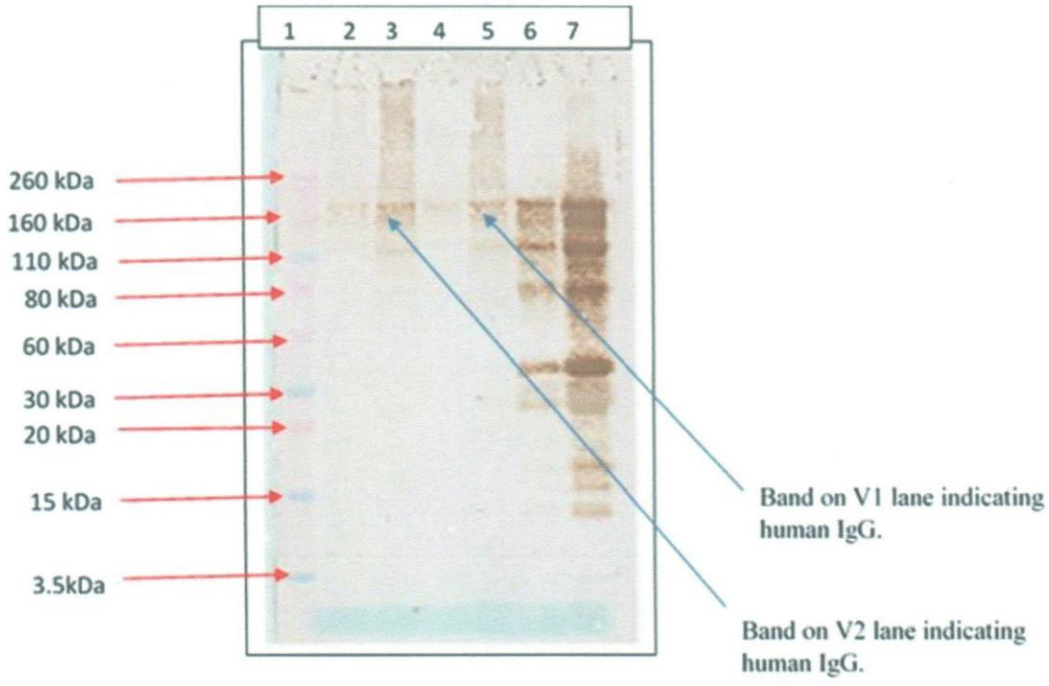

Figure 4.9: Western Blotting for detection of specific antibodies in day 11 culture supernatants purified IgG for V1 and V2. Lane 1 is molecular weight marker, lane 2 is1.824 µg of V2, lane 3 is 18.24 µg of V2, and Lane 4 is 2.0 µg of V1. Lane 5 is 19.92 µg of V1 and lane 6 purified IgG set as positive control.

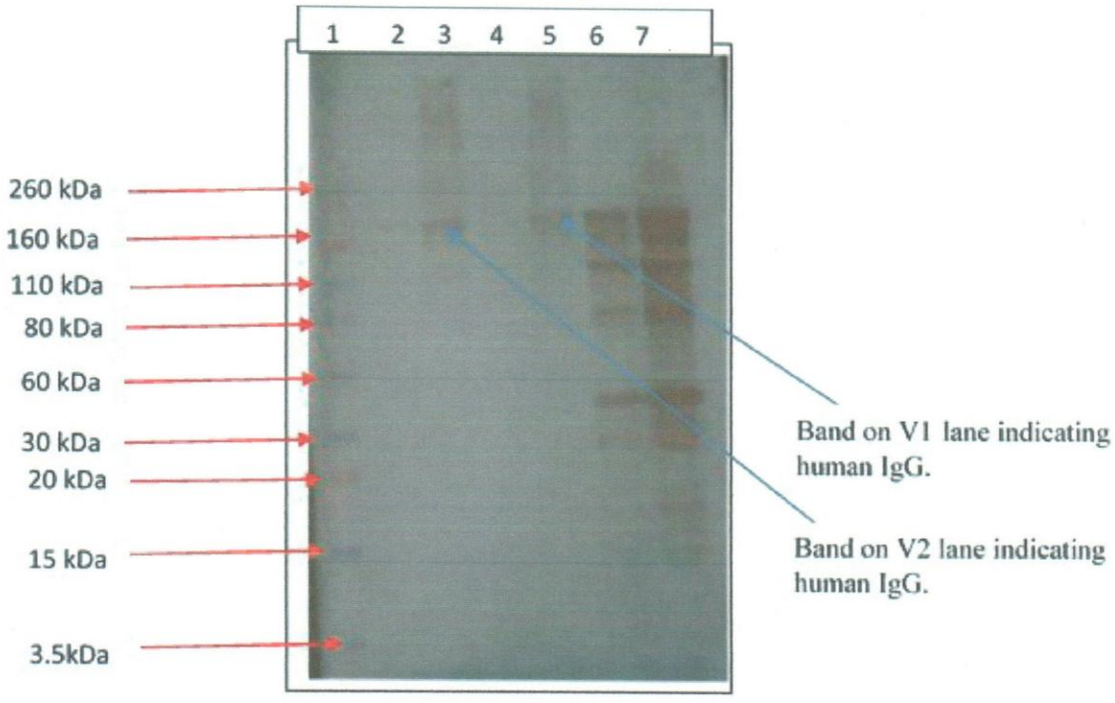

Figure 4.10: Western Blotting for detection of specific antibodies in day 25 culture supernatants purified IgG for V1 and V2. Lane 1 is molecular weight marker, lane 2 is 0.434 µg of V2, lane 3 is 4.34 µg of V2, and Lane 4 is 0.234 µg of V1. Lane 5 is 2.34 µg of V1, lane 6 purified IgG set as positive control.

4.7 Discussion

Antibodies can be found in blood and tissue fluids, and can protect against diseases by binding and neutralizing disease causing microorganisms or by preparing them for destruction by the immune cells. There is increasing usage of antibodies in human diagnostics and therapeutics. This makes the establishment of protocols for the identification, isolation and characterization of antibodies with unique specificity very important, especially in the African setting where active research into etiology and management of infectious diseases is needed. For *in vitro* human antibodies production, Immortalization of B cells is an important procedure to obtain long lived antibody secreting cells. The use of Epstein Barr Virus (EBV) to immortalize B cells in human antibody production have been described (Miyashita *et al.*, 1995; Wang, 2011). The objectives of this study were to immortalize B cells in freshly isolated human PBMCs from malaria exposed individuals, to demonstrate the presence of B cell clones that actively secrete human antibodies by purifying and characterizing expressed antibodies from culture supernatants and to further screen for malaria specific antibodies.

In this study we used an EBV-based kit to immortalize B cells in PBMCs from two malaria exposed individuals. B cells that were successfully transformed proliferated and produced good amounts of antibodies. There was very little malaria-specific antibody production despite the PBMC donors' haven been exposed in the short term to malaria parasites (Figures 4.3, 4.4, 4.5 and 4.6). This observation suggests that despite the possible repeated exposure of the two study volunteers to *P. falciparum*, only a small fraction of their B cell repertoire are specific to the parasite. Similar findings have been described in a previous study with PBMCs from previously exposed individuals (Berford, 2001), but

the frequency of malaria-specific antibody-secreting cells seems to be related to the intensity of transmission as demonstrated by some other studies (Doolan *et al.*, 2009; Muellenbeck *et al.*, 2013). The two volunteers used in this study live in urban suburbs in Accra.

A possible reason for the low prevalence of malaria-specific B cells could be that, malaria infections have been shown to cause loss of memory B cells and antibody secreting cells. For example, Wykes and colleagues (2012) have shown that malarial infections cause a decrease in the proportion of dendritic cells (DCs) that express B cell survival factor (BAFF) resulting in a decreased ability of these DCs to support B cell differentiation and survival of antibody secreting cells (ASCs). They showed in their experimental rodent malaria model that malaria infection leads to a loss of both memory B cells and antibody secreting cells and thus in malaria endemic regions, these cells are compromised in number and in function due to frequent exposure to the parasite which affects BAFF expression by dendritic cells (Liu *et al.*, 2012).

On average, iB cells were maintained in culture for four (4) weeks and within this time human antibodies were detected with high production at 11 days but cells showed signs of decreased growth after four weeks, including a shrinking of clonal cell patches and reduced antibody secretion into culture supernatant (figure 4.3, 4.4 and table 4.2). This Observation was consistent with a study that EBV-containing culture supernatant (from B95-8 cells) which was used for the immortalization. In this study the iB cells rapidly proliferated and was cultured for four (4) weeks with indication of human antibodies production (O'Nions and Allday, 2004). According to another study done by Berford in 2001, that EBV-containing culture supernatant (from B95-8 cells) was also used for the immortalization

and the EBV-immortalized B cells were grown for two (2) weeks and after this time the cells did not grow which they explained that EBV was insufficient to immortalize the celis. The consistency in the present study and O'Nions & Allday (2004) study with respect to human antibody production could be explained by the stimulation of memory cells to *Plasmodium falciparum*, which have already undergone isotype switching and that memory cells were present in our pool of B cells population indicating that EBV can infect memory B cells *in vitro* (Ehlin-Henriksson *et al.*, 2003).

In addition, the life span of iB cells can be influenced by genetic factors, including telomere length. This was proven together with the fact that iB cells continue proliferating while some die in the same culture conditions which exclude the possibility that mortality of iB cells is due to accidental poor culture conditions (Sugimoto *et al.*, 2004).

Although we screened the iB cells supernatants without any detection of *Plasmodium falciparum*-specific B cell clones, we confirmed that our iB cells were immuno-competent as they secreted IgG, albeit of unknown specificity.

The human antibodies were characterized by Western blots with labeled secondary antibodies specific to human IgG. Upon staining SDS PAGE with Coomassie blue, bands appeared for both volunteers corresponding to 160 kDa which is the estimated size of the intact IgG protein (Schirrmann *et al.*, 2010). Other smaller bands between 60 and 80 kDa are most likely IgG breakdown products from the purification. The latter will imply that the elution conditions (with 2.5 – 3M Glycine solution) may have been too harsh despite the neutralization of eluates by the 1M Tris buffer, pH9. This is affirmed by the Western blot data which shows that, apart from the 160 kDa band and a fainter one below it, the

other dense smaller bands did not show since these breakdown products would most likely have lost their antibody recognition sites. The commercially acquired IgG that was added as a positive control also showed similar breakdown products on the Coomassie stain, but these smaller bands were also stained on the Western blot, implying that those fragments still had their antibody recognition sites intact. The reason for these differences between the commercial product and the purified IgGs from the two volunteers with respect to the detection of smaller bands by Western blot is unclear.

CHAPTER FIVE

5.0 CONCLUSION AND RECOMMENDATION

5.1 Conclusion

In this study EBV from *Dendritics*™ successfully immortalized B cells in PBMCs isolated from malaria exposed individuals and these cells could be maintained in *in vitro* culture for up to four weeks. The immortalized B cells cultured *in vitro* were immuno-competent as they produced human antibodies, detectable by both ELISA and Western blot analysis. *P. falciparum*-specific antibodies were however not found after about 20 rounds of testing of culture supernatants, suggesting that parasite-specific B cells were at a very low frequency in isolated PBMCs and culture conditions could not stimulate significant proliferation of these cells.

5.2 Recommendations

Immortalized B cells obtained in this study should be cultured in medium that has CpG to stimulate proliferation, and culture supernatants should be screened further for *Plasmodium falciparum* specific B cells . Any such identified cells should then be cloned and used for the production of monoclonal antibodies of desired specificity. Immortalized B cells in storage can also be screened further for antibodies against other infectious disease agents.

REFERENCES

1. Artavanis-Tsakonas, K. and Riley, E. M. (2002). Innate immune response to malaria: rapid induction of IFN-gamma from human NK cells by live Plasmodium falciparum-infected erythrocytes. *J Immunol, 169*(6), 2956-2963.
2. Banchereau, J. and Steinman, R. M. (1998). Dendritic cells and the control of immunity. *Nature, 392*(6673), 245-252.
3. Barrett, A. D. and Stanberry, L. R. (2009). *Vaccines for biodefense and emerging and neglected diseases*: Academic Press.
4. Berfod, L. (2001). Production of antibodies with specificity for variant surface antigens expressed by P. falciparum involved in the pathogenesis of pregnancy-associated malaria. Københavns Universitet Det Sundheds videnskabelige Fakultet Faculty of Health Sciences, Institut for International Sundhed, Immunology Department of International Health, *Immunology and CMPCMP*, 17-21.
5. Boschetti, E. (2002). Antibody separation by hydrophobic charge induction chromatography. *TRENDS in Biotechnology, 20*(8), 333-337.
6. Bray, R. and Garnham, P. (1982). The life-cycle of primate malaria parasites. *British medical bulletin, 38*(2), 117-122.
7. Carson, C., Quinnell, R. J., Day, M. J. and Courtenay, O. (2010). Comparison of monoclonal and polyclonal antibodies for the detection of canine IgG1 and IgG2, and associations with infection outcome in Leishmania infantum naturally infected dogs. *Vet Immunol Immunopathol, 133*(2-4), 264-268. doi: 10.1016/j.vetimm.2009.07.017

8. Carvalho, L. J., Ferreira-da-Cruz, M. F., Daniel-Ribeiro, C. T., Pelajo-Machado, M. and Lenzi, H. L. (2007). Germinal center architecture disturbance during Plasmodium berghei ANKA infection in CBA mice. *Malaria journal, 6*(1), 59.

9. Chappel, J. A. and Holder, A. A. (1993). Monoclonal antibodies that inhibit< i> Plasmodium falciparum</i> invasion in vitro recognise the first growth factor-like domain of merozoite surface protein-1. *Molecular and biochemical parasitology, 60*(2), 303-311.

10. Chaplin, D. D. (2010). *Overview of the immune response*. J Allergy Clin Immunol 125 (2) : S3 – S23.

11. Cheng, A. and Bell, D. (2006). Evidence behind the WHO guidelines: hospital care for children: what is the precision of rapid diagnostic tests for malaria? *Journal of tropical pediatrics, 52*(6), 386-389.

12. Centers for Disease Control and Preventions (2010). Disease: *Malaria.* (http://www.cdc.gov/malaria/about/disease.htm)l.

13. Chandele A., Mukerjee, P., Das, G., Ahmed, R. and Chauhan,V.(2011). Phenotypic and functional profiling of malaria-induced CD8 and CD4 T cells during blood-stage infection with *Plasmodium yoelii. Immunology*; 132(2): 273–286.

14. Cohen, J. I., Wang, F., Mannick, J. and Kieff, E. (1989). Epstein-Barr virus nuclear protein 2 is a key determinant of lymphocyte transformation. *Proceedings of the National Academy of Sciences, 86*(23), 9558-9562.

15. Cox-Singh, J., Davis, T. M., Lee, K.-S., Shamsul, S. S., Matusop, A., Ratnam, S., Rahman, H. A., Conway, D. J. and Singh, B. (2008). Plasmodium knowlesi malaria in

humans is widely distributed and potentially life threatening. *Clinical infectious diseases, 46*(2), 165-171.

16. Cox, F. E. G. (2010). History of the Discvery of the Malaria Parasite and their Vectors. *Parasite and Vectors*, 1 - 5.
17. DiLillo, D. J., Hamaguchi, Y., Ueda, Y., Yang, K., Uchida, J., Haas, K. M., Kelsoe, G. and Tedder, T. F. (2008). Maintenance of long-lived plasma cells and serological memory despite mature and memory B cell depletion during CD20 immunotherapy in mice. *J Immunol, 180*(1), 361-371.
18. Doolan, D. L., Dobano, C. and Baird, J. K. (2009). Acquired immunity to malaria. *Clinical microbiology reviews, 22*(1), 13-36.
19. Drakeley, C., Corran, P., Coleman, P., Tongren, J., McDonald, S., Carneiro, I., Malima, R., Lusingu, J., Manjurano, A. and Nkya, W. (2005). Estimating medium-and long-term trends in malaria transmission by using serological markers of malaria exposure. *Proceedings of the National Academy of Sciences of the United States of America, 102*(14), 5108-5113.
20. Ehlin-Henriksson, B., Gordon, J. and Klein, G. (2003). B-lymphocyte subpopulations are equally susceptible to Epstein-Barr virus infection, irrespective of immunoglobulin isotype expression. *Immunology, 108*(4), 427-430.
21. Fingeroth, J. D., Weis, J. J., Tedder, T. F., Strominger, J. L., Biro, P. A., and Fearon, D. T. (1984). Epstein-Barr virus receptor of human B lymphocytes is the C3d receptor CR2. *Proc Natl Acad Sci U S A, 81*(14), 4510-4514.

22. Florens, L., Washburn, M. P., Raine, J. D., Anthony, R. M., Grainger, M., Haynes, J. D., Moch, J. K., Muster, N., Sacci, J. B. and Tabb, D. L. (2002). A proteomic view of the Plasmodium falciparum life cycle. *Nature, 419*(6906), 520-526.

23. Goldbaum, F., Leoni, J., Wallach, J. and Fossati, C. (1993). Characterization of an 18-kilodalton Brucella cytoplasmic protein which appears to be a serological marker of active infection of both human and bovine brucellosis. *Journal of clinical microbiology, 31*(8), 2141-2145.

24. Goding, J. W. (1996). Monoclonal antibodies*: principles and practice*: Academic Press.

25. Greenwood, B. M., Fidock, D. A., Kyle, D. E., Kappe, S. H., Alonso, P. L., Collins, F. H. and Duffy, P. E. (2008). Malaria: progress, perils, and prospects for eradication. *The Journal of clinical investigation, 118*(4), 1266-1276.

26. Gupta, S., Snow, R.W., Donnelly, C.A., Marsh, K. & Newbold, C. (1999).Immunity to non-cerebral severe malaria is acquired after one or two infections. *Nature Medicine*; 5(3).

27. Hamilos, D. L. (1989). Antigen presenting cells. *Immunologic research, 8*(2), 98-117.

28. Hoffman, S. L. (1992). Diagnosis, treatment, and prevention of malaria. *Med Clin North Am, 76*(6), 1327-1355.

29. Honjo, T., Nagaoka, H., Shinkura, R. and Muramatsu, M. (2005). AID to overcome the limitations of genomic information. *Nat Immunol, 6*(7), 655-661. doi: 10.1038/ni1218

30. Human Blood Booster Kit. (2010). France: *Immeuble Lenne* 60.

31. Ibraghimov, A. R., and Rokhlin, O. V. (1985). Interaction of heavy and light chains of myeloma immunoglobulin with pooled normal chains: Quantitative analysis of association and restoration of idiotype. *Immunology letters, 9*(6), 313-319.

32. Iqbal, J., Hira, P. R., Sher, A., and Al-Enezi, A. A. (2001). Diagnosis of imported malaria by Plasmodium lactate dehydrogenase (pLDH) and histidine-rich protein 2 (PfHRP-2)-based immunocapture assays. *The American journal of tropical medicine and hygiene, 64*(1), 20-23.

33. Janeway, C. A., Travers, P., Walport, M., and Shlomchik, M. J. (2001). Immunobiology.

34. Jin, A., Ozawa, T., Tajiri, K., Obata, T., Kondo, S., Kinoshita, K., Kadowaki, S., Takahashi, K., Sugiyama, T., Kishi, H. and Muraguchi, A. (2009). A rapid and efficient single-cell manipulation method for screening antigen-specific antibody-secreting cells from human peripheral blood. *Nat Med, 15*(9), 1088-1092. doi: http://www.nature.com/nm/journal/v15/n9/suppinfo/nm.1966_S1.html

35. Karpas, A., Dremucheva, A. and Czepulkowski, B. H. (2001). A human myeloma cell line suitable for the generation of human monoclonal antibodies. *Proc Natl Acad Sci U S A, 98*(4), 1799-1804. doi: 10.1073/pnas.98.4.1799

36. Koenig, R. (1981). Indirect ELISA methods for the broad specificity detection of plant viruses. *Journal of General Virology, 55*(1), 53-62.

37. Kohler, G. and Milstein, C. (1975). Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature, 256*(5517), 495-497.

38. Lam, K. P., Kuhn, R. and Rajewsky, K. (1997). In vivo ablation of surface immunoglobulin on mature B cells by inducible gene targeting results in rapid cell death. *Cell, 90*(6), 1073-1083.

39. Langhorne, J., Ndungu, F. M., Sponaas, A.-M. and Marsh, K. (2008). Immunity to malaria: more questions than answers. *Nature immunology, 9*(7), 725-732.

40. Lantelme, E., Palermo, B., Granziero, L., Mantovani, S., Campanelli, R., Monafo, V., Lanzavecchia, A. and Giachino, C. (2000). Cutting edge: recombinase-activating gene expression and V (D) J recombination in CD4+ CD3low mature T lymphocytes. *The Journal of Immunology, 164*(7), 3455-3459.

41. LeBien, T. W. and Tedder, T. F. (2008). B lymphocytes: how they develop and function. *Blood, 112*(5), 1570-1580. doi: 10.1182/blood-2008-02-078071

42. Lee, R. J., Wu, J. S., Juang, R. H., Wu, Y. J., Lin, C. Y., Wu, S. Y., Huang, P. R., Chu, C. H., Chin, L. T. and Chen, H. M. (2010). Increasing hybridoma viability and antibody repertoire after the cell fusion by the use of human plasma as an alternative supplement. *J Immunol Methods, 361*(1-2), 89-97. doi: 10.1016/j.jim.2010.08.001

43. Lequin, R. M. (2005). Enzyme immunoassay (EIA)/enzyme-linked immunosorbent assay (ELISA). *Clinical chemistry, 51*(12), 2415-2418.

44. Liu, X. Q., Stacey, K. J., Horne-Debets, J. M., Cridland, J. A., Fischer, K., Narum, D., Mackay, F., Pierce, S. K. and Wykes, M. N. (2012). Malaria infection alters the expression of B cells activating factor resulting in diminished memory antibody responses and survival. *Eur J Immunol, 42*(12), 3291-3301. doi: 10.1002/eji.201242689

45. Louie, L. G. and King, M. C. (1991). A novel approach to establishing permanent lymphoblastoid cell lines: Epstein-Barr virus transformation of cryopreserved lymphocytes. *Am J Hum Genet, 48*(3), 637-638.

46. Macdonald, G. (1957).The Epidemiology and Control of Malaria. Oxford University Press, London, ch 4: p 33-43.

47. McCanece, K. L., Huether, S. E., Brashers, V. L. and Rote. N. S. (2010).Pathophysiology, Biologic Basis for Disease in Adults and Children. Pp 219-220. Six Edition. MOSBY Elsevier. 3251 Riverport lane. *Maryland Heights. Missouri* 63043.

48. Medicine (2004). The lifecycle of P. falciparum. *Frontline*; 21(24). (http://www.malariasite.com/malaria/LifeCycle.htm)

49. Miyakoda, M., Kimura, D., Masao, Y., Chinzei, C., Shibata, Y., Honma, K. and Yui, K.(2013).Malaria-Specific and Nonspecific Activation of CD8+ T Cells during Blood Stage of *Plasmodium berghei* Infection. *The American Association of Immunologists*

50. McHeyzer-Williams, L. J. and McHeyzer-Williams, M. G. (2005). Antigen-specific memory B cell development. *Annu. Rev. Immunol., 23*, 487-513.

51. Melchers, F. (2005). The pre-B cells receptor: selector of fitting immunoglobulin heavy chains for the B cells repertoire. *Nat Rev Immunol, 5*(7), 578-584. doi: 10.1038/nri1649

52. Melzner, I., Weniger, M., Menz, C. and Möller, P. (2006). Long-term clonal persistence and evolution of t(14;18)-bearing B cells in healthy individuals. *Leukemia, 20*, 158-162.

53. Milne, C. D. and Paige, C. J. (2006). IL-7: a key regulator of B lymphopoiesis. *Semin Immunol, 18*(1), 20-30. doi: 10.1016/j.smim.2005.10.003

54. Miyashita, E. M., Yang, B., Lam, K., Crawford, D. H. and Thorley-Lawson, D. A. (1995). A novel form of Epstein-Barr virus latency in normal B cells in vivo. *Cell, 80*(4), 593-601.

55. Moody, A. (2002). Rapid diagnostic tests for malaria parasites. *Clinical microbiology reviews, 15*(1), 66-78.

56. Muellenbeck, M. F., Ueberheide, B., Amulic, B., Epp, A., Fenyo, D., Busse, C. E., Esen, M., Theisen, M., Mordmüller, B. and Wardemann, H. (2013). Atypical and classical memory B cells produce Plasmodium falciparum neutralizing antibodies. *The Journal of Experimental Medicine, 210*(2), 389-399. doi: 10.1084/jem.20121970

57. O'Nions, J. and Allday, M. J. (2004). Proliferation and differentiation in isogenic populations of peripheral B cells activated by Epstein–Barr virus or T cell-derived mitogens. *Journal of general virology, 85*(4), 881-895.

58. Okazaki, I. M., Kotani, A. and Honjo, T. (2007). Role of AID in tumorigenesis. *Adv Immunol, 94*, 245-273. doi: 10.1016/S0065-2776(06)94008-5

59. Perlmann, P., and Troye-Blomberg, M. (2004). Malaria and the immune system in humans.

60. Piper, R., Vanderjagt, D., Holbrook, J. and Makler, M. (1996). Malaria lactate dehydrogenase: target for diagnosis and drug development. *Ann. Trop. Med. Parasitol, 90*, 433.

61. Pleass, R. J. and Holder, A. A. (2005). Antibody-based therapies for malaria. *Nature Reviews Microbiology, 3*(11), 893-899.

62. Porsch-Özcürümez, M., Kischel, N., Priebe, H., Splettstösser, W., Finke, E.-J. and Grunow, R. (2004). Comparison of enzyme-linked immunosorbent assay, Western blotting, microagglutination, indirect immunofluorescence assay, and flow cytometry for serological diagnosis of tularemia. *Clinical and diagnostic laboratory immunology, 11*(6), 1008-1015.

63. Radbruch, A., Muehlinghaus, G., Luger, E. O., Inamine, A., Smith, K. G., Dörner, T., and Hiepe, F. (2006). Competence and competition: the challenge of becoming a long-lived plasma cell. *Nature Reviews Immunology, 6*(10), 741-750.

64. Ramakrishna, S., Suresh, B., Kang, I. C. and Baek, K. H. (2010). Polyclonal and monoclonal antibodies specific for USP17, a proapoptotic deubiquitinating enzyme. *Hybridoma (Larchmt), 29*(4), 311-319. doi: 10.1089/hyb.2010.0005

65. Reidy, J. A. and Wheeler, V. A. (1992). Sample age and Epstein-Barr virus transformation of cryopreserved lymphocytes. *In Vitro Cell Dev Biol, 28A*(6), 383-384.

66. Richie, T. L. & Parekh, F. K. (2009).Malaria Vaccines for Biodefence and Emerging and Neglected Disease ch 65:P1309-1364.

67. Roestenberg, M., Teirlinck, A. C., McCall, M. B., Teelen, K., Makamdop, K. N., Wiersma, J., Arens, T., Beckers, P., van Gemert, G. and van de Vegte-Bolmer, M. (2013). Long-term protection against malaria after experimental sporozoite inoculation. *Experimental infection and protection against Plasmodium falciparum malaria in humans, 377*, 193.

68. Rosen, A., Gergely, P., Jondal, M., Klein, G. and Britton, S. (1977). Polyclonal Ig production after Epstein-Barr virus infection of human lymphocytes in vitro. *Nature, 267*(5606), 52-54.

69. Sakaguchi, N. and Melchers, F. (1986). Lambda 5, a new light-chain-related locus selectively expressed in pre-B lymphocytes. *Nature, 324*(6097), 579-582. doi: 10.1038/324579a0

70. Salgame, P., Abrams, J. S., Clayberger, C., Goldstein, H., Convit, J., Modlin, R. L. and Bloom, B. R. (1991). Differing lymphokine profiles of functional subsets of human CD4 and CD8 T cell clones. *Science, 254*(5029), 279-282.

71. Schatz, D. G., Oettinger, M. A. and Baltimore, D. (1989). The V(D)J recombination activating gene, RAG-1. *Cell, 59*(6), 1035-1048.

72. Schirrmann, T., Menzel, C., Hust, M., Prilop, J., Jostock, T. and Dübel, S. (2010). *Oligomeric forms of single chain immunoglobulin (scIgG).* Paper presented at the MAbs.

73. Schmitt, P., Splettstoesser, W., Porsch-Özcürümez, M., Finke, E.-J. and Grunow, R. (2005). A novel screening ELISA and a confirmatory Western blot useful for diagnosis and epidemiological studies of tularemia. *Epidemiology and infection, 133*(04), 759-766.

74. Shapiro-Shelef, M. and Calame, K. (2005). Regulation of plasma-cell development. *Nature Reviews Immunology, 5*(3), 230-242.

75. Spaulding, B., Winther, H., Jensen, S. S. and Boenish, T. (2006). Antibodies *IHC Staining Methods* (pp. 1 - 9).

76. Spickett, G. (2006). *Oxford handbook of clinical immunology and allergy*: Oxford University Press.

77. Steinitz, M. (2014). Production of human monoclonal antibodies by the epstein-barr virus method. *Methods Mol Biol, 1060*, 111-122. doi: 10.1007/978-1-62703-586-6_6

78. Stephens, R., Albano, F. R., Quin, S., Pascal, B. J., Harrison, V., Stockinger, B., Kioussis, D., Weltzien, H.-U. and Langhorne, J. (2005). Malaria-specific transgenic CD4+ T cells protect immunodeficient mice from lethal infection and demonstrate requirement for a protective threshold of antibody production for parasite clearance. *Blood, 106*(5), 1676-1684.

79. Sui, J., Hwang, W. C., Perez, S., Wei, G., Aird, D., Chen, L. M., Santelli, E., Stec, B., Cadwell, G., Ali, M., Wan, H., Murakami, A., Yammanuru, A., Han, T., Cox, N. J., Bankston, L. A., Donis, R. O., Liddington, R. C. and Marasco, W. A. (2009). Structural and functional bases for broad-spectrum neutralization of avian and human influenza A viruses. *Nat Struct Mol Biol, 16*(3), 265-273. doi: 10.1038/nsmb.1566

80. Sun, J. C., and Bevan, M. J. (2003). Defective CD8 T cell memory following acute infection without CD4 T cell help. *Science, 300*(5617), 339-342.

81. Taylor, R. R., Egan, A., McGuinness, D., Jepson, A., Adair, R., Drakely, C. and Riley, E. (1996). Selective recognition of malaria antigens by human serum antibodies is not genetically determined but demonstrates some features of clonal imprinting. *International immunology, 8*(6), 905-915.

82. Thorley-Lawson, D. A. (2001). Epstein-Barr virus: exploiting the immune system. *Nature Reviews Immunology, 1*(1), 75-82.

83. Thermo Fisher Scientific lnc. (2013).Overview of ELISA.

84. Tremblay, S. and Khandjian, E. W. (1998). Successful use of long-term frozen lymphocytes for the establishment of lymphoblastoid cell lines. *Clinical biochemistry, 31*(7), 555-556.

85. Udhayakumar, V., Kariuki, S., Kolczack, M., Girma, M., Roberts, J. M., Oloo, A. J., Nahlen, B. L. and Lal, A. A. (2001). Longitudinal study of natural immune responses to the Plasmodium falciparum apical membrane antigen (AMA-1) in a holoendemic region of malaria in western Kenya: Asembo Bay Cohort Project VIII. *The American journal of tropical medicine and hygiene, 65*(2), 100-107.

86. Understanding Malaria. (2007). *NIAID Science Education*, 1 - 19.

87. Veillette, A., Bookman, M. A., Horak, E. M. and Bolen, J. B. (1988). The CD4 and CD8 T cell surface antigens are associated with the internal membrane tyrosine-protein kinase p56< sup> lck</sup>. *Cell, 55*(2), 301-308.

88. Ventura, M., Gibaud, A., Le Pendu, J., Hillaire, D., Gerard, G., Vitrac, D. and Oriol, R. (1988). Use of a simple method for the Epstein-Barr virus transformation of lymphocytes from members of large families of Reunion Island. *Hum Hered, 38*(1), 36-43.

89. Voller, A., Bidwell, D. and Bartlett, A. (1976). Enzyme immunoassays in diagnostic medicine: Theory and practice*. *Bulletin of the World Health Organization, 53*(1), 55.

90. Wang, S. (2011). Advances in the production of human monoclonal antibodies. *Antibody Technology Journal, 1*.

91. Wang, X. and Hutt-Fletcher, L. M. (1998). Epstein-Barr virus lacking glycoprotein gp42 can bind to B cells but is not able to infect. *J Virol, 72*(1), 158-163.

92. Wellems, T. E., Hayton, K., and Fairhurst, R. M. (2009). The impact of malaria parasitism: from corpuscles to communities. *J Clin Invest, 119*(9), 2496-2505. doi: 10.1172/JCI38307

93. White, N. J. (2008). Plasmodium knowlesi: The Fifth Human Malaria Parasite. *Clinical Infectious Diseases, 46*(2), 172-173. doi: 10.1086/524889

94. Whiteside T. L. (2010). Immune responses to malignancies , *J Allergy Clin Immunol* 125 (2) : S272 – 283.

95. WHO media center (2013). "Malaria". Fact sheet N°94" (http://www.who.int/mediacentre/factsheets/fs094/en/).

96. World Health Organization/Tropical Disease Research (2012).*Malaria.* (http://www.who.int/tdr/diseases/malaria/en/).

97. Williamson, R., Persson, M. and Burton, D. (1991). Expression of a human monoclonal anti-(rhesus D) Fab fragment in Escherichia coli with the use of bacteriophage lambda vectors. *Biochem. J, 277*, 561-563.

APPENDICES

APPENDIX I

BUFFERS FOR PBMC'S ISOLATION

Ethanol Solution for Sterile Work

70 % Ethanol Solution

Volume: 1 L

Components	Amount
Ethanol	700 ml
De-ionized H_2O	300 ml

PROCEDURE:

1. Add 700 ml of ethanol and 300 ml of distilled water

2. Shake well

Dilution medium for PBMC Separation

RPMI -1640 medium, L-glutamin, Penicillin-streptomycin solution.

Volume: 500ml

Components	Amount
RPMI-1640 medium	500ml
Penicillin-streptomycin solution	5ml

PROCEDURE:

1. Take 500 ml of RPMI-1640 medium containing L-glutamine solution and shake well.

2. Take 5 ml off to discard and add 5ml of Penicillin-streptomycin solution and shake well

Fresh Cell Wash for PBMC Separation

5 % Foetal Calf Serum (FCS)

Volume: 500 ml

Components	**Amount**
RPMI-1640 medium	500 ml
Penicillin-streptomycin solution	5 ml
Foetal Calf Serum	25 ml

PROCEDURE:

1. Take 500 ml of RPMI-1640 medium which contains L-glutamine solution and shake well.

2. Take 5 ml off and add 5 ml of Penicillin-streptomycin solution and shake well.

3. Take 25 ml off and add 25 ml of Heat Inactivated Foetal Calf Serum and shake well.

4. Filter sterile with 0.22 µm Millipore filter.

APPENDIX II

BUFFERS FOR ELISA ANALYSIS.

Buffer	Composition	Amount
Wash buffer	PBS, tween-20	0.05 % tween in PBS
Blocking buffer	Skimmed milk	5 % skimmed milk in PBS
Diluent buffer	Skimmed milk	1 % skimmed milk in PBS

APPENDIX III

Buffers for human IgG purification

Buffer	Composition	Amount
Binding Buffer	Phosphate	0.1 M
Wash Buffer	Phosphate-buffered saline	0.1 M
Elution Buffer:	Glycine	0.1 M
Neutralization Buffer	Tris-HCl	1.0 M

APPENDIX IV

BUFFERS FOR SDS PAGE, COOMASSIE STAIN AND WESTERN BLOT

Coomassie staining solution

Composition of working solution	Component	Amount per 100 ml
0.05 % (w/v) Coomassie Brilliant Blue R-250	Coomassie Brilliant Blue R-250	50 mg
40 % (v/v) ethanol	Ethanol	40 ml
10 % (v/v) glacial acetic acid	Glacial acetic acid	10 ml
50 % (v/v) water	Water	50 ml

De-staining solution

Composition of working solution	Component	Amount per 100 ml
40 % (v/v) ethanol	Ethanol	40 ml
10 % (v/v) glacial acetic acid	Glacial acetic acid	10 ml
50 % (v/v) water	Water	50 ml

Transfer Buffer

Composition of working solution	Component	Amount per liter
25 mM Tris base	Tris base	3.0 g
150 mM glycine	Glycine	11.3 g
10% (v/v) methanol	Methanol	100 ml

Tank-Blotting Transfer Buffer

Composition of working solution	Component	Amount per liter
25 mM Tris base	Tris base	3.0 g
150 mM glycine	Glycine	11.3 g
20% (v/v) methanol	Methanol	200 ml