Genetic Aspects of Malaria: Human Leucocyte Antigen (Hla) Dqb Polymorphism and Antigen Recognition Patterns in Women at Delivery

Jude, D. BIGOGA[1], Simon METENOU[2], Christina GINSBERG[3], Armead H JOHNSON[3], Vicent, P.K. TITANJI[1], Rose, G.F. LEKE[3].

[1]Faculty of Science, Department of Life Sciences, University of Buea. [2]Faculty of Science, University of Yaounde 1, [3]Georgetown University medical school, Washington DC, USA. [3]Faculty of Medicine and Biomedical Sciences, University of Yaounde 1.

ABSTRACT

To investigate whether parasites' and patients' genetic diversity affect the outcome of malaria in women at delivery, HLA DQB typing and malaria antigen recognition studies were carried out. Accordingly, HLA typing was carried out on 35 women, 15 of them with malaria-infected placentas and the remaining 20 without. DNA probes were used to hybridize patients' placental blood leucocytes DNA to investigate polymorphisms at the HLA DQB1 locus. Eleven (31%) of the women were homozygous with a single identifiable allele. The remaining 24 (69%) were heterozygous with at least two identifiable alleles. Next, *Plasmodium falciparum* parasite proteins were isolated from infected placentas, separated by SDS-PAGE and immunoblotted to identify parasite antigens. The immunoblots were probed with the patients' antibodies and the patterns between malaria-infected and non-infected women compared. Protein antigen profiles showed up to 19 bands in some women. Antigens with molecular weights (MW) 22, 32 61, 86, 93 and 100KD were frequently recognized by infected women. Non-infected women recognized preferentially antigens with MW 13, 27, 58, 102, 143 and 223KD that may be predictive of protection in the parasite. The homozygous women would be more susceptible to malaria, whereas the heterozygous ones are likely to be more competent in handling the plethora of malaria antigens detected.

Key words: malaria, *P. falciparum*, HLA polymorphism, Placenta, immunoblot, Cameroon,

RESUMÉ

La typage du HLA DQB1 et la détection d'antigène palustre ont été menées pour comprendre l'influence de la diversité génétique des patients et des parasites sur l'issue du paludisme chez les femmes parturiente. Le HLA a été caractérisé à l'accouchement chez 35 femmes, dont 15 avec placenta infecté (P+ve) de *Plasmodium* et 20 avec placenta non infecté (P-ve). Les sondes-ADN ont été utilisées pour hybrider le DNA leucocytaire isolé de placenta et étudier le polymorphisme dans le locus DQB1 de HLA. Onze (31%) des 35 femmes étudiées étaient homozygotes avec une seule allèle identifiable. Les 24 (69%) autres étaient hétérozygotes avec au moins deux allèles identifiables. La deuxième partie de ce travail consistait en l'isolement des protéines de *Plasmodium falciparum* des P+ve et séparation par SDS-PAGE-immunoblot pour identification d'antigènes parasitaires. Les immunoblots étaient sondés avec les anticorps de patients et les résultats entre femmes infectées et femmes non infectées furent comparés. Le profile protéique a montré jusqu'à 19 bandes détectables chez certaines femmes. Les antigènes de poids moléculaire (PM) 22,32 61, 86, 93 et 100 KD étaient fréquemment reconnus par les femmes P+ve. Les femmes P-ve reconnaissaient préférentiellement les antigènes de PM 13, 27, 58, 102, 143 et 223 KD. Cette étude a montré que les femmes homozygotes seraient plus susceptibles au paludisme tandis que celles hétérozygotes sont plus à même de résister à la pléthore d'antigènes détectés. Les antigènes parasitaires préférentiellement reconnus par les femmes P+ve seraient prédictifs de la protection du parasite.

Mots clés: malaria, *P. falciparum*, HLA polymorphisme, Placenta, immunoblot, Cameroun,

INTRODUCTION

Malaria remains a major cause of morbidity and mortality worldwide. About half the world's population is under the continuous risk of malaria infection with over 90% of the global clinical and death cases occurring in tropical Africa (WHO, 1996). The most affected are children under the age of five and pregnant women. In the latter category, malaria jeopardizes the life of both mother and foetus. Immunological studies have shown that pregnancy predisposes women to malaria infection (Mutabingwa, 1994; McGregor, 1984; Brabin, 1984) due to a depression in the immune status of the mothers (Riley *et al*, 1989). In highly endemic areas, parasitization of the placenta is common (Menendez *et al*, 1993) and often associated with serious pregnancy complications such as stillbirths, abortions, low birth weights and the intra-uterine growth retardation of the foetus (Fievet *et al*, 1995; cot *et al*, 1993; Riley *et al*, 1989; Blacklock and Gordon, 1952). The malaria parasite appears deceptively simple but very complicated, exhibiting an infinite complexity and exuberant diversity of life. This makes it extremely difficult to understand its life and even its relationship with the host. Zuckerman (1966) presumed the existence of several antigens in infection with even a single species of *Plasmodium* (McGregor, 1971). Such antigenic diversity could be the result of the increased susceptibility to infection by the human host, and an impediment to vaccine development as the parasite carefully adapts itself to evade immune recognition by HLA. HLA is a genetic locus encoding cell surface molecules essential for reactions of immune recognition. It has one of the most polymorphic and polygenic genes known in humans. HLA DQB is the most polymorphic of all HLA class II molecules with its antigenic diversity limited to the peptide binding sites. They exhibit broad binding specificities, binding many different antigenic peptides. HLA polymorphism is driven by infectious agents, which include malaria parasites, and ensures that at least some members of a species will be able to respond to anyone of a large number of potential antigens (Gregersen, 1989; Janeway &Travers, 1994; Janis, 1994). Within this study, we investigate on the association of the mechanisms of HLA DQB polymorphism and the outcome of malaria towards contributing to the understanding of the genetic basis of immunity to malaria in women who deliver with malaria infection compared to non-infected patureats.

MATERIALS AND METHODS:

Study site and sample collection:
The study took place in Yaounde, the capital city of Cameroon. A total of 50 women were recruited for this study after they were fully consented. The national ethical committee of Cameroon provided the ethical clearance for this study. Placental blood (10ml in EDTA anticoagulant tubes) and placental biopsies (Rasheed et al, 1992) were collected from the 50 women who turned up for delivery at the Biyem-Assi and Yaounde central hospitals. A fraction of each blood sample was used for HLA DQB typing while the remainder and biopsies were used for antigen recognition studies.

1.   HLA typing

1.1- Isolation of Leucocyte DNA
Leucocyte DNA was isolated from each of the blood sample by the sucrose/triton procedure (Kawasaki, 1990; Higuchi, 1989) as follows: EDTA anticoagulated whole blood (500µl) was mixed with 0.5ml lysis buffer (0.32Msucrose, 10mMtris-HCl, 1% Triton X-100, 5mM $MgCl_2$), vortexed and centrifuged at 13000xg for 20s. Twice the supernatant was aspirated, pellet re-suspended in 1ml lysis buffer and centrifuged at 13000 x g for 20s. The supernatant was decanted and pellet re-suspended in 1ml PBS, centrifuged at 13000-x g for 20s and again, supernatant decanted and pellet re-suspended in buffer with non-ionic detergents (50mM KCl, 10mM Tris-HCl pH 8.3, 0.1mg/ml gelatin, 0.45%NP-40, 0.45%Tween 20) and proteinase K. Following incubation for 1hr at 60°c then at 95°C for 10min .to denature the proteinase K, 2.5µl was immediately used for amplification or stored at 20°C for future use.

1.2- DQB1 Amplification:
This was done following a Polymerase Chain Reaction (PCR) amplification of the second exon at the DQB locus using the INNO-LiPA HLA DQB amplification procedure (Gregersen, 1989). A total reaction volume of 45ìl (10ìl amplification buffer, 10µl DQB primer mix, 10ìl $MgCl_2$ 14.6µl autoclaved distilled water and 0.4µl 5U/µl Taq polymerase) was amplified through an initial denaturation cycle of 95°C for 5min, followed by a 35-cycle denaturation step at 95°C for 30s, a primer-annealing step at 58°C for 20s, elongation at 72°C for 30s, then a terminal elongation step at 72°C for 10min. The amplified products were electrophoresed on a 1.2% ethidium bromide treated agarose gel alongside a ΦX1747 DNA molecular weight marker to check for proper amplification. Controls consisting of tubes without template DNA were included. Observing under a UV illumination, only samples with bands positive for DBQ1 (250bp – 270bp) were typed.

1.3- DQB1 Typing

DQB1 typing was performed according to the INNO-LiPA HLA DQB typing protocol as follows: 10µl of the amplified DNA was mixed with 10µl denaturation solution (Alkaline solution containing EDTA) and allowed to denature at room temperature for 5min. The resulting single strands were reverse hybridized to DQB1 oligonucleotide probes immobilized as parallel lines on nitrocellulose membrane based strips. Streptavidin labeled with alkaline phosphatase was added and bound to any previously formed biotinylated hybrid. Following addition of the substrate [5-Bromo-4-Chloro-3-indolyl phosphate/Nitro Blue Tetrazolium salt (BCIP/NBT)], positively hybridizing probes formed a purple precipitate. The reaction was stopped by washing strips twice in distilled water. Positively hybridizing probes were identified and interpreted following the INNO-LiPA typing chart and reading table (Decorte et al, 1994).

2. Malaria antigen Recognition studies

2.1- Antigen preparation

Placental impression smears were made and microscopically examined for the presence of parasites. *Plasmodium falciparum* parasites were extracted from biopsies that had at least 5% parasitaemia by gently teasing the biopsy in a petri dish using forceps and washing in PBS. This was filtered over a sterile gauze pad into a clean tube. The residual tissue was discarded and the filtrate carefully layered on a ficoll-hypaque medium and centrifuged at 3000 x g for 25min. separation yielded plasma and PBS as supernatant, placental blood mononuclear cells (PBMCs) and mature schizonts at the interphase of ficoll and PBS, and infected red blood cells (IRBCs) and granulocytes as pellet. PBMCs and IRBCs were then carefully aspirated and layered on a six-step percoll gradient medium (55%, 45%, 35%, 30%, 25% and 10% from bottom to top of tube) prepared from a 90% percoll gradient (9parts percoll and 1part RPMI-1640). Each tube was centrifuged at 3000rpm for 25min and yielded separate fractions of normal red blood cells (NRBCs) as pellet, early pure red cells just above the 55%, IRBCs above 40% and pure parasites at the interphase of 10% and 20%. IRBCs were aspirated, dispensed in fresh tubes and washed twice by centrifuging in PBS. Pellet was used to prepare slides for microscopy to determine the final parasitaemia.

2.2- Imunobloting:

The isolated parasites were pooled and sonicated. Parasite proteins (diluted 1: 20 in SDS-PAGE gel loading buffer) were resolved alongside a rainbow™ molecular weight marker (Amersham-Life science) by SDS-PAGE. The separated proteins were immunoblotted (Sambrook et al, 1989), the unbound portions of the blot blocked in blocking buffer (5%non-fat milk, PBS, 0.5% Tween 20), washed twice in wash solution IPBS-Tween 20) for 5min each and then sliced into strips for the identification of parasite antigens. Each strip was probed with individual patient's antibodies (1:2000 in 1% non-fat milk) for 2hrs, washed twice for 5 min and a conjugate antibody (goat anti human IgG-Alkaline phosphatase conjugated) diluted 1:2000 in 1% non-fat milk/PBS/Tween 20 added for 1hr. Following another similar wash step, the substrate [5-Bromo-4-Chloro-3-indolyl phosphate/Nitro Blue Tetrazolium salt (BCIP/NBT)] was added. Within 15min of incubation in the dark, the antigen profiles between malaria-infected and non-infected women were compared. Control strips were probed with normal European serum from Europeans who had never contracted malaria

RESULTS

Eleven (31%) of the 35 women were found to be homozygous at the DQB1 locus having only one identifiable allele while the remaining 24 (69%) were found to be heterozygous having at least two identifiable alleles. Statistical distribution of the alleles amongst the successfully typed women depicted the following: (a) the alleles DQB1* 0602 (42.8%) and 0301 (34.8%)frequently occurred amongst the women; (b) Alleles DQB1* 0201, 03032, 0402 and 0501 were moderately represented in10% to 30% of the women; (c) Alleles DQB1*0604, 06041, 06051, 0607, 0608 and 0609 represented in less than 10% of the women. Some of the DQB1* alleles (0302, 0304, 0305, 0401,0502,05031, 05032, 0504, 06011, 06012 and 0606) were not found amongst the women.

The parasite protein antigen profiles revealed up to 19 detectable bands in women with parasitized placentas and 18 bands in the non-parasitized group. Antigens with molecular weights (MW) 22, 32, 61, 86, 93 and 100 KD were observed to be more frequent in women with infected placentas while the protein bands with MW 13, 27, 58, 102, 143 and 223 KD were preferentially recognized by women with infected placentas. Most remarkably, IgG used to reveal the antigen profile was generally found to have stronger affinities to antigens found exclusively in women with infected placentas as observed from the intense nature of the bands. Also, most of the women found to be homozygous at the DQB1 locus were found to be in the infected group while most of the heterozygous women were in the non-infected group.
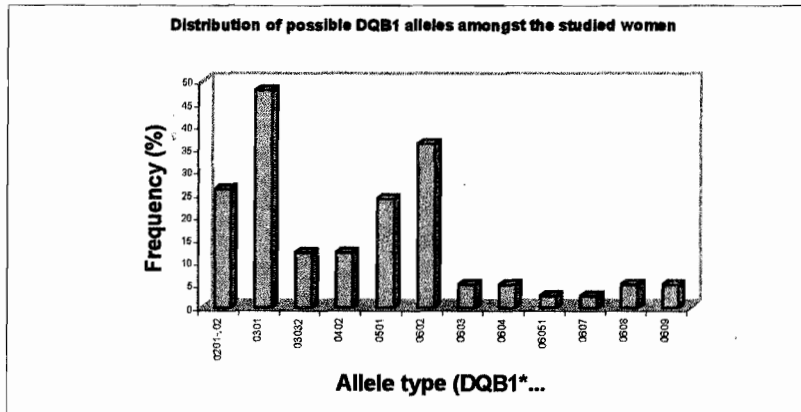
RESULTS

a) HLA typing

Fig 1: Distribution of possible DQB1 alleles in the studied subjects
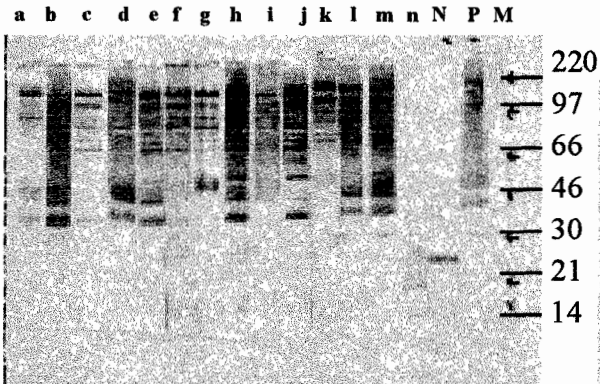
b) Malaria antigen recognition

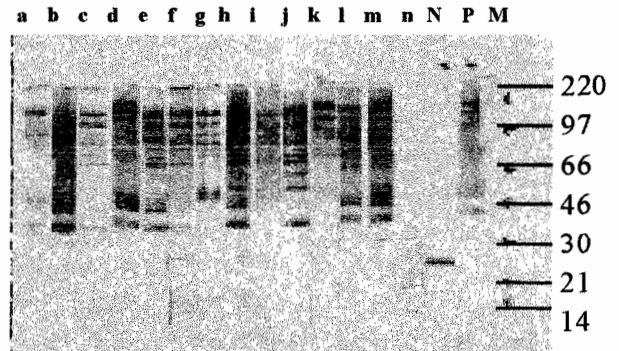**Fig 2a:** Malaria positive women at delivery

Fig 2b: malaria negative women at delivery

M = molecular weight marker; **a-n** = study subjects; **N** = Normal red blood cell protein;
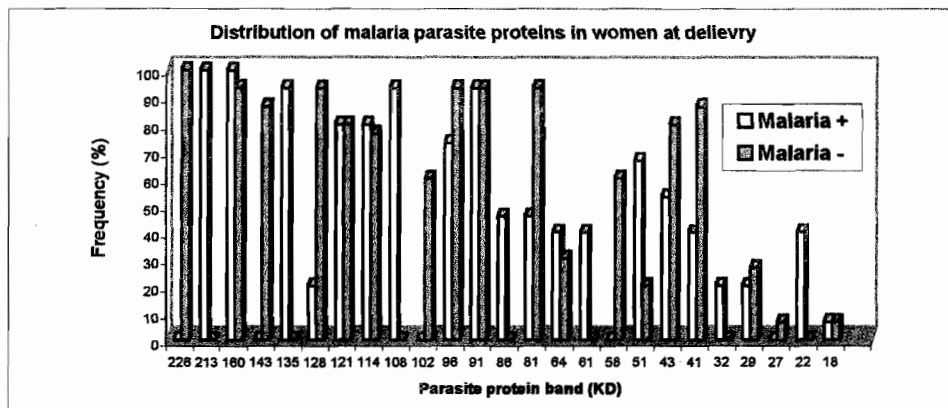**P** = Pooled serum for the studied subjects

Fig 2c: Frequency distribution of malaria parasite proteins in women at delivery

DISCUSSION

The findings of this study demonstrated genetic polymorphism at the HLA DQB1 locus and the existence of multiple malaria parasite protein bands. HLA polymorphism is known to affect antigen recognition by controlling peptide binding, and extending the range of antigenic peptides, which the immune system can respond to (Janeway and Travers, 1994). The multiple malaria parasite protein bands observed is akin to the presumptions of Zuckerman, (1966). This might suggest genetic variation in the parasite, antigenic diversity being one way by which parasites may evade immune recognition. HLA polymorphism thus provides strategies for overcoming such diversity.

Protein bands preferentially recognized by women with non-parasitized placentas could be predictive of protection, having no role in disease manifestation or may be, a higher degree of exposure is required to generate protective antibodies to these proteins (Rasheed *et al*, 1993). Immunoglobulin G (IgG) used to reveal the antigen profile was generally found to have stronger affinities to antigens found exclusively in women with infected placenta suggesting that the protein bands preferentially recognized by women with parasitized placentas may be highly implicated in disease manifestation.

Heterozygosity at the DQB1 locus in a proportion of these women (mostly non-infected) suggests genetic polymorphism, reflecting the versatility of HLA in handling the plethora of *Plasmodium* antigens, and therefore reduced susceptibility. Similarly, Hill et al, (1991) demonstrated that HLA B53 is associated with protection from severe anaemia and cerebral malaria and HLA that DRB1*1302 –DQB1* 0501 confers protection from severe anaemia, which is a clinically associated with malaria (Meyer *et al*, 1996). On the contrary, homozygosity at the DQB1 locus in some of these women (mostly infected) is restrictive, reflecting reduced chances of coping with the multiple malaria parasite antigens. However, it is worth mentioning that HLA DQB is only a small portion of HLA class II and therefore the association between HLA DQB1 alleles and the outcome of malaria cannot be over emphasized at this point due to the rather limited sample size. A wide range of polymorphism could be predicted if studies are carried out with other HLA class II regions and also considering a larger sample size and the level of malaria endemicity in the study area. As indicated by Mary *et al*, (1997), consideration should also be given to the ethnic group because HLA polymorphism is related to ethnicity and/or a given working population. A given HLA molecule may confer protection to individuals in one endemic area but not in other endemic areas

and protective malarial mechanisms may involve more than one HLA associations (Hill *et al*, 1991; Meyer *et al*, 1996). The parasite proteins if sequenced may provide useful information in designing peptides that can allow us rightly predict the binding motifs to HLA class II molecules.

REFERENCES

Blacklock, D. B. and Gordon R. M. (1925). Malaria infection as it occurs in late pregnancy; Its Relationship to Labour and early infancy. *Ann. Trop. Med. Parasitol.* 19:327-365.

Brabin B. J. (1983). An analysis of Malaria in pregnancy in Africa. *Bulletin of the World Health Organization,* 61: 1005-1015

Cot M. (1992). Effect of chloroquine chemoprophylaxis during pregnancy on birth weight. Results of a randomised trial. *Am. J. Trop. Med. Hyg.* 46: 21-27.

Buyse I., Decorte R., Cuppens H. (1993). Rapid DNA typing of class II HLA antigens using the polymerase chain reaction and reverse dot blot hybridization. *Tissue antigens.* 41 : 1-14

Decorte R., Buyse I.,Zamani M. (1994). Distribuion of HLA class II genes in caucasian population as determined by PCR and reverse dot-blot-typing. *In :Bar W, Friori A, Rossi U, Eds. Adv. In Forensic Haemogenetics 4 : 490-492.*

Fievet N., Michel C., Claire C., Bertrand M., Jean B., Beatrice D., Jean Pierre Y., Yvelines F., Florence M., Felix R., Jean P.V., Francis L., Philippe D. (1995). Malaria and Pregnancy in Cameroonian Primigravidae: Humoral and Cellular Immune Responses to *P. falciparum* Blood Stage Antigens. *Am. J. Trop. Med. Hyg.* 53(6): 612-617

Gregersen P. K. (1989). Biology of Disease HLA Polymorphism. Implications for genetic susceptibility to anti immune disease. *Lab. Invest.* 61: 5-19.

Higuchi R. (1989). *Simple and Rapid Preparation of Samples for PCR.* Erlich, H.A. Ed PCR Technology: Principles and Applications for DNA amplification. New York: Stoton press, p.31.

Hill A. V., Allsop C. E., Kwiatkowski D., Anstey N. M., Twumasi P., Rowe P. A., Bennett S., Brewster D., McMichael A. J. and Greenwood B. M. (1991). Common West African HLA antigens are associated with protection from severe malaria. *Nature*. 352; 595 - 600.

Janis Kuby (1994). *Immunology* W.H. Freeman and Co. New York, 210.

Janeway C. A. and Travers P. (1994) *Immunobiology: The Immune System in Health and Disease*. Current biology limited, London, California, Philadelphia.

Kawasaki E. S. (1990). Sample Preparation for Blood, Cells and Other Fluids in PCR Protocols: A Guide to Methods and Applications. Innis M A., Gelfand D. H., Sninsky J. J., and White T.J. Eds. Academic Press, New York, 146.

Mary S. L., Donnenberg A. D., Noel R. R. (1997). *Handbook of human immunology*. CRC press. Bocaraton. New York. 523.

McGregor I. A. (1971). Immunity to Plasmodial infections. Consideration for factors relevant to malaria in Man. *Int. Rev. Trop. Med.* 4:1-5.

McGregor I. A. (1984). Epidemiology, Malaria and Pregnancy. *Am. J. Trop. Med. Hyg.*33: 571-525.

Menendez C., Todd J., Alonso P. I., Francis V., and Greenwood B. M. (1993). Malaria chemoprophylaxis, infection of the placenta and birth weight in Gambian primigravidae. *Trans. Roy. Soc. Trop. Med. Hyg.* 88: 311-312

Meyer C. G. and Kremsner P. G. (1996). Malaria and Onchocerciasis: On HLA and related matters. *Parasitol. Today.* 12(5): 179-186.

Mutabingwa T. K. (1994) Malaria and Pregnancy: Epidemiology, Pathophysiology and Control Options. *Acta Tropica.* 57: 239-254.

Rasheed F. N., Bulmer J. N., Morrison L., Jail M. F. B., Hassan-King M., Riley E. M., Greenwood B. M. (1992). Isolation of maternal mononuclear cells from placentas for use in *in vitro* functional assays. *J. Immunol. Methods.* 142(6): 185-193.

Rasheed F. N., Bulmer J. N., Dunn D. T., Menendez C., Jawla M. F. B., Jepson A., Jakobsen P. H., and Greenwood B. M. (1993). Suppressed peripheral and placental blood lymphoproliferative responses in first pregnancies. *Am. J. Trop. Med. Hyg.* 48(2): 154-160

Riley E. M., Schneider G., Sambou I., Greenwood B. M. (1989). Suppression of cell mediated immune responses to malaria antigens in pregnant Gambian women. *Am. J. Trop. Med. Hyg.* 40: 141 - 144.

Riley E. M., (1992). MHC and Malaria. The relationship between HLA Class II alleles and immune responses to *P. falciparum*. *Intern. Immunol.* 4: 1055 - 1063.

Sambrook J., Fritsch E. F. and Maniatis (1989). Molecular cloning. *A laboratory manual.* New York: Cold spring Harbor Laboratory press.

World Health Organization (1996). World malaria situation in 1993. *Wkly Epid. Rec.* 69: 309 - 316.