IMMUNOLOGICAL INVESTIGATION IN NIGERIANS INFECTED WITH CHLOROQUINE RESISTANT *PLASMODIUM FALCIPARUM*

BY

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DOCTOR OF PHILOSOPHY
UNIVERSITY OF IBADAN

AUGUST, 1991

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DEDICATION

THIS WORK IS DEDICATED TO THE FOLLOWING

ALMIGHTY GOD
for His Mercies

MY WIFE, GRACE
for her endurance and love

MY CHILDREN, UKO, UBONG, EMEM
and UTIBE-ABASI
for their understanding and Love.

MY MOTHER, MRS. NENE BEN UMO-OTONG
for caring.

MY FATHER, CHIEF B. UMO-OTONG
for his encouragement and deep concern over my endeavours.
He is really a father.

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ABSTRACT

In most populations where malaria is endemic, chloroquine has been used as a first line of treatment against P. falciparum. However, with the emergence of chloroquine resistant P. falciparum (CRPF) malaria in many countries including Nigeria, éfforts are being intensified on immune intervention in malaria control. Prospective vaccine candidates are being identified, but the changing antigenic components of the parasite, manifested in each emerging strain, is hampering efforts in immune intervention. Host genetic variation in immune response against some important malaria antigenic entities, is also constituting a serious drawback. Therefore there is the need for the evaluation of chloroquine susceptibility of P. falciparum and seroepidemiological surveys, in respect of immune response to prospective malaria vaccine candidates, in populations where P. falciparum infection is endemic.

Isolates from 102 Nigerian children with acute infections were used in evaluating chloroquine sensitivity of P. falciparum using schizont inhibition assay (in vitro) technique. Sixty-two of these patients were drawn from Calabar and 40 from Ibadan. The patients were recruited at the respective Teaching Hospitals. Thirty-three of the patients in Calabar were selected (based on standard criteria) for simultaneous in vivo monitoring of susceptibility

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of the parasite species to standard doses of chloroquine (C25) using the WHO extended 14-day assessment method. Serum samples of all the patients and of 80 age/sex-matched apparently healthy controls were also analysed for some immunological parameters associated with malaria infection.

The results indicate a higher prevalence level of CRPF in Calabar (59.2%) when compared to Ibadan (35.3%), by *in vitro* methods, with a much lower cumulative Ec99 (effective concentration of chloroquine that inhibits 99% of schizont maturation) in the latter than in the former population (2.4×10^{-6}M Vs 4.6×10^{-6}M). A prevalence level of 53.6% (*in vivo*) in Calabar was recorded giving a high correlation between *in vitro* (59.2%) and *in vivo* methods of assessment used in that population. On the overall, children infected with chloroquine sensitive *P. falciparum* showed a higher geometric mean parasite density on the day of diagnosis (DO), ($P < 0.01$ in Calabar and $P < 0.05$ in Ibadan).

Eighty percent of patients and 73.3% of the control subjects were seropositive for total blood stage antigen (ELISA). Higher seroreactivity against these antigens was observed in children infected with CRPF than in those infected with the sensitive strains, suggesting that a marker for CRPf may be embodied within the CRPf strain of the parasite. There was no difference in IFA titres

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between individuals infected with CRPf and those infected with the chloroquine sensitive strain.

Forty-four percent of the patients and 29% of the control subjects were seropositive against circumsporozoite (CS) protein. The mean OD_{492} of anti-CS antibody reactivity between the two groups of infected patients studied, both in Calabar and in Ibadan, were not significantly different, indicating that all the patients had apparently similar sporozoite inoculation rates.

Mean levels of total serum IgM, IgG and its 4 subclasses, and that of the alternative complement pathway factor B (Bf) were higher in malaria patients as compared to the controls. Differences in each of these parameters were also observed between subjects infected with CRPf and those infected with the sensitive strain, however, these differences were significant only in respect of IgG3 subclass where the mean level was higher in subjects infected with CRPf. Mean serum C3 level was lower but not significantly, but C4 level and CH50 activity were significantly lower in patients than in the controls.

Generally, it was observed that serum levels of some of the investigated humoral immune parameters varied slightly from previously reported levels within the same population, thus reflecting possible changing antigenic

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stimuli with time.

The immunological relevance of the varying CRPf prevalence levels in the two geographical populations studied and of the differences in immunological profiles in the two groups of infected patients investigated are evaluated. The findings have potential implications in the prospective malaria immune intervention in Nigeria.

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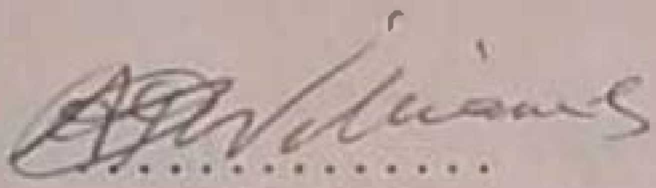
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CHAPTER 1

GENERAL INTRODUCTION

1.1. BACKGROUND INFORMATION

In Nigeria, as well as most developing tropical countries of the world, malaria is endemic, and is characterized by high mortality and morbidity in vulnerable individuals. It is estimated that 110 million clinical cases of malaria and between one and two million deaths associated with P. falciparum occur every year; (Bjorkman and Phillipson-Howard, 1990).

Malaria in humans is caused by a blood-borne protozoan parasite of the genus Plasmodium and is transmitted through the bite of infected female Anopheline mosquito. Four plasmodial species, P. vivax, P. ovale, P. malariae and P. falciparum infect man, but P. falciparum is the most lethal of all (Perlman et al, 1984). Due to the fact that Plasmodium is a relatively host specific organism the geographical distribution of the human-infecting species varies extensively, as does the degree of prevalence from one country to the other. In particular, African countries and some other tropical countries have a high prevalence of P. falciparum, which like most other plasmodial species had hitherto been very susceptible to chloroquine. However, the

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therapeutic use of chloroquine is unfortunately being hampered by the development of drug resistant *P. falciparum* strains.

Chloroquine resistant strains of *P. falciparum* (CRPf) had been recognised in South America and South East Asia during the late 1950's and 1960's respectively (Cook, 1988), but appeared in the East and Central Africa twenty years later, (Kean, 1979; Fogh, Jepson and Effersoe, 1979). Subsequently, cases of chloroquine resistant malaria were reported from the Republic of Cameroun by Sansonetti *et al*, (1985) and Brasseur *et al* (1988). In the Eastern States of Nigeria reports of chloroquine failures were made in the late 1970s. A suspected case of chloroquine resistant malaria was first reported from Imo State in 1979 (Eke,1979), but the first confirmed cases of CRPf malaria were reported later from Enugu, an Eastern capital, in 1986 (Greenberg *et al*, 1987; Jackson *et al* 1987). It would therefore appear that from East and Central Africa, the spread of chloroquine resistant malaria presumably continued into West African countries, including Nigeria and seems to have made the greatest impact in the Eastern border states of Nigeria. A trend of chloroquine resistance development in some African countries including Nigeria is presented in tables 1.1, 1.2 and 1.3 and in figs. 1.1, 1.2 and 1.3.

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TABLE 1.1

Trend of development of chloroquine resistant P.falciparum malaria in some East and Central African Countries

Country where malaria was contacted	Year of report/ contact	Assessment method used	Percentage prevalence level observed	Subject(s) Infected	Reference
Kenya	1978	In vivo/ In vitro	-	A non-immune tourist	Fogh, Jepson and Effersoe, (1979)
Kenya	1979	In vivo	-	A non-immune tourist	Kean, (1979)
Tanzania	1979	In vivo/ In vitro	-	A non-immune tourist	Campbel et al (1979)
Kenya	1982	In vivo/ In vitro	35% by In vivo assessment	Indigeneous children population	Spencer, Kaseje and Koech, (1983)
Congo	1985	In vivo/ In vitro	-	3 Indigeneous children, 1 indigeneous adult and 3 non-immune indigenes on home visit from France	LeBras et al (1985)
Cameroun	1985	In vivo/ In vitro	-	Two non-immune French boys on a visit.	Sansonett et al (1985)
Cameroun	1985	In vitro	46.3%	Indigeneous children population	LeBrass et al, (1986)
Cameroun	1985 1986	In vivo/ In vitro	-	Indigeneous children population	Brasseur et al (1988)
Gabon	1986	In vitro In vivo	-	Indigeneous children population	Simon et al (1986)
Somalia	1989	In vivo/ In vitro	V. low prevalence level	Indigeneous children population	Wersema et al (1988)
Cameroun	1985	In vitro In vivo	39% R III prevalence level	Indigeneous children population	Oduola et al (1989)

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TABLE 1.2

Incursion and Dissemination of CRPf Malaria in West Africa excluding Nigeria

Country	Year of Report	Assessment Method	Subjects infected	Reference
Senegal	1984	Therapeutic failures, followed by *in vitro* assessment	An indigenous child	Brandicourt *et al* 1986
Benin Republic	1986	Therapeutic failures, followed with *in vitro* evaluation	2 French expatriates and 5 non-immune indigenes on visits	LeBras *et al* (1986)
Ghana	1986	*In vivo*/*In vitro*	A 10 years old Indigene with HbSS genotype	Neequaye (1986)
Gambia	1987	*In vivo*/*In vitro*	Indigenous children population	Menon, *et al* (1987)

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TABLE 1-3

Genesis of Chloroquine resistant malaria in Nigeria

Town or City of contact	Year of report	Assessment method used	Percentage prevalence level observed	Subject(s) infected	Reference
Owerri (Imo State)	1979	Therapeutic failure	-	An indigeneous adult	Eke, (1979)
Lagos (Lagos State)	1985	Therapeutic failure	-	An indigeneous adult	Ekanem (1985)
Enugu (Anambra State)	1986	*In vivo*/ *In vitro*	-	A non-immune Nigerian (HbSS genotype and pregnant) on home visit from U.S.A	Greenberg *et al* (1987)
Ibadan (Oyo State)	1986	*In vivo*/ *In vitro*	0% *in vivo* 7.1% *in vitro*	Indigenous children population	Salako and Aderounmu (1987)
Enugu (Anambra State)	1987	*In vivo*/ *In vitro*	-	Non-immune visitor	Jackson *et al* (1987)
Various Cities	1987	*In vivo*/ *In vitro*	23% by *in vitro*	Nigerians visiting Britain	de Francis Coopera *et al* 1987.
Agbani Anambra State)	1988	*In vivo*/	41.2%	Indigeneous	Ezedinachi *et al* (1988)
Oban (Cross River State and Igbo-Ora (Oyo State)	1990	*In vivo*/ Oban; *In vitro*/, Igbo-Ora.	63% in Oban 0% in Igbo-Ora by *in vivo* assessment	Indigeneous children population	Ekanem *et al* (1990)

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Fig 1-1

The emergence of chloroquine resistant *P. falciparum* malaria in Africa, adapted from Cook, (1988).

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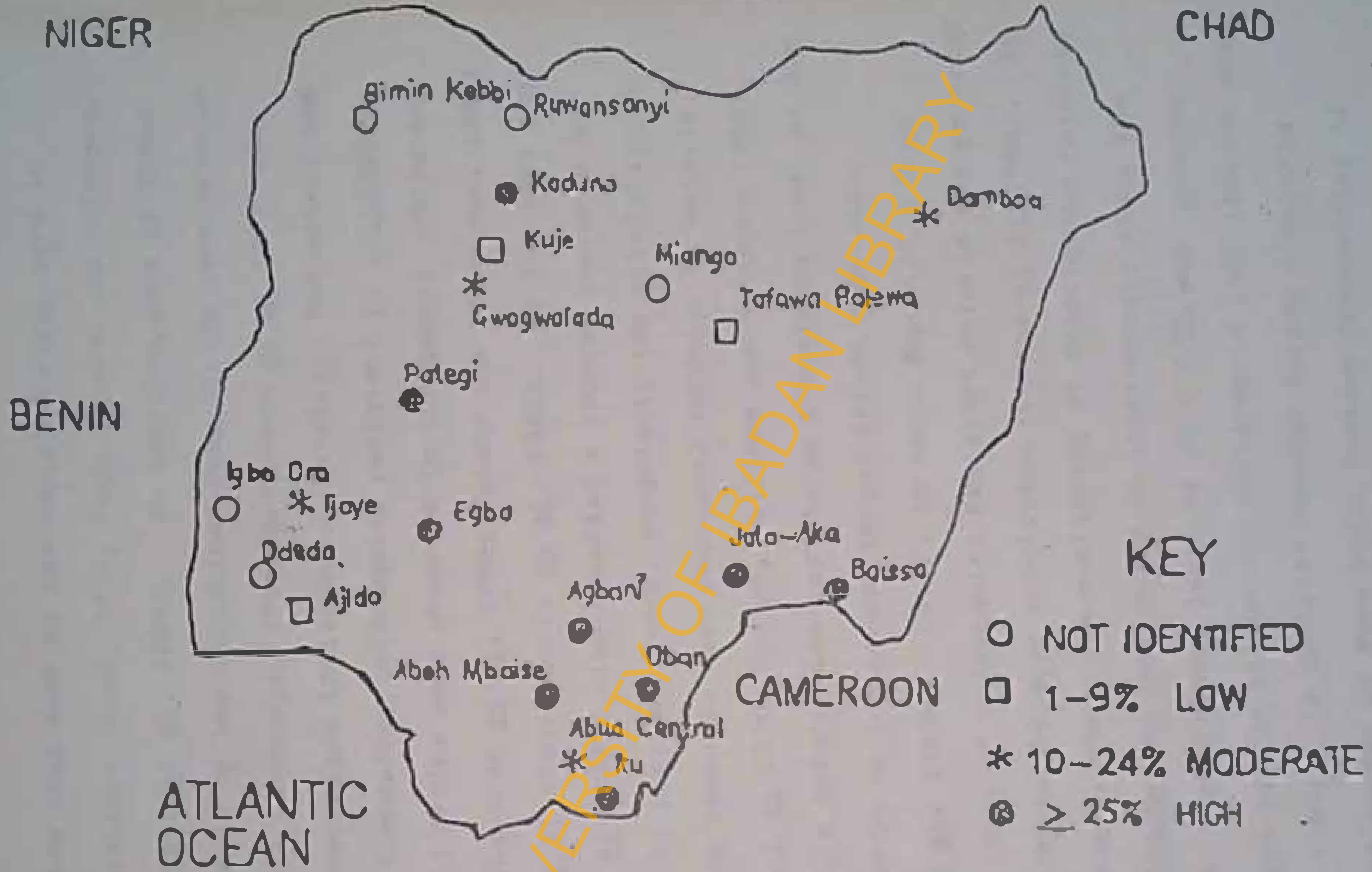

Fig. 1.2: Level of early and late parasitologic failures to chloroquine therapy in Nigerian children under five years of age, July 1987-December 1989. Adapted from Fed. Min. Health, National Malaria Therapy Surveillance Network, Lagos, Nigeria.

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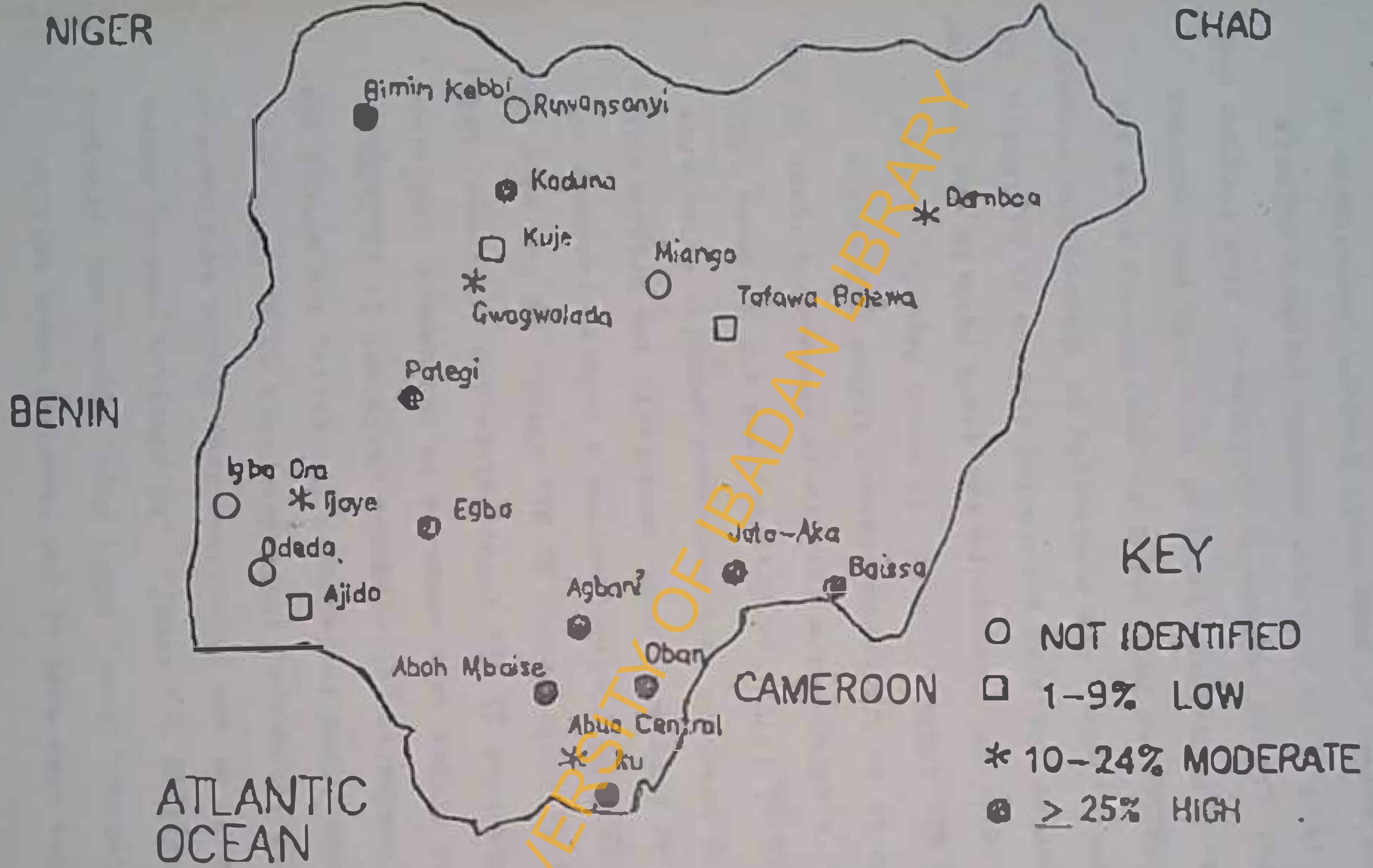

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Fig. 1.2: Level of early and late parasitologic failures to chloroquine therapy in Nigerian children under five years of age, July 1987-December 1989. Adapted from Fed. Min. Health. National Malaria Therapy Surveillance Network, Lagos, Nigeria.

The tables show that most of the reported cases were in non-immune visitors (Kean, 1979; Fogh, Jepson and Efforsoe, 1979; Sansonetti *et al*, 1985). In addition, some of these earliest reports of the infections from Africa were made in Hb-ss patients (Neeguaye, 1986; Greenberg *et al* 1987) who are immunocompromised individuals (Gavrilis, Rothenberg and Guy 1974). Thereafter, chloroquine resistant *P. falciparum* infections in Africa were observed in indigenous children population (Le-Bras *et al*, 1986; Salako and Aderounmu, 1987; Brasseaur *et al* 1988; Oduola *et al*, 1989). At the same time Stekette *et al* (1987) also reported a reduced response to chloroquine by pregnant women, especially the primigravids, another group known to have decreased immunity to malaria (McFarlane *et al* 1970; Ladipo, Williams and Salimonu 1980).

In 1982, a chloroquine resistance prevalence level of 25% was recorded in an indigenous Kenyan infant population (Spencer *et al*, 1983a). Later, in Benin Republic, a high prevalence level of chloroquine resistant malaria was recorded in an indigenous children population (LeBras *et al* 1986). In Ibadan, Nigeria, where the monitoring of chloroquine response of *P. falciparum* had been going on continuously since the early 1980s, a prevalence level of only 7.1% was recorded for the first time in indigenous children in 1986 (Salako and Aderounmu, 1987). In contrast however, during a malaria surveillance survey of some South Eastern Communities of

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Nigeria, Ezedinachi et al (1988), recorded a 41.2% chloroquine resistance prevalence level in a predominantly children population. Furthermore, Ekanem et al, (1990) compared the chloroquine responses of children in Igbo-Ora and Oban, both of which are cluster communities in the West and South Eastern Nigeria respectively, and found that while chloroquine resistance prevalence level was 63.6% in Oban, children in Igbo-Ora were responding fully to treatment with the drug.

Calabar, a Nigerian town in the South Eastern part of the country, shares a common border with the Republic of Cameroun. The two countries are linked by air, sea and land. Cross migration between the people of Nigeria and the Republic of Cameroun via Calabar is difficult to control due to the presence of many access routes through creeks and bush paths. Whether or not the high prevalence level of chloroquine resistant P.falciparum malaria in Calabar is a result of importation from the neighbouring Cameroun Republic remains to be elucidated. Ibadan is appreciably close to the Republic of Benin where a very high incidence of chloroquine resistance was reported in 1986 (LeBras et al 1986), yet the prevalence level of resistance in Ibadan still remains relatively low (Ekanem et al, 1990).

While these observed individual population differences in response to chloroquine therapy may be due to other factors,

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host immunogenetic influence should also be considered important. There are known factors influencing host-parasite relationship generally and these include host genetic constitution, which also determines pre-existing host immunity to the parasite. Evidence abounds showing that IgG plays a significant role in humoral immune responses and protection against malaria (Cohen and McGregor, 1961; Edozien, Gilles and Udeozo, 1962; Perlmann et al, 1984). It has also been observed that malaria antibody accounts for a significant proportion of the total IgG concentrations (McGregor, 1972; Salimonu et al, 1982), which is mainly contributed by IgG 1 fraction of the immunoglobulin, (Salimonu et al, 1982). Subsequently the latter authors suggested that in humoral immune responses to certain antigens, there is a selection for antibodies of one particular subclass. Similarly, Wahlgren et al (1986a) observed that antibodies produced against a P.falciparum major surface glycoprotein (m.wt = 195,000 KD) were frequently of IgG 2 isotype. In Swedish malarial patients, most of whom were CRPf-infected, Wahlgren et al (1983) observed a relative increase in IgG 2 malaria-sepcific antibodies as compared to immune Liberians, who had increased IgG 3 antibody levels. Yount et al (1968) had observed a preponderance of IgG 1 subclass production against some antigenic determinants whereas antibodies to certain polysaccharides were of the IgG 2 isotype. Host genetic influence in immunoglobulin

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production had earlier on been reported by Voller, (1962). The subsequent individual variability in antibody isotypes may thus influence the outcome of the host immune responses. Thus Granoff and Munson (1986) had observed that IgG 2 m(n) negative markers were associated with a seven-fold risk of vaccine failure following Hib-prp immunization. Moreover, Americans infected with human immunodeficiency virus (HIV) show an increased production of IgG 1 and IgG 3 (Aucounturier et al 1986) whereas the Nigerian HIV infected individuals appear to reflect an increased IgG 2 production (Uko et al, 1990), the difference of which may account for the varying immunopathological outcome of HIV infections in these two populations. Furthermore, increased susceptibility to pyogenic infections had been associated with selective IgG subclass deficiency. Of particular interest is the finding in adults of a correlation between levels of serum IgG 2 and the antibody response to bacterial polysaccharide (Oxelius 1974; Rynnel-Dogoo et al, 1986; Siber et al, 1990). Each immunoglobulin isotype exhibits a unique profile of effector funtion (Jefferis and Kumarantne, 1990), but the association of IgG subclass in the pathogenesis of malaria has not been adequately investigated. In particular, the possible differences in antibody responses against CRPf and the CSPf strains have not been studied.

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Although hypocomplementaemia in malaria has been widely reported, (Kidwai et al, 1986; Greenwood and Brueton, 1974) no specific roles in protection (Cohen and Butcher, 1969) or pathogenesis (Williams, Rosen and Hoff, 1973) have been ascribed to the complement system. However, since the role of complement system is closely associated with immunoglobulin isotypes (Spiegelberg, 1974) this study was also designed to investigate the possible changes in the levels of immunoglobulin isotypes and of the components of the complement system, in association with the emergence of CRPf in Nigeria.

Immune responses against some putative malaria vaccine candidates appear to be MHC-restricted in experimental animals (Good et al, 1986; Del Guidice 1986) and possibly in man as well (Sinigaglia et al 1990). The antigenic variation of P.falciparum, which is associated with the emerging strains of the parasite, may affect either the epitope, changing the specificity of a response, or the MHC-binding region, changing the potential to generate any response at all (Marx, 1987). In seroepidemiological surveys of seroreactivities in some malaria endemic dwellers, Del-Guidice et al,(1987) observed that host genetic factors, possibly MHC-related, play a role in immune response to the P.falciparum cirmus-porozoite protein. The authors postulated that the existence of such genetic regulation of immune response to the putative malaria vaccine candidate may predispose some individuals

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to responding better to P.falciparum sporozoite challenge than others. In other seroepidemiological surveys from various populations using RESA/Pf 155-another putative P.falciparum vaccine candidate protein-, significant individual variations in immune responses against this vaccine candidate have been observed (Chizzolini et al, 1989; Petersen et al, 1990; Bjorkman et al 1990).

The role of the mosquito vector in the transmission of plasmodial species, strains or isolates has been examined (James et al, 1932; Ramsdale and Colussi, 1975; and Bruce-Chwatt and Zulueta, 1980). Shute and Maryon in 1951 found that P.falciparum from Nigeria was able to infect Anopheles stephensis readily, but Anopheles labran chiae only poorly. The latter species however, proved to be a good vector of European strains of P.falciparum (WHO, 1987a). Warren et al (1976) also found that the new world mosquito, A. albimanus was able to transmit parasites from Central and South America easily, but was very poorly susceptible to parasites from Africa. In Nepal, it was reported that while the transmission of chloroquine sensitive P.falciparum by the local Anopheline mosquito species goes on, there seems to be no transmission of the resistant strains, in spite of their apparent presence as a result of human migration (WHO, 1987a). While environmental and other host genetic factors cannot be excluded, these observations suggest a specific correlation between

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chloroquine resistant parasite and certain vectors or vector populations. A new strain of malaria parasite could be adapted to heterelogous Anopheline species, but such development would probably take ages to manifest (WHO, 1987a). In the light of all the above, it is therefore likely that the African or Nigerian strain(s) of chloroquine resistant P.falciparum may be indigenous and not a result of importation from South America or South East Asia.

The resistance of Plasmodia to drugs is thought to be attributable to selection, under drug pressure, or resistant mutants, and such mutation is then transferred by classical Mendelian inheritance during sexual reproduction of the parasite in the mosquito (Walliker, 1982). Furthermore, antigenic diversity among parasite clones in the parasite population can also be selected for by immune pressure, due to the possible action of schizont agglutinins as has been suggested in P.knowlesi infections (Brown and Brown, 1965; Butcher and Cohen 1972). Schizont agglutinins are also present in P.gallinaceum infections. Todorovic et al, (1968) and Klotz et al (1987) demonstrated in P.knowlesi, that mutations could occur rapidly in the asexual erythrocytic parasite under immune pressure, because of the number of parasites during a single infection. This effect cannot however be tested in human malaria infections because of the risk to vlunteers and the consequent need for early treatment (Miller, 1988). If present in human infections, could there

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be any individual variation in the production of this variant selection agglutinins?

According to Bruce-Chwatt, (1985) the stages in plasmodial life-cycle, particularly in the blood, are associated with a significant antigenic heterogeneity. Clinical experience has shown that immunity to one strain of P.falciparum does not confer protection from other strains of the parasite (Edozien, Gilles and Udeozo, 1962; Jeffery, 1966; Voller and Richards 1970; McBride, 1982). Jeffery (1980) had also noted that acquired immunity to P.falciparum has a distinct strain specificity. It would therefore be expected that knowledge of the antigenic diversity of the existing and emerging strains of the parasite is important, if an effective vaccine against all strains is to be produced. The prevailing need for a design of a widely representative vaccine against malaria should therefore take into consideration the strain specificities of the P.falciparum parasite along with the preponderance of individual humoral immune response. This can be done in part, through the assessment of factors associated with chloroquine sensitivity of P.falciparum, not only at the antigenic level of the parasite, but also at the level of humoral immune responses to the parasite strains, by the host population, who are likely to benefit from a prospective malaria vaccine.

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1.2. AIMS AND OBJECTIVES OF THE STUDY

a. To assess the current CRPf malaria status in Nigeria, based on two geographical locations - Calabar in the South Eastern part and Ibadan in the West of Nigeria.

b. To assess the levels of humoral immune factors including those of the complement system, in individuals infected with either the chloroquine resistant or the chloroquine sensitive strain of the parasite species.

c. To examine the pattern of seroreactivities against some putative malaria vaccine candidates in the two Nigerian populations under investigation.

1.3 SIGNIFICANCE OF THE STUDY

Of the existing antimalarial chemotherapeutic and chemoprophylactic agents, chloroquine is considered the least expensive, and is reasonably easy to use (Wernsdorfer and Knozutour 1980; Ratnapala 1984; Phillips *et al* 1986; WHO, 1986a; Cook 1988). Furthermore, it has been observed that resistance to chloroquine induces resistance to other drugs and confers on the parasite some biological advantages such as rapid transmission (Bruce-Chwatt, 1985, Wilkinson *et al* 1976). In the light of these, the present development of chloroquine resistant *P.falciparum* presents a great health threat to the inhabitants of the very large portion of malaria endemic world. It has been severally accepted that immune intervention may bring a lasting solution to the

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problem of malaria control. Prospective vaccine candidates are being identified, but the changing antigenic components of the parasite, manifested in each emerging strain, is hampering efforts in malaria control through immune intervention. Host genetic variation in immune responses, against some important malaria antigenic entities, is also constituting a major draw-back. In view of these constraints, it is envisaged that:

1. Evaluation of chloroquine susceptibility of P.falciparum in populations where malaria is endemic may reveal the development and/or the prevalence level of chloroquine resistant malaria, and incite the need for appropriate measures. Geographical aggregation of chloroquine resistant P.falciparum infection, irrespective of apparent severity, is well recognised, and therefore an approach towards a better understanding of the pathogenesis of the disease is to determine the nature of the underlying factors predisposing to susceptibility. Moreover, as suggested by WHO (1984a), specific questions that might be resolved by monitoring the prevalence of chloroquine resistant malaria include:-

    a) Is resistance present in an area or a population?

    b) Has resistance in an area or population reached a critical level requiring some specific action?

    c) Is there a difference in drug response between parasite populations in different places?

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d) What are the factors and their variations that may be associated with individual variation in drug response?

2. Seroepidemiological surveys against the putative malaria vaccine candidates in various malaria endemic populations may reveal the level of immune responsiveness of various populations against these prospective vaccine candidates. Such information will augur well for prospective immune intervention in different malaria endemic populations, including Nigeria.

3. Ascertaining the total malaria antibody levels, the classes, and subclasses of immunoglobulins associated with the resistant strain ineffectivity, within the malaria endemic population of Nigeria, may make for a better understanding of the immunopathogenesis of the disease. In particular, it may be interesting to know the amount of individual variability in IgG subclass response to particular P.falciparum strain(s), that may exist in a population that has been at equal risk of infection. Such knowledge may help to explain further, the apparently worsening malaria situation in the country, especially in the South Eastern states. It may possibly reveal a peculiar humoral immune response characteristic elicited by the emerging chloroquine resistant strain, thereby providing a serological marker

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for identifying the prevalence of CRPf malaria. This may perhaps, remove the need for the more cumbersome, *in vitro* response test method, which requires the use of an incubator - a facility that is hardly available for most field studies.

4. Knowledge of the activity of the complement system and of the serum levels of some complement components in chloroquine resistant *P.falciparum*-infected individuals, as compared with what is already known in chloroquine sensitive malaria-infected patients, may improve the current limited understanding of the role of the complement system in malaria infections generally.

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CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

Malaria, a disease of man and other vertebrates, is caused by a blood inhabiting protozoan parasite of the genus *Plasmodium*, and is transmitted through the bite of infected female *Anopheles* mosquitoes (Wernsdorfer, 1980). The rodent malaria is caused by *P.berghei*, *P. yoelii*, *P. chabaudi* and *P. vinkei*. *P. knowlesi*, *P. simium* and *P. cynomolgi* are some of the plasmodial species that cause simian (non-human primate) malarias. *P.gallinaceum* and *P. lophurae* cause the disease in birds, while *P.agamae* infect reptiles. Human malaria is caused by four species of *Plasmodium* viz; *P. ovale*, *P. vivax*, *P. malariae* and *P. falciparum*. None of the other Plasmodial species except those found in some monkeys can be transmitted to man. This high host specificity is believed to indicate a long association between man and the four particular species of *Plasmodium* that infect man (Bruce-Chwatt and Zulueta, 1980).

Sandosham (1965) defined malaria as a disease characterised by fever, anaemia, enlargement of the spleen and pigmentation of the tissues. In endemic areas, the disease is the cause of most febrile illnesses and the frequency of splenomegaly in children is used as an index of

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prevalence and transmission of malaria (Davey *et al*, 1961). Data released by the World Health Organisation in 1985 show that no fewer than 6.5 million cases of malaria were recorded in 1982 (WHO, 1985). These figures represent only a part of the true picture, as data from tropical Africa were not included because of the incomplete and uncertain reporting from the large endemic areas of the African continent. Of the total world population (1983) of some 4.7 billion, about 2.2 billion live in places where the incidence of malaria has been virtually eliminated or reduced in varying degrees, but almost 400 million people in rural tropical areas are exposed to its full brunt. No fewer than 373 million inhabitants of sub-Saharan Africa live in endemic areas where *P.falciparum* is the prevalent species.

Though restricted to the tropical and warm temperate regions, malaria patients may present in any part of the world as a result of modern population movement associated with rapid air travel (Bowman and Rand, 1980). The epidemiology of malaria and its transmission have been shown to vary greatly and can be altered by fluctuation in rainfall, migration or acute changes in nutritional status (WHO, 1989). Development of new strains, resistant to drugs, has also affected the epidemiology of the disease. For instance, malaria which was virtually eradicated in Sri-Lanka in 1963,

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resurged in 1967/1968 (Bruce-Chwatt 1968; Bowman and Rand, 1980) and in 1990 (Wijesundera et al, 1990), following the emergence of chloroquine resistant strains of the parasite.

The World Health Organisation has been involved in malaria eradication since 1957 (Williams, 1971). Major advances have been made in the development of insecticides and synthetic antimalarials. However the situation is not improving due to the development of resistance of malaria parasites to drugs and the Anopheline vectors to insecticides. By 1980, about 51 Anopheline species of malaria vectors had become resistant to DDT and other insecticides (Bruce-Chwatt 1985), making it clear that in rural areas of developing countries, control measures will need to take more account of all available methods, including the wider use of antimalaria drugs for the treatment of the disease and for its prevention (WHO, 1980). In recent years the problem of drug resistance has reached threatening dimensions in Eastern Asia, South America, and more recently in Africa (Campbell et al 1979, Kean 1979). Another worrying issue is that resistance of P. falciparum to chloroquine seems to stimulate resistance to other compounds, and appears to confer on the resistant strain, a greater capacity for transmission (Peters, 1984; Wilkinson et al, 1976). The emergence of drug resistant malaria therefore has retarded the malaria eradication program

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initiated by the World Health Organisation many years ago.

In Nigeria, as in other malaria endemic West African countries, the predominant malaria infection is the malignant tertian type caused by P.falciparum. P. falciparum constitutes 90 - 95% of malaria infections in Nigeria (Akinwolere and Williams 1989; Ekanem et al 1990). Based on earlier reports, the intensity of transmission however, varies from area to area and from season to season. In the dry season (November - March) the intensity is low, with an average sporozoite innoculation rate of 0.01 against a mean rate of 0.25, during the rainy season (April - October), (Bruce-Chwatt, 1962). The emergence of chloroquine resistant P.falciparum has apparently worsened the malaria situation in Nigeria (Umotong et al, 1991; Ekanem et al, 1990), the most thickly populated country of all the malaria endemic African countries, but the degree of the problem is yet to be evaluated.

2.2 LIFE CYCLE

The four species of malaria parasites that infect man differ morphologically, but have similar patterns of life cycle. Their natural reservoir is man and they are transferred from man to man by various species of Anopheles mosquito. The parasite therefore has two interdependent life cycles, an extrinsic or exogeneous one in the mosquito (sporogony) and an intrinsic or endogeneous one (schizogony) in man.

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The former which is a sexual cycle is referred to as definite and the latter which is an asexual cycle is referred to as intermediate. The asexual cycle occurs in two different compartments, one in the tissue and the other in the red blood cells. They are referred to as exoerythrocytic and erythrocytic schizogony respectively (Bowman and Rand, 1980; Garnham, 1984; Pratt, 1977; Wyler, 1982). With the exception of P.malariae where higher non-human primates may be involved occasionally, Plasmodium species have man as the only natural vertebrate host (Garnham, 1984). The malarial life cycle is presented in Fig. 2.1.

2.2.1 Sporogony

Sporogony takes place in Anopheles mosquitoes, following ingestion of a blood-meal containing micro- and macrogametocytes, which mate within the arthropod and ultimately give rise to sporozoites. Natural infection in the vertebrate host is transmitted by the bite of infected female Anopheles mosquito that inject sporozoites. Sporozoites are carried in the blood to the liver, where they bind to and invade the parenchymal cells by mechanisms that are not yet fully understood (Perrin et al, 1982). The sporozoites then divide asexually to form merozoites. This exoerythrocytic developmental stage takes approximately 10 days and is followed by the rupture of the infected cells, releasing thousands of exoerythrocytic merozoites into the circulation.

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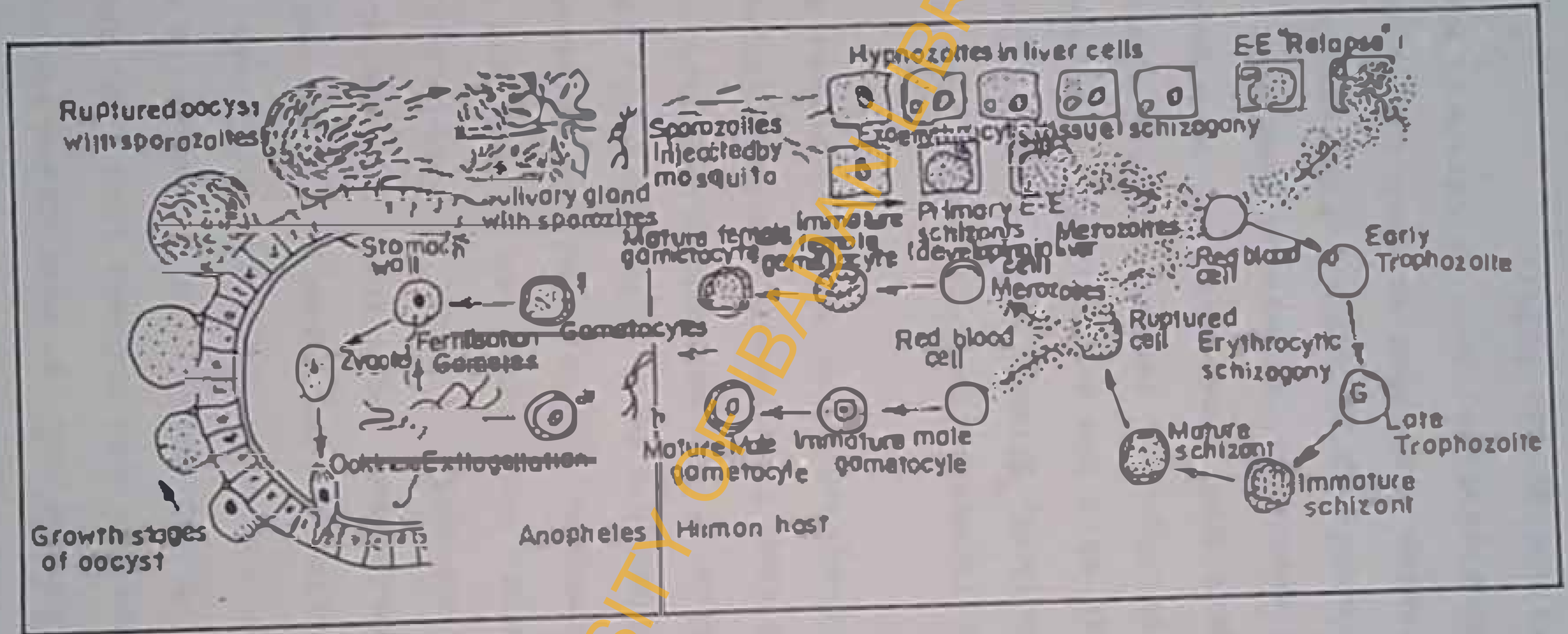

Fig. 2.1 Cycle of malaria parasite development. Reproduced from Bruce-Chwot, (1985).

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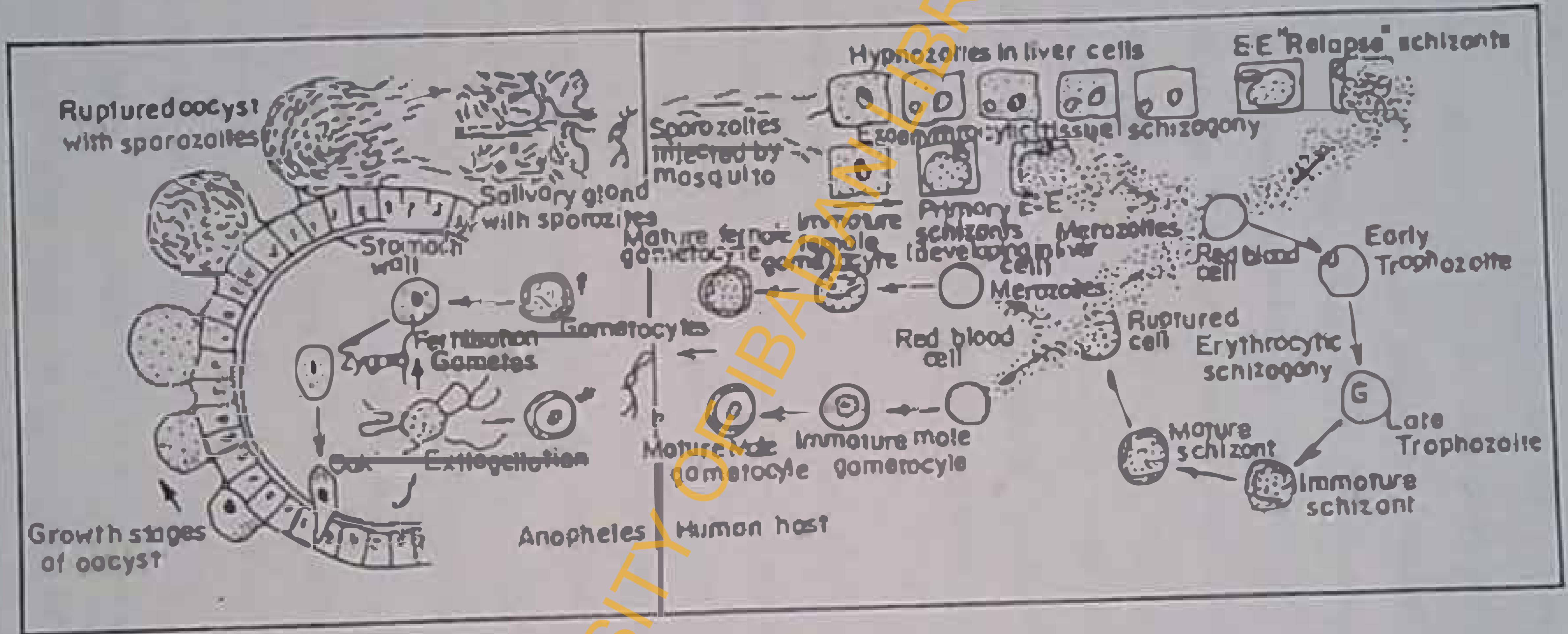

Fig. 2.1 Cycle of malaria parasite development. Reproduced from Bruce-Chwat, (1985).

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following penetration of erythrocytes, the organism grows and almost fills the cell in most cases, before the nucleus divides by accelerated mitosis into two, then subsequently into 4, 8, 16 or 32. The organism is now called a schizont as from when the nucleus divides into two. The pigment collects into a few pieces or masses and the cytoplasm condenses around the final nuclei to produce uninucleate merozoites. When the erythrocyte is distended and ruptures, the merozoites escape into the plasma along with pyrogens and the familiar yellow-black malaria pigment (Bowman and Rand, 1980; Garnham, 1984). The extracellular life of the merozoites is short - only a few seconds - and then they invade new erythrocytes by attachment to and invagination of the erythrocyte surface membrane.

The asexual erythrocytic parasite causes all the symptoms of malaria (WHO, 1986b). The level of parasitaemia is correlated with the severity of the disease, (Field and Niven, 1937), but, recent reports from malaria endemic areas suggest that some hosts may be asymptomatic in the presence of apparently pyrogenic levels of parasitaemia (Greenwood, et al 1987). Furthermore, whether differences in parasite virulence or host factor modulate the frequency of complication, such as cerebral malaria, remains unknown (Miller et al, 1986).

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2.2.2. Pre-erythrocytic Development

It seems to be well established that all sporozoites of *P. falciparum* and *P. malariae* trigger immediate tissue schizogony in hepatocytes. There appears to be little variation in the prepatent period of both species, suggesting a highly uniform and synchronous tissue schizogony. In contrast, there are two types of tissue schizogony in relapsing forms of malaria due to *P. vivax* and *P. ovale* - one that is immediate and the other that occurs periodically owing to the presence of dormant liver forms or hypnozoites. These hypnozoites are activated into tissue schizogony at different times (Krotoski *et al* 1982; Bray, 1984).

2.2.3. Blood Schizogony:

The merozoites released from the liver enter peripheral blood erythrocytes where two separate erythrocytic phases of the life cycle are undergone.

In the erythrocytes, the merozoites are first visible as tiny rings which feed on the protein portion of the haemoglobin by phagocytosis, leaving the haem portion as grains of pigment which remain scattered in the cytoplasm. The parasite at this stage is usually uninucleated and is termed a trophozoite. Within about a day and depending on the species, the central vacuole disappears as the trophozoite grows.

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The ring forms and the older trophozoites of *P. falciparum* usually disappear from peripheral blood after 24 hours and are sequestered in the capillaries of some internal organs, such as the brain, the heart, and the intestines, as well as in the bone marrow, where further development takes placé. After several generations of merozoites have been produced, some of them develop into sexually differentiated forms called gametocytes.

2.2.4 Gamecytosis

Young gametocytes grow at half the speed of asexual forms and quickly show sexual dimorphism, the cytoplasm of the macrogametocyte (female gamete) staining a fairly dense blue with Giemsa, and the nucleus remaining condensed and heavily stained. The cytoplasm of the micro-gametocyte on the other hand is much paler and the nucleus spreads over a large area, sometimes more than half of the cytoplasm. The two sexes do not, as a rule, enter the blood simultaneously, the microgametocytes often appearing a day or two later and often in smaller numbers (Garnham, 1984). The mature gametocytes can survive for several days but cannot develop further morphologically unless they are ingested by a suitable female *Anopheles* mosquito in which the sexual cycle continues (Bowman and Rand, 1980; Wyler, 1982).

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2.3 PATHOLOGY

The clinical picture of malaria consists of bouts of fever accompanied by other symptoms and alternating with periods of freedom from any feeling of illness (Bruce-Chwatt 1980). These other symptoms may include headache, anorexia, nausea and vomitting, shivering (rigors), intense thirst, profuse sweatings, enlargement of the spleen and pigmentation of the tissues (Bowman and Rand 1980; Boonpucknavig, Srichaikul and Punyagupta 1984). The clinical manifestation sequel to the innoculation of sporozoites represents the primary attack, while the subsequent attacks are known as relapses. The duration of the primary attack, the length of the latent periods, as well as the liability to relapses vary with the species of *Plasmodium*, as well as individual immune status (WHO, 1986b).

The main organs affected by *Plasmodial* infection are the spleen, liver and bone marrow. All become full of infected erythrocytes, red cell debris and malaria pigments; the spleen and liver become enlarged and their tissue hyperplastic. Nephrosis of the kidney may also occur but this happens mainly following *P. malariae* infections (Okerengwo, 1980). In the brain, the accumulation of large numbers of parasitized red cells may block the cerebral capillaries which may lead to focal necrosis, haemorrhage and death (Edington, 1967; Ikpatt, *et al*, 1990).

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However, studies by Bruce-Chwatt (1980) suggest that cerebral malaria (CM) is a form of disseminated vasculomyelopathy, a hyperegic response of a central nervous system to the antigenic challenge of *P. falciparum*.. More recent findings however suggest that tumour necrosis factor (TNF) may be implicated in CM (Grau *et al*, 1987; Peyron *et al*, 1990). One of the many activities of TNF is to sensitize neutrophils and macrophages (Larrick *et al*, 1987) to agents that induce them to secret superoxide and oxygen radicals. Subsequently, oxidant stress may predispose to CM. However the reasons why some individuals are more prone to CM than others (Clark, 1987) still remain elusive.

The most severe manifestation of malaria occurs in later infancy, between the ages of 3 and 5 years (Greenwood *et al*, 1987). The great majority of children in this age group show parasitaemia which can attain levels as high as 100,000 per ul. Thus, malaria is responsible for considerable morbid state in this group of children. Above the age of 5 years, the effect of acquired immunity becomes increasingly apparent in children. This is indicated by decreased morbidity in the presence of parasites, decreased parasite counts and subsequently, decreased rate

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and apparent decrease in spleen size. This state of partial immunity exists throughout adult life provided there is continued antigenic stimulation by frequent exposures to infections. Nevertheless some adults in endemic areas are more vulnerable to malaria-associated illnesses than others. Genetic variation in immune responses against some putative protective malaria proteins has been observed in many endemic areas (Bjorkman et al, 1990). However, knowledge of the genetic factors, which may possibly be immunological in nature, associated with this differential protection are still elusive.

2.4 CHEMOTHERAPY

The first effective antimalarial drug used in modern times was quinine, which is the chief alkaloid of the back of Cinchona, a tree indigenous to certain parts of America. Although quinine has been synthesized, the procedure is too complex and too expensive to provide practical source of the drug. Moreover, radical cure with quinine alone is difficult to achieve as the drug can cause serious toxicity (Ratnapala et al, 1984). In 1944, chloroquine was found to be an outstanding anti-malarial compound, faster in therapeutic action than quinine and less toxic. Chloroquine is used for clinical cure of malaria because it interrupts the erythrocytic schizogony

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2.5. CHLOROQUINE RESISTANT P. FALCIPARUM

2.5.1. Introduction:

Drug resistance in malaria is defined as "the ability of a parasite strain to survive and/or to multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended but within the limits of tolerance of the subject", (WHO, 1965). Although this definition can be extended to all species of the malaria parasite and all useful dosages of blood or tissue schizontocides, gametocytocides, and sporontocides, in practice, it is most commonly applied to the resistance of P. falciparum to the blood schizontocides, particularly the aminoquinolines (WHO, 1965).

Many mechanisms have been postulated for the development of chloroquine resistance. These include drug pressure (Peters, 1987), extensive use of subcurative dosages (Wernsdorfer and Payne, 1990), and migration and increased virulence of the resistant parasite (Warhurst, 1986). Selection of mutant clones in the parasite population. may also be achieved by immune pressure (Todorovic et al, 1968; Klotz et al, 1987). If drug pressure was responsible

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for the triggering and spread of resistance universally, high levels of resistance would be expected throughout malarious countries. However, in Liberia, studies confirmed high chloroquine sensitivity in an area with intense and haphazard use of chloroquine for over 20 years (Bjorkman _et al_, 1985), with resistance first encountered in 1988 at the same time as it occured in adjacent areas without this substantial drug pressure. Furthermore, the distribution and intensity of CRPf is frequently unequal within a country, despite uniform drug use patterns. It could be suggested that some element of importation, as described by Clyde (1987) is needed to trigger the development of drug resistance. Alternatively, population variation in the prevalence of drug resistant malaria could be attributable to the effect of immune pressure. It has been shown in experimental malaria that antigenic diversity amongst parasite clones could be selected by immune pressure due to the action of schizont agglutinins (Brown and Brown, 1965; Butcher and Cohen, 1972; Todorovic, _et al_, 1968; Klotz _et al_ 1987). Unfortunately, the effect of immune pressure on mutant selection cannot be tested in humans (Miller, 1988).

2.5.2. Mechanism of Chloroquine Resistance

A number of theories have been proposed to explain the mechanism of chloroquine resistance (Fitch, 1983, Krogslad and Schlesinger, 1987). A more recent proposal

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to explain chloroquine resistance mechanism however, was made by Martins *et al*, 1987. They postulated that the mechanism by which *P. falciparum* acquires multidrug resistance may be similar to that by which neoplastic mammalian cells develop simultaneous resistance to multiple structurally unrelated drugs. Such multi-drug resistance in neoplastic cells has been correlated with an increase in the number of glycoproteins in the cell membrane, which serve to pump many different hydrophobic molecules out of the cells, such that they fail to attain intracellular toxic concentration. This view on the mechanism of chloroquine resistance has recently been supported by Wilson *et al* (1989); Ye *et al*, (1989); Welem *et al*, (1990); and Fork *et al* (1990).

2.5.3. Characteristics of chloroquine resistant *P. falciparum*:

While pyrimethamine resistant clones of *P. chabaudi adami* appear to have selective disadvantage compared with the sensitive strain, the converse is true with chloroquine resistant strains of this parasite species. Similarly, studies have indicated that chloroquine resistant *P. falciparum* is more infectious to the *Anopheles* vector (Wilkinson *et al* 1976) and grows faster in culture than the sensitive strain (Thaithong, 1983). This characteristic seems to place the resistant strain of the parasite at a biological advantage and appears to explain its rapid geographical spread.

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P. falciparum is the only species of malaria parasite in which there is an indication that the duration of blood schizogony and merozoite yield vary, the former being shorter and the latter higher in chloroquine-resistant isolates as compared to chloroquine-sensitive strains (Rosario, 1981; Thaithong, 1983).

2.6. MALARIA IMMUNOLOGY

The ability of a host to resist a malaria infection is determined not only by immunological mechanisms but also by innate characteristics (WHO, 1987a).

2.6.1. Mechanism of innate resistance:

In the course of evolution, vertebrate hosts including man, have developed a variety of mechanisms of resistance against malaria. Some of such mechanisms are expressed regardless of the environment and regardless of previous exposure to the parasites. These mechanisms depend ultimately on the genetic constitution of the host, and may also relate to a requirement for the parasite (e.g. an erythrocyte receptor or a nutritional requirement) or to a substance within the host that is in some way deleterious to the parasite. The importance of host factors in the development of plasmodial infection is exemplified in the fact that only two species of non-human primates (*Aotus trivirgatus* and *Saimiri sciureus*) are highly susceptible to human malaria species, *P. falciparum*, but others, including the chimpanzee can be rendered susceptible

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by splenectomy, (Perrin _et_ _al_, 1982).

In endemic areas, malaria has exerted selective pressure favouring genetic traits expressed at the level of the red blood cells that confer resistance to malaria (Luzatto, 1979). Such genes, therefore occur with relatively high frequency in areas presently or formerly endemic for malaria.

The first evidence that the red cell itself influences merozoite invasion was the observation of the apparent preferential infection of red blood cell sub-populations by _P. vivax_ and _P. malariae_, which preferentially invade reticulocytes and mature red blood cells, respectively (Kitchen, 1939). In contrast, _P. falciparum_ merozoites can infect all sub-populations of red blood cells and the resulting parasitaemia may exceed 50% with subsequent high mortality (Bruce-Chwatt, 1948). It has also been shown using _in vitro_ cultures of erythrocytic stages of _P. falciparum_, that when both human and _Aotus_ monkey red cells are mixed in the same culture, merozoites preferentially bind to and develop within the human cells, suggesting that properties of the red cells play a role in the host specificity of various plasmodial species (Trigg, 1975).

It has been suggested that attachment and penetration of merozoites depend on a receptor on the red cell membrane and that merozoites contain ligand-like substances on their surfaces complimentary to this receptor (McGee 1953).

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Treatment of human red blood cells which are normally susceptible to invasion by P. knowlesi, by pronase, but not by neuraminidase or trypsin, blocks invasion by P. knowlesi merozoites (Miller et al, 1975). This experiment suggested that invasion of red cells is mediated by specific receptors. The observation that Duffy blood group negative human erythrocytes are resistant to P. knowlesi (Miller, 1975) and P. vivax (Miller, 1978) merozoite invasion, provides further evidence that specific receptors on the red cell membrane are involved in merozoite invasion. It was therefore postulated that the relative insusceptibility of West African and American Blacks to infection with P. vivax is due to the extreme rarity of Duffy blood group determinants (Fy^a and Fy^b) in these populations (Miller, 1978, Spencer, 1978), while they are common in other racial groups. Human red cells, including Duffy negative cells, are all invaded by P. falciparum merozoites. There is however, experimental evidence that the rare $En^a(-)$ cells are more resistant to P. falciparum merozoite invasion. These experiments suggested that P. falciparum uses as receptors, an essential structural component of the red cell membrane (glycophorin) (Cartron et al, 1983).

Impairment or retardation of parasite growth has been observed in red blood cells containing fetal haemoglobin (HbF). Red blood cells of new born infants that contain a

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large proportion of HbF do not support optimal growth of _P. falciparum._ This factor may contribute to the relatively low frequency of malaria infections in babies. In adults with hereditary persistence of HbF, red cells are invaded efficiently, but parasite growth is impaired in the HbF containing cells (Pasvol _et al_, 1977). These findings suggest that haemoglobin composition may influence the resistance to malaria, of individuals with HbF-containing red blood cells.

In thalassemia and G-6-PD deficiency, which are prevalent in areas endemic for malaria, resistance to malaria infection has been difficult to demonstrate experimentally. Luzzatto (1969) however had shown that in females heterozygous for G-6-PD deficiency, there is a reduction in the percentage of infected cella within the population of deficient RBC.

In the caae of sickle cell anaemia (HbS), the mechanism of resistance against parasite survival has not been unequivocally determined. Experimental studies have shown that protection, which acts at the red cell level, is afforded by the HbS trait. Friedman (1979) and Pasvol (1978) in two independent studies demonstrated that when _P. falciparum_ parasites were grown in HbS-containing red blood cella, they developed normally at normal oxygen tension, but failed to mature or cause red cell lyais at low oxygen tension. These workera therefore suggested that

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a similar effect may occur *in vivo*, where red blood cells containing mature parasites become trapped in the capillaries of the deep organs in an environment of relatively low oxygen tension. However, it has been suggested that HbS homozygotes as opposed to heterozygotes, do not enjoy protection against malaria, (WHO, 1975) since many fatal malaria infections have occured in the homozygous sickle cell subjects.

There is morphologic evidence that during their intraerythrocytic development, *Plasmodia* alter the structure of the host cells and modify the biologic properties of the red cell membrane. Red blood cells containing *P. falciparum* and *P. malariae* develop electron dense excrescences at the cell surfaces, called knobs (Trager *et al*, 1966). The number of knobs increases as the parasite matures. Knobs associated with *P. falciparum* adhere to endothelial cells and form focal junctions with the endothelial cell membrane. This phenomenon probably contributes to the observed sequestration of mature erythrocytic forms of *P. falciparum* in the deep organs and may be one of the mechanisms involved in the pathogenesis of cerebral malaria (Kilejian, Abati and Trager, 1977). Although cerebral malaria in endemic areas occurs mostly in the least immune age groups (1-5 years) (Osunkoya and Williams, 1980), and in the non-immune (WHO. 1975), not all subjects in these groups are equally vulnerable to cerebral

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malaria. Parasite and/or host genetic factors that predispose to this complication are not well understood.

In the chloroquine resistance prevalent Thai province of Chantaburi, the introduction of effective mefloquine therapy led to an almost complete diappearance of cerebral malaria (Miller, 1989). Ikpatt et al (1990), in a recent survey of cerebral malaria cases in Calabar, Nigeria, observed that the central nervous system complication was on the increase. They attributed this increase to the widespread emergence of chloroquine resistant *P. falciparum* malaria. Whether the knobs in CRPf-infected red cells have been modified for increased CNS cytoadherence, or the host has reacted (Bruce-Chwatt, 1980) differently in response to the CRPf strain thereby predisposing to cerebral malaria, is yet to be elucidated.

To set the stage, malaria and its impact vary with the area of the world (Miller, 1989). In endemic Africa, malaria has a heavy impact on children and primigravid women. Multiple studies have consistently found a major effect of malaria control on mortality in children aged 1-4 years (Molineaux, 1980; Greenwood et al, 1987). In contrast, the disease in Madang, Papua New Guinea, appears quite different, causing little mortality in children (Stace et al, 1982). Since both regions are hyperendemic for *P. falciparum* malaria, Miller (1989) suggested that the New Guinean host may be more innately resistant than

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their African counterparts. This possiblity is adduced from the fact that 95% of the New Guinean population have a deletion of one alpha haemoglobin gene (Oppenheimer *et al*, 1984) and 30% of them have ovalocytosis which suppresses parasitaemia (Miller, 1989).

2.6.2. Acquired immunity to malaria

Human malaria immunity is acquired only after repeated exposures to infection for many years and is directed mainly against the erythrocytic stage of the parasite (Cohen, 1977), which causes all the symptoms. Furthermore, irradiated sporozoite vaccines protect animals (Nussenweig *et al*, 1969) and humans (Rieckman *et al*, 1974; Clyde *et al*, 1975) against challenge with live sporozoites. Response to malaria antigenic challenge can be crudely divided into antibody-dependent immunity and antibody-independent (cell-mediated) immunity. Few, if any parasite, will generate cell mediated immune response in the absence of antibody response (Troye-Blomberg, 1988).

(i) Role of serum antibody in malarial immunity

Coggeshall and Kumn (1937) were the first to demonstrate experimentally that immune serum might have a potential role in malaria immunity. They demonstrated that the sera of rhesus monkeys with chronic *P. knowlesi* infections conferred passive immunity to monkeys with acute *P. knowlesi* infections. This phenomenon has been confirmed by other workers in birds and mammals (Manwell and Goldstein, 1940;

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Taliaferro and Taliaferro 1940). The demonstration of such passive transfer of malaria immunity in man was achieved by the work of Cohen *et al* (1961). They extracted IgG from a pool of sera collected from adults living in The Gambia, West Africa, which is holoendemic for malaria. The IgG fraction was administered over several days to children suffering from *P. falciparum* malaria. Consequently parasitaemia was suppressed and clinical symptoms alleviated. The conferred immunity however was temporary, as most of the children contracted infections a few months later. IgM fraction from the same pool of sera and also gammaglobulin fraction from Europeans did not have this therapeutic effect. Edozien *et al* (1962), working in Nigeria, confirmed the Gambian findings and further demonstrated the suppressive effect of IgG from cord blood on parasitaemia.

Using *in vitro* techniques however, Cohen and Butcher (1970) observed that both IgG and IgM from immune monkeys could suppress the growth of *P. knowlesi.* These workers concluded that antibodies in IgG fraction alone seem likely to be protective in *P. falciparum* infections in man. Neither IgA nor IgE could exhibit *in vitro* suppression of parasite growth. Findings from *in vitro* experimentations with putative malaria vaccine candidate proteins have supported the use of antibodies in immune protection against malaria. Hollingdale *et al* (1984) observed that monoclonal antibodies raised against (NANP)n, a synthetic circumsporozoite (CS) protein, could inhibit the *in vitro* penetration

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of cultured hepatocytes by sporozoites. In many epidemiological surveys, anti-CS antibodies have been found to correlate with protection against infections in endemic regions (Esposito et al, 1988; Marsh et al, 1988; Snow et al, 1989). However, sero-epidemiological reports from some other endemic regions show that anti-CS antibodies are not protective (Hoffman et al, 1987; Pang et al, 1988; Webster, 1988). These two conflicting reports are inspite of the fact that native CS protein epitopes, as well as those of its synthetic products, are well conserved in different geographical isolates of the parasite species (de la Cruz et al, 1987; Dell Portillo, 1987; Lockyer and Schwarz, 1987; Weber and Hockmeyer, 1985; Zavala et al, 1985).

The protective role of antibody against the ring-infected erythrocyte surface antigen (RESA), a putative blood stage vaccine candidate, was first demonstrated simultaneously by Perlmann et al (1984) and Wahlin et al 1984). These antibodies were also found to be mainly of the IgG class (Perlmann et al, 1984). Following this discovery, other seroepidemiological studies have confirmed the protective value of anti-RESA antibodies, (Petersen et al, 1990, Collins et al 1988, Perlmann et al 1989, Wahlgren et al, 1986b, Chizzolini et al 1989, Troye-Blomberg et al 1989). Like CS protein, RESA epitopes of *P. falciparum* are invariant (conserved) in various isolates studied and RESA antibodies from different parts of Africa, Columbia and

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Sweden were found to react similarly with erythrocytes infected with a Tanzanian P. falciparum strain kept in culture for many years (Perlmann et al, 1987).

In contrast to the above observations, seroepidemiological studies in other endemic populations indicate that anti-RESA antibodies have no demonstrable protective value against malaria (Deloron et al 1987, Marah et al, 1989, Bjorkmann et al, 1990). In populations where studies show that antibodies against the putative vaccine candidates are protective as well as in those populations where otherwise is the case, individual immune response restrictions have been observed (Resenberg and Wirtz, 1990; Del Guidice et al 1987; Webster et al, 1988; Petersen et al, 1990; Bjorkmann et al, 1990). There is therefore immunological evidence that some individuals may not be able to respond to P. falciparum prospective vaccine candidate proteins. Such evidence haa been reported from Africa (Del Guidice et al, 1987) and Thailand (Wabster et al, 1988).

(ii) The role of antibody subclass in immunity against malaria

The inability to produce antibody of the optimally protective isotype can result in a selective immunodeficiency state (Jefferis and Kumararatne, 1990). This is particularly apparent for responses to certain bacterial carbohydrate antigens that are normally of the IgG2 isotype (Yount et al, 1968). There is evidence to suggest that the proportions of each subclass produced, following antigenic stimulation at

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local sites, may differ, due to selective localization of B-lymphocyte subsets and/or selective stimulation of B-cell subsets by factors present in the local environment. Furthermore, in vitro studies show that interleukins (IL) can modulate or regulate IgG subclass production (Flores-Romo et al, 1990; Ishizaka et ál, 1990). Thus IL-4, in vitro, stimulates isotype . switch to the production of IgG1 and IgE and suppresses the production of IgG3, IgG2b and IgG2a. Gamma-interferon, (γ-IFN) in vitro, stimulates IgG2a and suppresses IgG3 and IgG2b production.

Immunity to P. yoelii is believed to be related to the major histocompatibility complex (MHC) class 1a antigen expression on the reticulocytes (Kumar and Miller, 1990). Brake et al (1988) demonstrated that a T-cell clone transferred protection against P. chabaudi adami infection in nude mice. This T-cell clone secreted both γ-IFN and IL-2 and therefore was of the Th 1 phenotype. However, only one of ten clones tested was protective.

In view of the possible control of IgG subclass production by interleukins and the possible dependence of IL production on the MHC, it is arguable that both individual genetic variability and the antigenic epitope will influence the IgG subclass produced. Since the biological effector functions of the different IgG subclasses vary greatly (Spiegelberg, 1974), the serum concentrations of antibodies belonging to a given subclass may reflect their clinical

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and/or protective roles in the course of an infection (Jefferis and Kumarara, 1990). Subclass of antibodies formed in different phases of infection in different genetic groups of people, may be instrumental to providing protection (Wahlgren *et al*, 1983). On the contrary, antibodies of the subclasses IgG2 and IgG4 might be inhibitory or may not activate the complement system, (Jefferis and Kumarara, 1990).

2.6.3. The Complement System

The complement system comprises about 21 discrete proteins, all of which circulate in the blood plasma. The main biological roles of complement are inflammatory responses to tissue injury and the destruction of viable infectious agents. The complement system or its components therefore, is involved in many aspects of health and diseases. Activation of complement results in diverse biological consequences which include increased vascular permeability, anaphylaxis, chemotaxis, enhanced phagocytosis and digestion, irreversible membrane damage, dissolution of immune complexes and possibly also modulation of some aspects of lymphocyte function (Lachmann and Peters, 1982; Weilher *et al*, 1982; Sundsomo, 1983). Complement activation is a self regulating process during which fragments of complement components, with nascent biological properties, are generated and subsequently catabolized (Lachmann and Peters, 1982).

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(i) Complement activation

It is now well established that there are two major pathways of complement activation, each dependent primarily although not entirely, on the sequential conversion of proteolytic zymogens to proteases. These are called the classical and the alternative (or properdin) pathways of complement activation. The molecular mechanisms of both pathways of complement activation together with their biological consequences have been reviewed extensively by others; (Lachmann and Peters, 1982; Green, 1983; Reid and Porter, 1983). The essential aspects of the main compartments of the complement system are summarized in Fig. 2.2.

The classical pathway complement (CPC) activation is the better understood general mechanism of the complement system. It is initiated predominantly by antigen-antibody complexes of "appropriate" composition, although other triggers are known (Porter and Reid, 1979). Initiation of the Alternative Pathway (APC) of complement activation does not require a specific trigger analogous to that responsible for classical pathway activation. Instead, there is a continuous *in vitro* low level of APC activity, which in the presence of suitable target cells, or other membrane structures, may be rapidly amplified (Green, 1983; Muller-Eberhard and Schreiber, 1990; Fearon, 1980).

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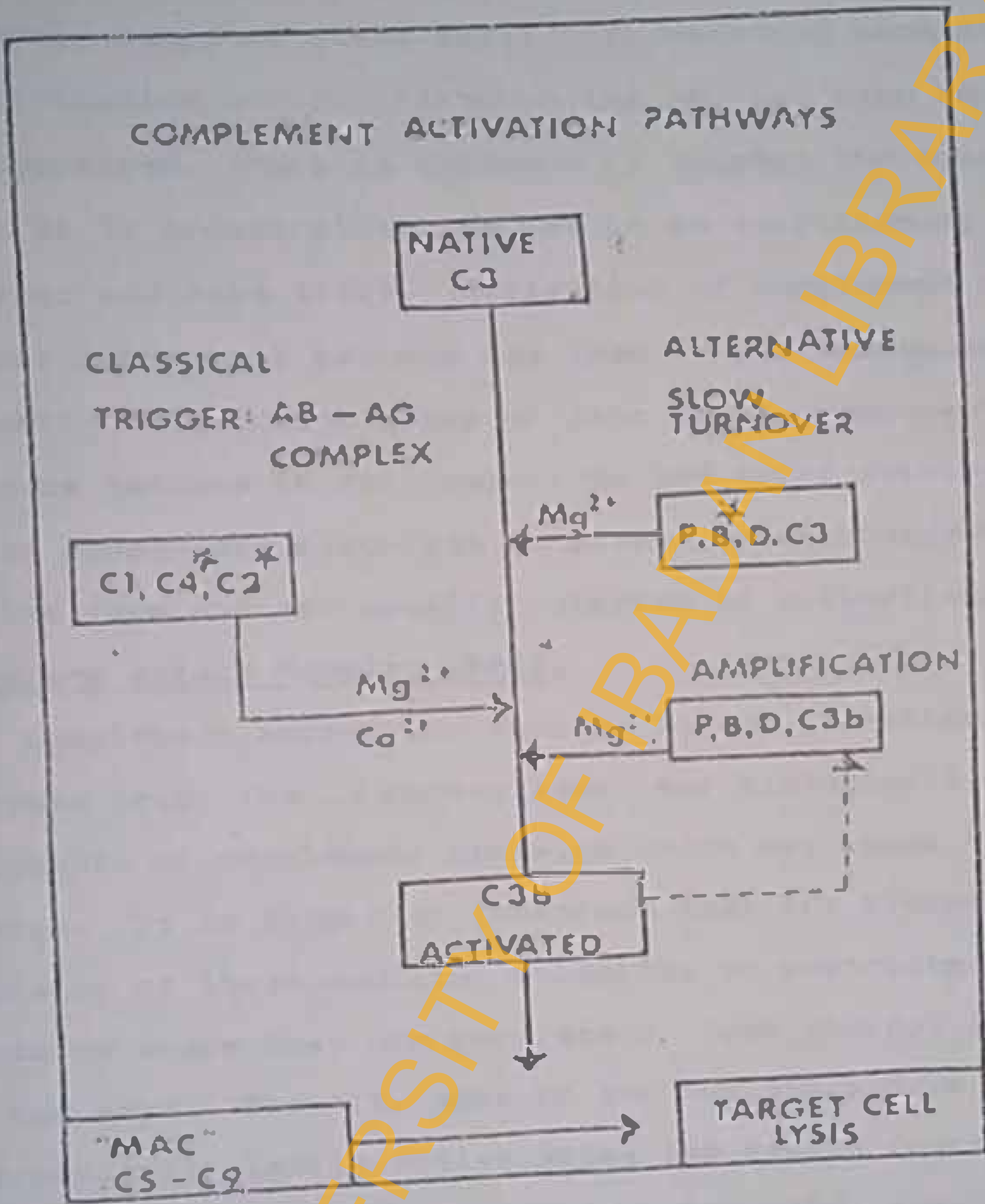

Figure 2.2.

Schematic diagram showing the relationship between the main pathways of complement activation. The MHC associated complement components are indicated by asterisks and are essential for efficient conversion of C3 to its activated form, C3b. Adapted from Umotong, (1983).

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Homeostatic mechanisms maintain APC activity in its severely dampened state until circumstances permitting amplification occur. Although the APC has been recently rediscovered, there is evidence to support the hypothesis that it is ancestral to the CPC in an evolutionary sense (Porter and Reid 1979). Activation of complement by either complement pathway may lead to the subsequent assembly of a common group of late acting components which produce lesions in cell membranes and cause cytolysis. These components circulate in plasma in an unassembled but active form and are usually referred to collectively as the Membrane attack complex (MAC).

Complement activation results in the formation of potent enzymes (e.g. the C3 convertases) and biologically active fragments of complement proteins which may cause tissue damage. It is important therefore that the biochemical activity of these mediator molecules be restricted to sites close to where they are generated. Such control is achieved in two ways. Firstly, some of the mediators have intrinsically labile active sites and remain functional therefore for only brief periods. Secondly, they may be specifically inactivated or inhibited by extrinsic control proteins e.g. C1 inhibitor and C3b inactivator (Muller-Eberhard, 1979).

(ii) Complement assays

Functional assays for both pathways are usually based on quantitation of erythrocyte lysis, (Gee, 1983).

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Classical pathway activity is assayed by measuring the ability of serum or plasma specimens to lyse a suspension of erythrocytes senaitized with anti-erythrocyte antibody in the presence of calcium and magnesium ions. Sheep erythrocytes sensitized with rabbit antibody are most often used. Alternative pathway activity is assayed by measuring the lytic properties of serum or plasma for unsensitized rabbit or guinea pig erythrocytes in the presence of magnesium ions and in the absence of calcium ions. Both types of assay may be modified to measure the activity of individual complement components, although these are often now determined immunochemically if specific antisera are available.

Haemolytic classical pathway activity is assayed by determining the quantity of complement required to effect lysis of a defined proportion of a standardized suspension of erythrocytes, optimally sensitized with an appropriate antibody. When the proportion of erythrocytes lysed is 50%, the unit obtained is referred to as a 50% haemolytic unit (CH_{50}). Many experimental parameters influence the extent of complement mediated haemolysis in a given system. These are, reaction volume, erythrocyte concentration and source, Ca^{2+}, Mg^{2+} concentrations, ionic strenght, pH, temperature and time of incubation. Since these variables determine the haemolytic titre of the complement source, they must be controlled and standardized for a given assay. The generally

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accepted international CH_{50} unit for human and guinea pig complement is defined as the volume of undiluted serum required to lyse 50% of 5×10^8 sheep erythrocytes, optimally sensitized with antibody, in the presence of optimal concentrations of calcium and magnesium cations, at pH 7.3 and ionic strength 0.149, in 1 hour of incubation at 38°C, in a total reaction volume of 2.5 mls.

Normal human serum complement levels obtained using the CH_{50} unit defined above range between 30 and 60 for the Caucasians (Wetheral, 1982). A more sensitive unit based upon the lysis of 5×10^7 erythrocytes in a reaction volume of 5 mls is also often used and results in a higher normal range of 80 - 200 CH_{50} units (Wetheral, 1982).

The alternative complement pathway lysis of rabbit erythrocytes was first described by Platts Mills and Ishizaka (1974) and provides the basis for a simple method for determining alternative pathway activity in human serum and some other species. Since alternative pathway activity requires Mg^{2+} but not Ca^{2+}, the assay is performed in the presence of a buffer containing Mg^{2+} and EGTA (ethyleneglycoltetra-acetic acid). The EGTA selectively chelates Ca^{2+}, thereby inhibiting any interference by the classical pathway mechanism.

(iii) <u>The complement system and malaria</u>

Hypocomplementaemia in acute human malaria is well documented (Ree, 1976; Williamsons <u>et al</u>, 1974; Greenwood

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and Brueton 1974; Kidwai et al, 1985; Kidwai et al, 1986; Gupta, Sabharwal and Chugh, 1982). Except the study of Gupta, Sabharwal and Chugh (1982), in which the functional (haemolytic) complement activity was assessed, all other reports were based on the antigenic levels of some key complements proteins. Evaluation of the functional activity is necessary in order to investigate normal immunochemical levels of a component or components which may be functionally inactive (James et al, 1982). In spite of the apparent fall in the total complement activity and in the levels of some of its components during erythrocyte schizont rupture (McGregor, 1972), it has been reported that complement is not involved in protection against malaria, although the major class of protective antibody is IgG (Diggs et al, 1972). Contrary to this view however, Kidwai et al (1986) in a study of serum complement levels in cerebral malaria, noted that low serum C3 and C4 may be associated with a fatal outcome. This study was conducted on Indian children at a time when chloroquine resistant *P. falciparum* malaria was highly prevalent within the population. Furthermore, increased central nervous system involvement has very recently been reported in Calabar, Nigeria, with the emergence of CRPf malaria (Ikpatt et al, 1990). However, specific studies on the role of complement in respect of chloroquine resistant *P. falciparum* malaria has as yet not been reported.

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2.6.4. Cell mediated immune response in malaria:

While the importance of antibody in protective immunity was established long ago by Coggeshall and Kumm (1937) and confirmed by Manwell and Goldstein (19440), Taliaferro and Taliaferro, (1940); Cohen and McGregor (1961); Edozien, Gilles and Udeoze (1962), the role of the thymus in immunity against malaria has been elusive. However, Brown (1968) showed that thymectomy enhanced the severity of experimental malaria in rats. This observation was subsequently confirmed and extended by others who utilized various rodent models of malaria, as well as thymectomized and athymic nude mice (Brown, 1971, Allison and Clark, 1977). Together, these findings firmly established that the thymus plays an essential role in resistance to experimental malaria, and in all possibility, to the naturally occuring disease as well (Weidenz, 1990). Nevertheless, the exact protective mechanism of the T-cells still remain an elusion (Kabilan et al, 1990). While the early studies indicated that T-cells function as helper cells in the production of protective antibodies (Brown, 1971) later studies suggest that T-cells participate through cell mediated, antibody-independent immune response against malaria (Allison, 1983). But evidence obtained from experimental infections in rodents support the idea that both T-cell functions, possibly through $CD8^+$ and gammainterferon (γ-IFN), have a role in the development of immunity to

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malaria (Kumar *et al*, 1988; Schofield *et al*, 1987; Weiss *et al*, 1988). In human infections, both antibody-dependent and antibody-independent immune protection against the malaria parasite are controlled by T-cells of the CD4+ phenotype (Weidanze, and Long 1988). A recent study on humans by Troye-Blomberg *et al* (1990) has shown that parasite antigens corresponding to immunodominant T-cell epitopes can induce T-cell proliferation, γ-IFN secretion and interleukin (IL-4) expression. In this study, the authors found a significant association between the induction, by particular peptide antigens, of IL-4 in T-cells and the presence of antibodies to the same peptides in the plasma of T-cell donors. The authors therefore suggested that a relationship exists between the activation of IL-4 producing T-cell subsets and antibody production in human sytems, in which the immune response is induced by natural infection.

Analysis of murine regulatory CD4+ T-cell clones has revealed that they can be further divided into two subsets, based on their repertoire of lymphokine production (Troye-Blomberg *et al*, 1990). Upon activation, CD4+ cells designated T helper 1 (TH1) produce IL-2 and γ-IFN, and cells designated T helper 2 (TH2) produce lymphokines IL-4 and IL-5 (Mosmann *et al*, 196; Cherwinsky *et al*, 1987). Although the relationship between these two cell types is not clear (Troye-Blomberg, 1990), there is good evidence

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that TH1 cells mediate certain antibody-independent response (e.g. delayed type hypersensitivity) and TH2 cells provide help for specific antibody production (Mosmann et al, 1986; Cherwinsky et al, 1987). Analysis of the murine Plasmodium chabaudi malaria system supports a role for TH1 cells in early antibody - independent protection, whereas the final clearance of the parasite load coincides with the appearance of malaria-antigen specific TH2 cells and antibody-mediated effector mechanism (Langhorne et al, 1989). Although there is as yet no evidence for the existence of similar CD4$^+$ subsets in the human system, human CD4$^+$ T cells have been shown to be heterogeneous with regard to surface marker characteristics (Smith et al, 1986; Dohlsten et al, 1988) and cytokine production (Troye-Blomberg et al, 1990; Paliard et al, 1988). From experimental evidence, it appears that different plasmodial species induce different types of immune response in any given host. The result of early studies using B-cell deficient chickens supported the view that T-cells provide help to B cells so that the latter can synthesize antibodies. Plasmodium gallinaceum infections initiated by the injection of parasitized erythrocytes into immunologically intact chickens progressed rapidly and resolved spontaneously without mortality. The same infections in bursectomized chickens were fulminant and lethal (Ferris, Beamer and Stutz, 1973). Similar findings

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were obtained by Weinbaun, Evans and Tigelaar (1976) in experiments utilizing mice with B-cell deficiency and on life long treatment with anti-u chain antibody. B-cell deficient mice died when infected with the normally avirulent 17X strain of *P. yoelli*, whereas immunologically intact mice became ill but survived (Tigelaar *et al*, 1976; Roberts *et al*, 1977). Unexpected but interesting results were obtained by Grun and Weidan (1981) when B cell deficient mice were infected with another rodent malarial parasite, *P. chabaudi adami*. In this instance B cell deficient mice developed acute malaria in response to infection with parasitized erythrocytes, but instead of dying as did *P. yoelli* infected mice, they resolved their infections with kinetics of parasitaemia similar to those seen in immunologically intact mice. Athymic mice were unable to resolve such acute *P. chabaudi adami* infections and eventually died.

Cavacine *et al* (1986) used adoptive transfer of T-cells but not B-cells, from mice immune to the erythrocytic stage of *P. chabaudi adami* into nude mice and observed that these animals resolved an otherwise lethal infection with homologous parasite. Protection was best achieved using $CD4^{+}$ enriched population of splenic T cells from immune mice. Since the passive transfer of serum from reconstituted nude mice which had healed their infections spontaneously failed

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to convey protection, the authors concluded that resistance to acute P. chabaudi adami malaria was achieved by T-cell dependent cell mediated immunity. All these findings put together indicate that certain resistance mechanisms predominate in acute malaria infections in mice, caused by different plasmodial species i.e. cell mediated in case of P. chabaudi adami and antibody-mediated in acute P. yoelli infections. The influence of varying strains of plasmodial species on the outcome of host resistance mechanism has not been reported. Similarly, the contribution of differing host genetic factors on the outcome of resistance mechanism in malaria has not been extensively studied.

Genetic studies on the differences in response to infections with P. berghei in inbred strains of mice was carried out by Greenberg et al, (1975) who observed that inherent differences in innate resistance between strains of mice appeared to be controlled by genes at a single locus in some mice and in others, by genes at several loci. In a recent study, Chang et al (1989) observed that congenic mouse strains differed in their degrees of reactivity with two gp 195 synthetic repeat peptides-prime candidate antigens for the development of immunity to the asexual blood stages of P. falciparum. Similar work by Lew et al (1989) on congenic mice also showed that both T-cell and T-dependent B-cell response to certain Pf155/RESA peptides are MHC-class II restricted.

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Although similar MHC restriction in immune response to malaria antigen has not been demonstrated in humans, the lack of demonstrable responses in human studies has frequently been interpreted as reflecting such restriction (Troye-Blomberg et al, 1990). However, Sinigaglia et al (1990) observed that for CS protein, there is at least one T-cell determininat which is able to bind to and be recognised by most human MHC-class II molecules. These authors also identified several epitopes recognised by T-cell clones in association with different class II (HLA) isotypes and alleles on 190L polypeptide, derived from a conserved region of the P 190 merozoite surface protein. In seroepidemiological assessment of seroreactivities in dwellers of malaria endemic regions, Del-Guidice et al (1987) had also observed that host genetic factors (possibly MHC related) play a role in immune response against malaria antigens. They postulated that the existence of such genetic regulation of immune response to CS protein, may predispose some individuals to responding better to *P. falciparum* sporozoite antigen than others. Similar findings in respect of Pf155/RESA have also been demonstrated by Petersen et al (1990) and Bjorkman et al (1990). Thus, for optimal benefit from a subunit malarial vaccine, it is essential not only to define the sequences making up the immunodominant epitopes, but also to understand the nature

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of immune response elicited in various genetic groups.

2.6.5. <u>The major Histocompatibility Complex (MHC):</u>

Attempts to prevent rejection of tissue transplanted between genetically non-identical individuals of the same species have led to the discovery and definition of an important system of transplantation antigens. These molecules occur on the surfaces of many types of cells and provide the antigenic stimulus responsible for immunological rejection of incompatible donor tissues by the recipient. Transplantation antigens have been intensively investigated in mice, guinea pigs and humans and are usually called the histocompatibility antigens. MHC encompasses a tightly linked and highly polymorphic genes that function in immune response (Bodmer, 1987). Some of the MHC genes encode molecules which reside on the membranes of most nucleated cells especially lymphocytes and macrophages. These mediate the cellular interactions which distinguish "non self" tissue from "self" tissue and in this way function as antigenic regulator of the immune system. At least 16 operative genes have been defined, including the Class I (HLA-A,B,C) and Class II (HLA-DR, DQ, DP) cell surface glycoproteins as well as a class III region which encodes four complement components. The linkage of the HLA-A,B,C,DR and DQ genes on the 6th chromosome creates a unique HLA haplotype, giving rise to potentially more than a billion genetically different individuals (Bodmer, 1987).

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In addition to these three classes of MHC loci, gene determinants for the synthesis of several enzymes are also located within or near this chromosomal segment. The gene for 21 hydrozylase deficiency is known to be closely linked to the HLA B/DR region of the human MHC. This enzyme is important in steroid metabolism and its absence is the most frequent cause of congenital adrenal hyperplasia. Furthermore, there are other genes coding for tumor necrosis factor on the MHC (Spies et al, 1986; Muller et al, 1987). Tumor necrosis factor, secreted by activated macrophages and lymphocytes is associated with the development of resistance to malaria (Peyron et al, 1990) and is also associated with the severity of cerebral malaria (Gran and Lambert, 1988).

The class II MHC genes code for a series of products concerned with antigen presentation and therefore specific immune response. One of the striking features of this presentation is that it is only efficient when the antigen presenting cell and the responding cell possess a common allelic form of the class II sub-region of the major histocompatibility complex (Rosenthal and Shevach, 1973).

There is growing agreement that protein antigens are not presented intact by macrophages to T-cells, but are first broken down in the presenting cells into peptide fragments containing 12-20 amino acids (Marx, 1987). The class II histocompatibility protein molecule is in the form of

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peptide groove which can readily accomodate a peptide (antigen) containing 12-20 ammino acids (Marx, 1987). Since almost all of the polymorphic amino acids of the histocompatibility proteins are located in the area of the groove, changing them might well alter the molecule's ability to bind and present antigens to killer T-cells. When an antigenic determinant is unable to interact with the MHC molecules possessed by a given individual, T-cell clones specific for this particular combination of determinant and MHC molecules cannot be activated and therefore not all individuals will respond to any one antigenic determinant. For example, Good _et al_ (1986) and Del-Guidice _et al_ (1986) reported that polymers of NANP (the repetitive sequence which forms the dominat B cell epitope of _P. falciparum_ CS protein) were recognised by T-cells only in mice bearing $1A^b$ in the H-2 region, and only these mice made antibodies to the repeat peptide. Similarly among 15 strains of mice with different H-2 types and different genetic backgrounds, none responded to the PPPPNPD repeats of the _P. berghei_ CS protein (Romero et al, 1988) and only mice bearing $1A^k$ responded to the RESA/Pf 155 repeat (Lew _et al_, 1989). The implications of these findings is that if the same situation occurs in humans, a given subunit vaccine might be immunogenic in only a small proportion of individuals (Sinigaglia _et al_, 1990).

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From the practical point of view, having a clear idea of how histocompatibility molecules work could increase the ability of researchers to manipulate immune responses. Thus a particular residue could be changed or a suitable adjuvant added to a peptide antigen, to see how it changes MHC peptide binding or MHC T-cell recognition. The information gained may help explain why a particular MHC molecule variant is more or less susceptible to a disease than others. Such information for example may help in the design of vaccines for stimulating immunity (Marx, 1987).

2.6.6. Malaria antigens

The Plasmodium undergoes progressive transformations during its progress from the infective sporozoite injected by the bite of infective mosquito, through the enormously amplified exoerythrocytic schizogony in the liver of the vertebrate host, followed by the cascade-like multiplication of asexual stage in the blood, and then the appearance of sexually differentiated gametes which proceed, after fertilization, to the sequence, zygote, ookinete, oocyst and to the formation of countless new sporozoites in the body of the insect vector. Each of these stages has a well defined function, but though each has the same complement of genes, every stage expresses a different part of the Plasmodial genome, and during this series of events the parasite generates an enormous number of antigens. Some of

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these stimulate the protective immune response of the host, while others are immunologically irrelevant or harmful (McGregor, 1972; Bruce-Chwatt, 1985). The production of a malaria vaccine will require the identification, characterization, and use of those antigens which stimulate effectively the protective response of the host against a homologous parasite species. Although the principle of this procedure was known a long time ago, its practicability became obvious only in 1976 when Trager and Jensen first succeeded in maintaining _in vitro_ cultures of _P. falciparum_ (Trager and Jensen, 1976), while Milstein and Kohler (1975) developed the hybridoma technique for producing sensitive monoclonal antibodies.

Generally, the path of development of malaria vaccine starts with identifying the antigens which induce the greatest specific immune response in animals (together with an absence of toxic effects). Next, studies are carried out in primates susceptible to infection with human plasmodia. Thereafter, seroepidemiological surveys in humans agaisnt these prospective vaccine candidates are carried out and finally vaccination in humans would be tried. Very many isolated antigens of relative molecular weights are now obtained in pure forms through affinity chromatography using monoclonal antibodies, polyacrylamide gel

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electrophoresis and other advanced biotechnology methods (Brown et al, 1981; Freeman and Holder, 1983; Perlmann et al, 1984). Furthermore, large scale plasmodial culture systems are now produced in gram quantities from crude plasmodial antigens.

Further steps comprise cloning of genes that code for the protective antigens, analysis of their nucleotide composition, deduction of the amino acid sequence of the encoded molecule, and production of the relevant peptides by recombinant DNA methods or by solid phase synthesis.

Based on these possibilities, three types of vaccine against *P. falciparum* are presently under investigation and trials, and their eventual uses can be foreseen (Bruce-Chwatt, 1985).

A merozoite or more accurately asexual erythrocytic vaccine, inducing immunity against blood forms of the parasite, would act as a therapeutic compound and meet the problem of drug resistance. A sporozoite vaccine, preventing the infection might replace chemoprophylactic compounds and be useful for protection of non-immune visitors to malarious areas and some vulnerable groups of indigenous populations. A gamete-reactive vaccine used on a large scale might prevent the transmission of malaria in epidemic areas or where the danger of resurgence is high (Bruce-Chwatt, 1987).

(A) Sporozoite Antigen

Malaria sporozoites possess a major surface antigen,

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the circumsporozoite (CS) protein, which uniformly surrounds their external coat. Despite their short stay in circulating blood, sporozoites induce a strong immune response that is characteristically species and stage-specific (Cochrane, 1980). A milestone in the identification of malaria antigens has.been the isolation in the early 1980's of CS protein from the outer surface of sporozoites in the salivary glands of infected *Anopheles* mosquitoes (Nussenzweig, et al, 1986). The protein structure was clarified in 1983 by cloning CS genes, first in *P. knowlesi* of monkeys, and a year later in the human *P. falciparum* (Dame *et al*, 1984). The CS protein of *P. falciparum* has a large central domain of 412 amino acids, which comprises nearly half the polypeptide chain. These tandem repeats of amino acids, are flanked by non-repetitive sequence (Nussenzweig and Nussenzweig 1985). All the monoclonal antibodies so far produced against circumsporozoites appear to recognise this repetitive sequence in the CS protein (Zavala *et al*, 1983), as do naturally acquired antibodies from malaria endemic areas (Zavala *et al*, 1985). The repetitive domain of the *Plasmodium falciparum* CS protein consists of four amino acids (Asn-Ala-Asn-Pro = NANP) repeated several times (Dame *et al* 1984; Enea *et al*, 1984), which is well conserved in all the isolates from different geographical regions so far investigated (Zavala *et al*, 1985;

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Weber and Hockmeyer 1985; Lockyer and Schwarx 1987). The CS synthetic peptide includes immunodominant B-cell epitopes. Naturally occuring antibodies recognizing the immunodominant CS proteins of P. falciparum have been reported from numerous countries including The Gambia, Thailand, Tanzania, Indonesia, Kenya and Burkina Faso (Nardin et al, 1979; Burkot et al, 1989). Mice and rabbits produce high antibody titres when immunized with synthetic peptide coupled to tetanus toxoid as a carrier and absorbed on to aluminium hydroxide (Zavala and Hollingdale, 1985). However, there are conflicting reports on the presence of T-cell epitope on circumsporozoite protein. While it has been suggested by Schofield and Uadia (1990) that the response to native (sporozoite as opposed to recombinant) CS protein is T-independent, Good and Zevering (1990) have strong evidence that CS-specific T-cells can facilitate a boosting or secondary antibody response by sporozoite specific B-cells. It is therefore believed that while the response to sporozoites may be in part T-independent, a significant part of the response appears to be T-dependent (Good and Zavering, 1990).

In 1941, Mulligain and his colleagues in India found that killed sporozoites of avian Plasmodia were highly immunogenic. In the U.S.A., Heidelberger and his co-workers (1946) attempted to vaccinate man against P. vivax malaria, but to no avail. In 1973 however, Clyde end othera (1973)

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reasonably succeeded in immunizing three human volunteers, by using many millions of *P. vivax* and *P. falciparum* sporozoites obtained from experimentally infected *Anopheles* previously exposed to intensive X-ray irradiation (to attenuate the virulence of the parasite). This was the first indication that active sporozoite immunization might be possible.

The CS synthetic peptide, when injected into experimental animals induced the formation of specific antibodies that reacted with live *P. falciparum* sporozoites and prevented their invasion of cultured human hepatocytes (Young *et al*, 1985, Hollingdale *et al*, 1984). More recent studies by Burkot *et al* (1989) have also shown that *P. falciparum* CS protein may be so immunogenic in humans that a peak immune response can be reached after a limited number of exposure. Analysis of Tanzanian sera by Del-Giudice *et al*, (1987), Indonesian sera by Hoffman *et al*, 1986; The Gambian sera by Snow *et al* (1989), and sera from indigenous dwellers of Burkina Faso by Esposito *et al* (1988) showed that CS antibodies are correlated with protection. However, later field studies by Hoffman *et al* (1987) in Kenya and other similar studies by Pang *et al* (1988) and Webster *et al* (1988) both in Thailand, and Burkot *et al* (1989) in Papua New Guinea, indicated that these antibodies are not protective after all. Moreover, the half life of anti-CS antibody is reported to be as long as several years

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n Africans (Druilhse et al 1986) and as short as 27 days n the Thais (Webster et al 1987). Furthermore, it has been suggested that genetic restriction may play a role in the ability of certain individuals to mount an antibody response in humans (Burkot et al, 1989, Rosenberg and Wirtz, 1990), as it was demonstrated in mice (Del-Giudice et al 1986, Good et al, 1986).

Both the prevalence rates and the level of reactivity to CS protein increase with age and with exposure to infections (Hoffman et al, 1986; Campbell et al, 1987; Chizzolini et al, 1989; Snow et al 1989; Deloron and Cot, 1990). Thus, in areas of low endemicity, children seldom test positive against CS protein. In Madang area of Papua New Guinea, with innoculation rates of between 44 and 293 bites by P. falciparum sporozoite-infected mosquitoes per year, (Burkot et al, 1988), a maximum immune response was achieved in the first five years of life (Burkot et al, 1989).

Both recombinant and synthetic peptide of CS protein of P. falciparum have been of tremendous use as antigens in serologic assays and appear to have potentials for detecting antibodies in people living in areas endemic for malaria. Their use in this regard includes:

(i) their use as an indicator of the level of parasite transmission (Sporozoite innoculation rate) in a study community (Campbell et al, 1987; Snow et al 1989).

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(ii) the application in investigating the possible relationship between sporozoite antibodies and protection against malaria infections (Espisito *et al*; 1988; Snow *et al*, 1989; Burkot *et al*, 1989).

(iii) the measurement of immune responses against CS protein which has also been employed as a possible indicator of genetic restriction in immune response against malaria (Snow *et al*, 1989; Rosenberg and Wirtz 1990).

(B) Blood Stage Antigens

The exoerythrocytic shizogony in the liver is short-lived in *P. falciparum* infections. It is followed by the cascade-like multiplication of the asexual stage in the blood, where the most abundant amount of malaria antigens is produced (Bruce-Chwatt, 1985). However, to date, none of these antigens appearsto be dominant as a target of protective immunity against malaria, rather, several antigens appear to be capable of inducing immune response that limit the growth or development of *P. falciparum in vitro.* Conflicting reports have been accumulated regarding the protective value of some of these antigens *in vivo.* Like immune responses against the circumsporozoite protein, some blood stage malaria antigens are believed to be genetic-restricted as well (Molineaux and Gramiccia, 1980; Bjorkman *et ol*, 1990). Parasite polymorphism may also contribute to immune response restriction against malaria (Good *et al*, 1988). Considering

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these possibilities in line with the emergence of chloroquine resistant strains of P. falciparum, it is likely that maximum efficiency will be achieved only with a malarial vaccine that combines several of these antigenic epitopes (Perlmann et al, 1989).

(a) Soluble Malaria Antigens

Using aqueous extracts prepared from blood, heavily infected with mature, or nearly mature asexual forms, or serum from patients with dense parasitaemia, Turner and McGregor (1969) identified two groups of blood stage antigens which they termed α and β antigens. Wilson et al (1969) extended these studies and classified P. falciparum antigens as 'L' (Labile), 'R' (resistant), and 'S' (stable), on the basis of their susceptibility to heat. Wilson et al (1969) also observed that the previously identified α and β were similar to S and L antigens respectively. S-antigens were found in the sera of 57% of Gambian children with severe P. falciparum malaria by McGregor et al(1968). In Nigerians, Williams (1971) also found malaria soluble antigens (MSSA) in sera, which he described as being similar to the S-antigens of Wilson et al (1969). He observed also that incidence of MSSA in Nigerian adults varied from 0.5% to 6%.

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S-antigens recovered from the sera of patients studied during consecutive parasitaemic episodes were frequently of the same serologic specificity when the episode occured within one month, but were usually of different specificities when the interval was longer (McGregor _et al_, 1968). These workers found as well that persistence of circulating S-antigens following a malarial episode was influenced by a number of factors. In general, the higher the titre of S-antibody present at the time of treatment, the longer the antigen persisted. The parasitaemia density achieved was probably also important, for antigen was found to remain detectable for two weeks or longer in significantly more patients who presented with parasitaemias of more than 200, 000 per mm^3 than in patients with lower parasitaemias. Furthermore, specific S-antigens were found to induce antibody responses in some children but not in others, suggesting that factors other than immunogenecity of the antigens were involved in the immune response.

Studies by Wilson (1980), and Anders, Brown and Edwards, (1983) showed that S-antigens are antigenically diverse, with heterogeneous sizes ranging between relative molecular mass (Mr) 120,000 - 250,000, with different and large numbers of repeated sequences of amino acids. Immunoprecipitation of S-antigens from lysates of parasites, metabolically labelled with ^{3}H-glycine showed that the

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antigen is first produced at the beginning of schizogony. Labelling intensified as schizonts matured, until the S-antigen is released into the medium, when infected red cells rupture (WHO, 1984b). No conclusive evidence however has been adduced in support or otherwise of the involvement of anti-S-antigen antibody in protective immunity.

(b) Ring Infected Erythrocyte Surface Antigen (RESA)

A cell-fixing method for immunofluorescence screening led Perlmann *et al* (1984) and Coppel *et al* (1984) to independently identify a previously undescribed antigen on the surface of ring-infected erythrocytes. Monolayers of human erythrocytes infected with *P. falciparum* ring stages were fixed with 1% gluteraldehyde and air dried. Sera from malarial patients reacted positively in immunofluorescent assay (IFA) when tested against erythrocyte membranes of the ring stage parasitized cells. Uninfected erythrocytes did not react. Gluteraldehyde fixation was found to be necessary to prevent accessibility of antibodies to intracellular parasite antigens. Air drying of the fixed cells was also an absolute requirment to detect the reactivity of these antigens.

Coppel *et al* (1985) described the antigen aa red cell erythrocyte surface antigen (RESA) and its molecular weight

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was determined to be 155,000 daltons (Perlmann *et al*, 1984; Wahlin *et al*, 1984; Coppel *et al* 1984), probably identical with the glycophorin binding protein of *P. falciparum* of Mr 155,000, described earlier by Perkins (1984). Perlmann *et al* (1984) and Coppel *et al* (1984) also reported that RESA-Pf155 antigen was formed at the late schizont stage and was present on the merozoite surface. During merozoite invasion, the antigen is subsequently deposited on the red blood cell membrane, where it occupies the apical bilayer, hence the reactive epitopes are not fully accessible (on the red blood cell membrane) to the immune system. Moreover, presence of antigen is apparent only in the very early ring stage (WHO, 1984b), and immunofluoresence reactivity is malaria species-specific. Thus, *P. chabaudi*-infected erythrocytes containing ring forms, reacted similarly with antiserum to this parasite only,but not with antisera to *P. yoelli* or *P. vivax* (WHO, 1984a).

On further analysis of RESA/Pf155, Favaloro *et al* (1986) observed that the antigen contains two regions of tandemly repeated amino acid sequences, one in the carboxy-terminal region (3 repeat region), predominantly consisting of the octamer Glu-Clu-Asn-Val-Glu-His-Asp-Ala (EENVEHDA) and the related tetramer Glu-Glu-Asn-Val (EENV), and the other at middle of the molecule (5' repeat region) consisting of the undecamer Asp-Asp-Glu-His-Val-Glu-Pro-Thr-Val-Ala (ODEH8EEPTVA), and related sequences. A schematic presentation

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of the antigen units is in Fig. 2.3.

The 5' and 3' repeats display some immunological cross reactivity (Cowman et al, 1985). Thus, both rabbit and human antibodies to these repeat sequences can equally inhibit merozoite invasion (Berzins et al, 1985). However, the 3' repeats have been found to be more immunogenic than the 5' repeats (Collins et al 1988).

Several immunodominant B-cell epitopes of *P.falciparum* RESA/Pf115 are located in the molecular regions containing these amino acid repeats (Miller et al, 1986). More recently, T-cell epitopes have also been mapped to the same repeat regions on RESA/Pf155 by Kabilan et al (1988) and Troye-Blomberg et al (1988). Thus Pf155/RESA has been found to be capable of inducing *in vitro* proliferation, interleukin (IL)-2 release and gamma interferon (γ -IFN) production in T-cells from individuals primed to this antigen by natural infection (Troye-Blomberg et al, 1985). Although antibodies contribute to immune protection against malaria, priming of T-cells is important for the development and maintenance of immunity (Good and Miller, 1989; Troye-Blomberg and Perlmann 1988; Weidanz and Long, 1988), and T-cell derived γ-IFN is believed to be an important mediator of cellular effector mechanism (Kabilan et al 1990). Consequently, peptides corresponding to the repeat regions of RESA/Pf155 are considered prime candidates for effective

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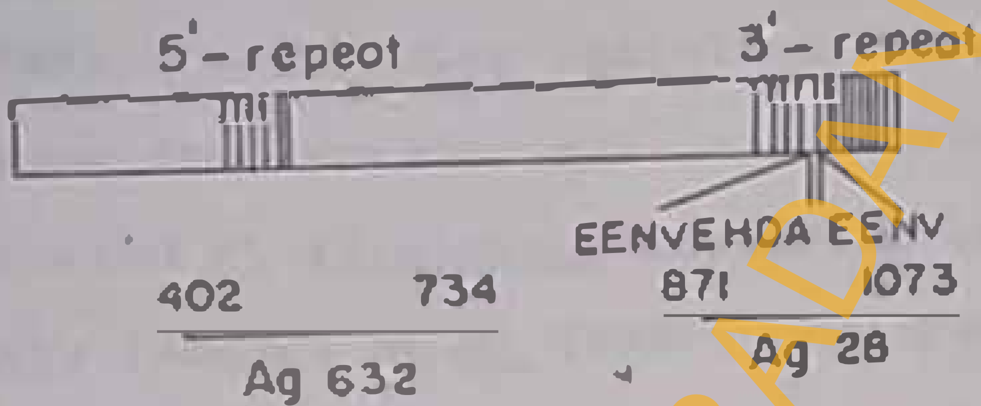

Fig. 2.3 Schematic presentation of Pf155/RESA. The amino acid positions of FP Ag632 and FP Ag28 are shown. The 5' repeat block consists of the consensus sequence DDEHVEEPTVA repeated twice and five similar sequences with one or two deletions each. The 3' repeat block at the C terminus consists of the sequence EENVEHDA repeated 5 times and the sequence EENV repeated 29 times plus eight variants of the latter. Adapted from Wahlin et al (1990).

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blood stage vaccine against malaria. Investigations by various workers (Coppel *et al*, 1984; Berzins *et al*, 1986) have shown that majority of antibodies to Pf155/RESA are directed against epitopes within the repeat regions. *In vitro* studies have also shown that human antibodies to this antigen are extremely efficient in inhibiting *P. falciparum* reinvasion (Perlmann *et al*, 1984; Wahlin *et al*, 1984). Rabbit antibodies against a synthetic peptide representing the repeated sub-unit EENVEHDA of Pf155/RESA also inhibited *P. falciparum* merozoite reinvasion *in vitro* efficiently (Anders *et al* 1990). Furthermore β-galactosidase fusion proteins of DNA clones encoding this antigen sequence partially protected *Aotus* monkeys from *P. falciparum* challenge. Protection in these animals also correlated with the presence of antibodies reactive with Pf155/RESA repeat sequences, including the octopeptide (Collins *et al*, 1986). Evaluation of the presence of potentially protective anti-Pf155/RESA antibodies in human immune sera, as well as in antisera from experimental animals by means of *in vitro* invasion inhibition assay, have however shown that antibodies are often not efficient inhibitors as components in total immunoglobulin preparations, but become very efficient when affinity purified (Perlmann *et al* 1987). This difference could be due to the presence, in the sera, of both reinvasion inhibiting antibodies and of antibodies promoting reinvasion.

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The existence of this latter type of antibodies were recently demonstrated by using monoclonocal antibodies to an asparagine rich protein (Ag 106) of P. falciparum. Antibodies to certain epitopes efficiently inhibited merozoite reinvasion, whereas antibodies to other epitopes in the same antigen were very efficient promoters of reinvasion (Franzen et al, 1989). More recently, Wahlin et al, (1990) observed that these merozoite invasion inhibition antibodies were anti-idiotypic. Whether or not parasite polymorphism or individual immune response restriction contribute to the production of these merozoite invasion inhibition antibodies has not been elucidated.

In children living in malaria endemic areas of Africa, a correlation was found between high levels of antibodies to Pf155/RESA and acquired immunity to P. falciparum (Wahlgren et al 1985; 1986b). Many other workers also agreed that anti-Pf155/RESA antibodies have some protective value against P. falciparum infections (Chizziloni et al 1989; Collins et al, 1988; Perlmann et al, 1989). However, studies by a few other workers do not corroborate these views (Deloron et al, 1987; Marsh et al, 1989). In a longitudinal consecutive determination of seroreactivities of Pf155/RESA antigen and to its repetitive amino acid sequence in adult men from a holoendemic area of Liberia, genetic restriction was suggested as responsible for

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individual variation in immune responses against the antigens (Bjorkman et al, 1990). Genetic regulation of Pf155/RESA epitope-specific host responses has also been noted by Perlmann et al,(1989) and Petersen et al, (1990) during longitudinal studies of individuals living in malaria endemic African villages. While studying antibody responses against Pf155/RESA derived peptides in monozygotic twins, Troye-Blomberg et al (1990) noticed that these antibodies and some of the T-cell responses could be paired within the twin pairs, indicating a genetic regulation of the B-cell responses. These patterns of immune responses against RESA/Pf155 and its peptides are suggested to be due to differences in MHC and/or allotype restriction (Perlmann et al, 1989, Troye-Blomberg et al, 1990).

Parasite polymorphism may also contribute to immune response restriction (Good et al, 1988), although the immunodominant repetitive region of Pf155/RESA are invariant in different *P. falciparum* strains, from various geographical locations so far studied (Pavaloro et al, 1986; Perlmann et al, 1987; 1989). Results of preliminary serological studies of Pf155 have also given no indications of antigenic diversity (Perlmann et al 1984).

The invariant nature of Pf155/RESA antigen therefore makes this protein and its immunodominant repetitive peptides, an important prospective blood stage vaccine candidate Perlmann et al, 1987).

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Moreover, to circumvent some of the problems specifically associated with Pf155/RESA sub-unit vaccines, particularly the possible genetic restriction of the host's immune responses, these vaccines may still need to be made poly-valent by the inclusion of several parasite antigens (Perlmann et al 1989) or by use of appropriate adjuvants (Bruce-Chwatt, 1987).

(C) Other Blood Stage Antigens:

(i) High molecular weight schizont surface glyco-protein KDa 185 - 220):

This group of blood stage antigens are proteolytically processed at about the time of schizont rupture to generate the majority of antigens detected on the surface of merozoites (Perrin and Dayal, 1982; Holder and Freeman 1982; WHO, 1989). These antigens have been variously referred to as precursor of merozoite membrane antigen (PMMA), pf 195, P 190, gp 185, and merozoite surface antigen (Msa - 1, MSA - 2) (WHO 1988). Of these, the Pf 195, known in some laboratories as P190 (Hall et al, 1984) is the most extensively studied. Although amino acid repeat sequences are present in this molecule, many other parts of the structure are also antigenic, so that the repeat portions do not seem to be immunodominant (Ridley, 1988). Considerable antigenic diversity exists in this molecule among different strains of P. falciparum (WHO, 1989), and it is therefore not on the priority list as a vaccine

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candidate protein.

(ii) Knob-associated antigens:

Erythrocytes infected with *P. falciparum* also acquire the functional property of cytoadherence to endothelial cells, as the parasite develops from the ring to the trophozoite stage. Asexual *P. falciparum* parasite forms are detected in peripheral blood smears only as immature ring stages. Trophozoite- and schizont-infected erythrocytes are sequestered in deep tissues by specific attachment to endothelial cells lining the venules and capillaries (Luse and Miller, 1971). These mature parasitized cells may block flow in such organs as the brain and so give rise to the classical neurological symptoms of cerebral malaria. This cytoadherence phenomenon, between the surfaces of *P. falciparum-infected* erythrocytes and capillary endothelial cells, is mediated by knob-like protrusions of the erythrocyte membrane (Luse and Miller, 1971; Trager, Rudzinska and Bradbury, 1969) and the underlying cytoskeleton (Leec *et al* 1984). Laboratory-derived knobless variants (K^-) of *P. falciparum* do not attach to endothelial cells *in vivo* (that is, mature K^- parasite infected cells appear in peripheral blood), (Barnwell, Howard and Miller, 1984). The K^- parasites also do not attach *in vitro* to endothelial cells or melanoma cells (Udeinya *et al* 1983), which have been used as targets in a

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model of this cytoadherence phenomenon. Expression of knobs on the erythrocyte membrane can be linked directly to enhanced parasite survival since K$^-$ parasites are much less virulent in Aotus monkeys than knob-bearing (K$^+$) parasites from the same isolates (Barnwell, Howard and Miller, 1984). It has been suggested that through expression of knobs and cytoadherence, mature P. falcioarum parasites avoid passages through the spleen, thereby avoiding exposure to localized specific and non-specific mechanisms that would remove the altered erythrocytes (David et al, 1983).

A malarial protein of about KDa 300, expressed on the surface of P. falciparum-infected erythrocytes has been identified as the likely cytoadherence moiety, on the basis of three properties. These are that:

(i) the capacity of antisera to block or reverse cytoadherence in vitro matches the capacity of the sera to react with this protein at the surface of infected cells (Leech et al, 1984).

(ii) the protein molecule itself is exquisitely sensitive to trypsic cleavage at the cell surface, parallel with the loss of cytoadherence seen with trypsin-treated infected cells (Leech et al, 1984).

(iii) K$^-$ parasite variants which do not cytoadhere also do not express this protein at the cell surface (Aley Sherwood and Howard, 1984).

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Ikpatt *et al* (1990) noted that cerebral malaria cases in Calabar seem to have increased with the emergence of CRPf in the area.

A similar observation has also been reported from Thailand by Miller (1989). Whether or not these emerging strains of *P. falciparum* are predominantly knob-associated, or express knob-protein variants with greater capacity for adherence is yet to be elucidated. Based on studies conducted on *P. flaciparum* from many geographical areas, the putative cytoadherence protein has however been found not to be invariant (Leech, Barnwell and Miller, 1984; Udeinya *et al* 1983; Marsh and Howard 1986), and this would invalidate its potentials as a prospective vaccine candidate. However, an antigenically invariant epitope was identified on the surfaces of infected erythrocyte isolates from Gambian patients (Marsh and Howard, 1986). This conserved epitope, once identified, may be an important antigen for use in vaccines. The identification by Barnwell and Ockhenhous (1985) and Roberta *et al*, (1985) of ligands for cytoadherence may assist in the purification of the cytoadherence molecules and the particular domain involved in its function.

(C) Gamete Antigens:

Target antigens of transmission-blocking immunity in malaria were first identified in the avian parasite,

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P. gallinaceum, using monoclonal antibodies which reacted with surface proteins of extracellular gametes of the parasite (Kaushal et al, 1983). Those of the human parasite P. falciparum were identified shortly afterwards by Rener et al (1983) and later on by Vermeulen et al (1985).

It is believed that the immune response elicited against these antigens will limit transmission of infection by suppressing the development of sexual and sporongenic stages (WHO, 1990). The rationale for transmission-blocking vaccine development rests on the expectation that such vaccine will reduce malaria transmission levels, at least under certain epidemiological conditions and if used in combination with sporozoite or asexual blood-stage vaccines, will prevent the propagation of any vaccine-resistant mutant parasite (WHO, 1990).

2.6.7. Antigenic Variation in P. falciparum:

Antigenic variation is a process by which an infected organism gains a selective advantage by changing its antigenic profile, thus avoiding elimination by the host immune system (Homel, Davis and Oligino, 1983). Antigenic variation in malaria explains why immunity against the disease is hardly sterile.

Repeated antigenic variation has been demonstrated in the simian malaria parasite P. knowlesi, by Brown and Brown (1965) and by Brown et al (1968). Voller and Rossan

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(1969) confirmed the existence of antigenic variation in P. knowlesi when they found evidence of such variation in another monkey malaria, P. cynomolgi bastianelli. Furthermore, antibody resistant parasite populations have been isolated from a rodent malaria P. berghei, by Briggs et al (1986).

In human malaria however, the first indications of the existence of antigenic variation were the findings by Wilson et al (1969) and McGregor and Wilson (1971), of several different S-antigens of P. falciparum. More antigenic differences in P. falciparum isolates were also demonstrated by McBride, Walliker and Morgan (1982). Other bases of P. falciparum variability have been indicated by protein variants (Fenton, Walker and Walliker, 1985), enzyme variants (Sanderson, Walliker and Millez, 1981) differences in response to drugs (Peters, 1982), as well as by the sequencing of many genes (Kempt et al, 1986). These parameters may likely co-exist in a variant strain of the parasite, since, for example, a drug-resistant strain may manifest with specific protein changes (Creasy et al, 1990).

Antigenic variation of P. falciparum may affect either the epitope, changing the specificity of an immune response, or the MHC-binding region thus changing the potential to generate any response at all. Knowledge of the antigenic diversity among populations of malaria parasites

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and of the extent and nature of this diversity, within a parasite species, becomes increasingly relevant, as malaria control measures become more sophisticated and more selectively targeted towards the molecular components of the causative parasites. There is evidence that some variants of these characters occur at different geographical areas (Walliker, 1985). In an elaborate study of genetic diversity of P. falciparum from Thailand, Zimbabwe and Brazil, Creasy et al, (1990) used 20 variant markers to demonstrate that there exist geographical variations in the frequencies with which many variants occur. These authors recommended studies to determine the extent of these variations which occur in small communities, and to determine whether or not such changes occur over a given period of time (e.g. from the start to the end of a transmission season or from one year to another). They postulated that such geographical variations involving antigenic components of a parasite species would affect the state of immune response in different populations.

In a study of in vitro growth inhibition of P. falciparum by sera from different regions of the Philippines, Sy et al (1990) observed that sera from most areas exhibited greater growth inhibition of homologous than heterologous strains. They suggested that although geographically quite close, the parasites still exhibited some immunological differences.

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Both the knowledge of the extent of antigenic variation and of the probable associated immune response variation, in different malaria endemic populations, will be of immense relevance in immune intervention in malaria control.

2.6.8. ELISA for antibody against total blood stage antigen of *P. falciparum*

Antibodies against *P. falciparum* are not fully protective against the disease, partly due to the fact that the specific antigens are intracellular (Greenwood and Whittle (1981). Acetone treatment of blood stage infected red blood cells renders most of these antigens accessible to ~~in vitro~~ antibody binding. Since some of these antigens may also not be exposed by acetone fixation, it is likely that IFA may not estimate the total malaria antibody. Sonicates of blood stage-infected human red blood cells, coated on to ELISA plates have therefore been used as antigens in evaluating total blood stage antibodies (Wahlgren *et al*, 1986b; March *et al* 1989). These workers showed that there was no positive correlation between total blood antibody titres by ELISA and the blood stage antibody titres determined by IFA.

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CHAPTER 3

EVALUATION OF CHLOROQUINE SENSITIVITY OF *PLASMODIUM FALCIPARUM* IN CALABAR AND IBADAN

3.1 INTRODUCTION

Drug resistance is probably one of the best studied characteristics of intraspecies diversity in malaria parasite (WHO, 1987b). Accurate and reasonably simple test systems are available for use in evaluating chloroquine sensitivity of malaria parasites, thereby making it possible to monitor, with some accuracy, the geographical spread and distribution of drug resistance. Monitoring of chloroquine sensitivity of *P. falciparum* in areas where malaria is endemic is therefore considered a necessary prerequisite for appropriate control measures, (WHO, 1987b).

Although the emergence of chloroquine resistant *P. falciparum* (CRPf) in the South Eastern States of Nigeria had been speculated for a long time, no *in vitro* chloroquine response studies had been carried out in order to confirm cases of drug treatment failures reported from these areas. The study reported here was therefore undertaken to establish for the first time, the prevalence level of CRPf malaria in a South Eastern State population, using the "W.H.O." approved *in vitro* method. Calabar was chosen because of its nearness to the Republic of Cameroun, one of the African countries from where CRPf malaria was initially reported (Sansonetti *et al*, 1985).

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The study was also designed to compare by *in vitro* method, the current prevalence level of CRPf malaria between Calabar and Ibadan a town in S. W. Nigeria, where there had been little or no reported incidences of CRPf malaria. This study was also envisaged to serve as a prerequisite to the assessment of the nature of humoral immune responses that may be elicited against the emerging Nigerian strain(s)of CRPf in the two Nigerian localities investigated.

3.2 Materials and Methods

3.2.1. Study Area:

The study was conducted in two different geographical locations in Nigeria - Calabar and Ibadan. Calabar and Ibadan are located at 4.5N, 8.22E and 7.23N, 3.56E respectively, and both cities experience identical climatic conditions. The rainy season starts from April/ May and ends in October, while the dry season spans between November and April. The two areas are also holo-endemic for malaria transmission, with *P. falciparum* contributing 90 - 95% of malaria cases (Ekanem *et al*, 1990).

3.2.2. Patients and control subjects:

Patients selected for both *in vivo* and *in vitro* studies satisfied the following conditions:-

(i) No history of taking antimalarials within the previous 14 days and this was verified by the Dill Glazko urine test (Lilijveld and Kortmann 1979).

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(ii) Pure *P. falciparum* infections with parasite density $\geq$ 500 per ul.

(iii) Subjects selected for *in vivo* study as well, were confirmed not too sick to take oral medication.

(iv) All patients were assessed by clinical examination, carried out by a consultant physician.

(i) Screening of patients

Children aged between 5 months and 12 years,(mean = 4.9 $\pm$ 3.8 years) who reported with fever at the children's emergency unit of the University of Calabar Teaching Hospital, between August and October, 1989, were screened for parasitaemia. Those with adequate parasite counts were further screened to ensure that they satisfied other criteria listed above. *In vitro* studies were carried out on the isolates of all the patients recruited, while *in vivo* studies were simultaneously carried out on some of them.

In Ibadan, recruitment of subjects was undertaken between August and October, 1990. Blood was collected from children,(mean age = 6.5 $\pm$ 5.1 years) who were identified suitable for enrolment by clinicians. This was done in the out-patient clinic, children's emergency clinic and the clinical pharmacology unit of the University College Hospital, Ibadan. Only *in vitro* studies were carried out on isolates from patients enrolled at Ibadan.

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(ii) Screening for control subjects

Control subjects were recruited from apparently healthy children appearing for elective, day-care surgeries due to anatomical disorders e.g. hernias. Such children were screened for malaria parasitaemia, and those found to be parasite-free were enrolled for the study. The age ranges of the controls were 6 months - 13 years, (mean = 5.1 ± 3.9 years) in Calabar, and 6 months - 13 years, (mean = 6.3 ± 5.0 years) in Ibadan.

3.2.3. Examination of Blood Slides

Giemsa-stained thick blood films were examined by oil-immersion microscopy. As the fields were examined, white blood cells (wbc) and asexual parasites were counted using two hand tally counters. Counting was stopped when 500 parasites or 1,000 leucocytes had been counted, whichever figure appeared first. The parasite density was then calculated as:

$$\frac{\text{number of parasites counted}}{\text{number of leucocytes counted}} \times 6{,}000, \text{ parasites per mm}^3 \text{ of blood.}$$

6,000 wbc was taken as the number representing the normal white cell count within the present study population (Ekanem *et al*, 1990).

Leishman-stained blood films were examined by oil-immersion microscopy to determine the parasite species.

3.2.4. Dill Glazko Urine Test

To ascertain that subjects enrolled into the

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study had not taken 4-aminoquinolines within the previous 14 days, Dill-Glazko urine test was performed on each subject's urine sample, prior to enrolment. The test was performed as described by Lelijveld and Kortmann (1970).

3.2.5. Chloroquine Response Evaluation

(i) *In vivo* study

The extended 14-day World Health Organisation Field Test (WHO, 1973; Lemnge and Inambao, 1988) was used to determine the *in vivo* response of *P. falciparum* to chloroquine. The *in vivo* study was carried out only in Calabar. Children enrolled into the study had parasitaemia ≥ 500 per mm^3 and were not too sick to take oral medication. Each child was weighed and given 25 mg of chloroquine base (as chloroquine sulphate) per kg body weight, over three days as follows: 10mg/kg body weight on day 0 (D0), 10mg/kg body weight on day 1 (D1) and 5mg/kg body weight on day 2 (D2). The drug was given orally by a physician in the clinic, and any child who vomitted within 30 minutes of therapy was excluded from the *in vivo* study. All enrolled patients were hospitalized for at least 4 days and on discharge were followed up by home visits. Thick blood films and axillary temperature readings were also taken on D2, D7 and D14. Blood films were stained by Giemsa and parasite densities were evaluated as described in section 2.3 (above).

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Interpretation of *in vivo* results

The interpretation of *in vivo* results was based on the WHO recommendation of 1984 (WHO, 1984a) which states as follows:

i. If no asexual parasites are found by D7 of therapy the infection is either sensitive or resistant (R) at the R1 level. Failure of parasite to reappear by D14 is considered a complete cure (sensitive to chloroquine).

ii. Disappearance of asexual parasite on D2, but reappearance by D7 is also classified as R1 level of resistance.

iii. Reduction of asexual parasitaemia to 25% or less (without clearance) on D2 indicates RII level of resistance.

iv. Reduction of asexual parasitaemia by less than 75% or an increase on the original count by D2 indicates an RIII level of resistance.

The final judgement however took into consideration the clinical state of the patient. This was assessed by a consultant physician.

(ii) *In vitro* study

WHO Microtest Kits

These kits were used for the *in vitro* assessment of the response of *P. falciparum* to chloroquine. Each kit contained the following:

- A tissue culture plate (12 x 8 wells) predosed with chloroquine, and sufficient to run twelve tests.
- Eppendorf pipette, 50 ul dispenser

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- Sterile RPMI 1640 powder - medium, in 125mg sachets
- Sterile HEPES, 7.2% in 20mls aliquots
- Sterile $NaHCO_3$ solution, 2.4% in 20mls aliquots
- Flexivial, containing sterile double distilled water, in 10ml aliquots.
- Millipore filter, 0.22 um pore size and 25mm diamter
- 15ml Falcon tubes with screw caps
- 6ml Falcom tubes with press cap
- 20ml sterile disposable syringes
- 1ml sterile disposable tuberculin syringes
- 1½" x 20" sterile gauge needles
- Frosted edged microscopic slides
- Scapel blade with holder
- Steel forceps
- Aluminium foil (roll of 30m x 30cm).
- 100 ul sterile, heparin-treated capillary tubes
- 1ml sterile graduated pipettes
- Plastic covered wire and racks
- Non-sterile untreated 50 ul capillary tubes
- Mouth piece for capillary tube
- Glass writing (diamond) pencil
- Pure paraffin candle
- Instruction booklet for the use of the micro-test kit
- Photograph of pre- and post-cultured P. falciparum.

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The RPMI 1640 medium, HEPES and $NaHCO_3$ solutions and the chloroquine pre-dosed microtitre plates were kept at 4°C in a refrigerator until needed for use.

For *in vitro* chloroquine response test, RPMI 1640 medium, HEPES and $NaHCO_3$ were reconstituted as directed by WHO (1982). The test was performed using the technique of Rieckman *et al* (1978), as follows: 10ul of patient's blood was obtained through a finger prick and dispensed into 0.9ml culture medium in each of the 6ml Falcon tubes. Each tube was closed with a plastic stopper and gently agitated to suspend the red blood cells. The blood-medium mixture was then left at room temperature for 2 - 3 hours.

The 96 - multi well, chloroquine predosed plates were removed from the refrigerator and allowed to equilibrate at room temperature. Each plate contained 8 rows of wells, labelled A - H and each row had 12 wells designed for 12 tests. Wells 'A' served as controls and contained no chloroquine; wells B - H contained varying doses (pmole) of chloroquine as follows: 1,2,4,5.7,8,16,32. 50ul of a sample/ medium mixture was applied to each well on the assigned row (A-H) using an Eppendorf pipette. A fresh pipette tit was used for the application of each sample. After sample application, the lid was placed on the micro-titre plate and the plate gently shaken for a few seconds to ensure that the drug deposited in the wells were completely dissolved. The plates were then incubated according to the method of Trager and Jensen (1976) for

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24 - 28 hours.

After 24 hours, some erythrocytes were taken from a control well and a thick blood film made. This was rapidly dried by a hot air blower and stained with 10% Giemsa for 5 minutes. If on microscopic examination of the smear it was found that 10% or more of the asexual parasites (trophozoites) had become schizonts, with at least 3 nuclei, incubation was stopped, otherwise examination was repeated at 26 and finally at 28 hours.

At the end of the incubation period, as much of the supernatant fluid as possible was removed using a sterile Pasteur pipette, and a thick blood film was made from the contents of each well, air-dried and stained with Giemsa. The number of schizonts with 3 or more nuclei in each well was counted against 200 asexual parasites. A successful test was taken as one with 20 or more schizonts in the control well, regarded as 100% maturation. The degree of schizont maturation inhibition was expressed as a percentage using the following formula:-

$$100 - \frac{\text{Schizont count at a given chloroquine concentration}}{\text{Schizont count in the control well}} \times 100$$

Minimum inhibitory concentration (MIC) was defined as the lowest concentration of the drug at which no schizonts were observed. The presence of schizonts in $\geq$ 5.7 pmole/well, equivalent to 1.14×10^{-6} M/L of blood was considered to indicate chloroquine resistance (Timmerman et al, 1982).

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The quantitative regression analysis method of evaluation (Wernsdorfer, 1983) was also used to analyse the results. This method is more precise and provides the basic parameters of:

(a) inhibition at (Probit) concentration

(b) the slope b of the regression

(c) variance of the slope

(d) effective concentration at any chosen level.

Other guidelines used in the interpretation of the *in vitro* results are:

(a) isolates with an EC 99 (effective drug concentration at which 99% of parasite growth is inhibited) below 1.0×10^{-6} M were considered fully sensitive to chloroquine (Grab and Wernsdorfer, 1983).

(b) isolates showing an EC 99 of 1.0×10^{-6} M or more were considered to be chloroquine resistant (Grab and Wernsdorfer, 1983).

Changes in the sensivity of isolates from the same geographical areas could be visualized. A move of the regression to the right or a flattening of the regression is indicative of diminishing drug sensitivity.

3.3 Results

3.3.1. Screening in Calabar

A total of 806 children who reported at the children's emergency unit with fever were screened for

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parasitaemia. Three hundred and sixty-four (45.2%) of them were slide positive. Thirty-three (8.8%) of the positive cases (aged between 5 months and 12 years, mean = 4.9 ± 3.8 years) fulfilled the enrolment criteria for the *in vivo* study. The *in vivo* study was performed simultaneously with the *in vitro* tests of the corresponding parasite isolates.

A total of 62 children including those selected for *in vivo/in vitro* study, were selected for the overall *in vitro* study in Calabar. These included 28 females and 34 males.

3.3.2. Screening in Ibadan

Only the *in vitro* study was conducted in Ibadan. Screening of patients in this area, was performed in the Clinical Pharmacology Department, Out-Patient Department and the Children's Ward of the University College Hospital, Ibadan by physicians, and suitable subjects for the enrolment into the study were bled for *in vitro* tests. A total of 40 children, 19 females and 21 males, were enrolled for the *in vitro* study at Ibadan.

3.3.3. Simultaneous *in vivo/in vitro* chloroquine Response Study

Twenty-eight (84.8%) of the 33 children completed the study. In 13 (46.6%), of them (table 3.1) parasitaemia was cleared and the patients remained free of parasites all through the 14-day observation period. They were thus

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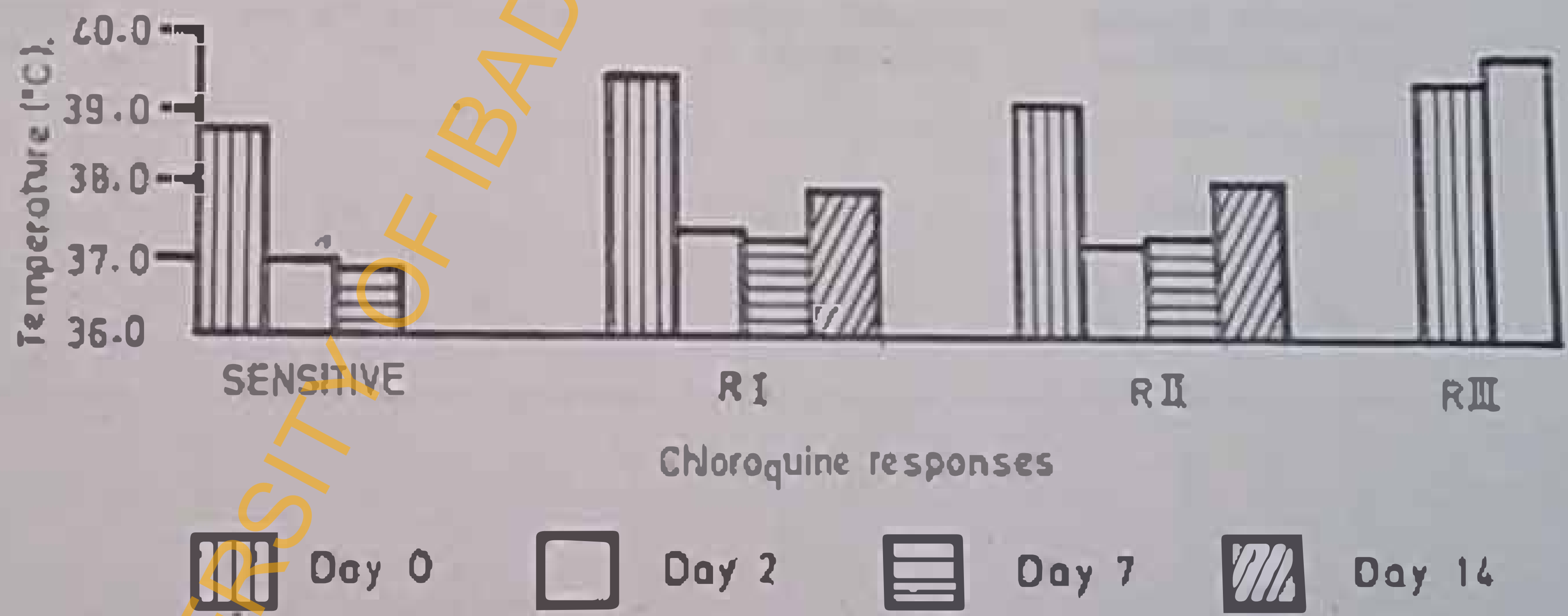

FIG. 3.1 MEAN AXILLARY TEMPERATURES Vs *IN VIVO* CHLOROQUINE RESPONSE BY DAY OF STUDY, CALABAR.

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TABLE 3.1

A summary of *in vitro* and *in vivo* chloroquine responses of *P - falciparum* in Calabar

ASSESSMENT METHOD	TOTAL NUMBER STUDIED	NUMBER SUCCESSFUL (%)	NUMBER SENSITIVE TO CHLOROQUINE (%)	NUMBER RESISTANT CHLOROQUINE (%)
In vivo	33	28 (84.8)	13 (46.4)	15 (53.6)
In vitro	33	27 (81.8)	11 (40.7)	16 (59.3)

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classified as showing chloroquine sensitive responses (complete cure). In 15 (53.6%) of the children, either the D0 parasite load was not reduced by at least 75% on D2 or there was a resurgence of parasites on D7 and/or D14. These were regarded as parasitological failures. Their chloroquine resistance levels were as follows:- RI (5 cases), RII (6 cases) and RIII (4 cases). Their clinical responses were good except in the RII group, where the D2 mean temperature rose above the D0 mean temperature. The mean temperature of the RII group also rose considerably on D14 (Fig. 3.1).

The pattern of *in vitro* sensitivity of *P. falciparum* to chloroquine is shown in Tables 3.1 & 3.2. Twenty-seven (81.7%) of the 33 cultures were successful. Eleven (40.7%) of the isolates were sensitive at chloroquine concentrations $\leq$ 5.7 (pmol per well). On the other hand, 16 (59.3%) of the isolates showed schizont maturation in chloroquine concentrations of more than 5.7 pmol per well: (6 isolates at 8 pmol/well; 5 at 16 pmol/well and 5 at $\geq$ 32 pmol/well), and were regarded as *in vitro* resistance cases (Tables 3.3). The cumulative EC 99 (Effective concentration at which 99% of the parasite growth was inhibited) of the isolates was 4.6×10^{-6} M per litre of blood (23 pmol per well) (Fig.3.2).

Twelve (80%) isolates from the 15 *in vivo* resistance cases were also resistant *in vitro*. All the isolates from

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cases showing high grade *in vivo* resistance (RII and RIII) showed *in vitro* resistance with MIC $\geqslant$ 8 pmol per well.

Two isolates from the 13 patients showing full responses to chloroquine were however resistant to the drug *in vitro*. One of these isolates matured at chloroquine concentration of $\geqslant$ 32 pmol/well and the other at 8 pmol per well.

Six *in vitro* cultures were not successful: one of the isolates was taken from an RIII response subject, 3 from subjects who exhibited full response to chloroquine *in vivo* and 2 were obtained from children who did not complete the *in vivo* study. Isolates of 3 successful *in vitro* cultures were also from subjects who defaulted in the *in vivo* study.

The mean temperature values of subjects whose isolates showed *in vitro* chloroquine sensitivity was lower than in subjects whose isolates showed chloroquine resistance responses, but the difference was not statistically significant. The corresponding geometric mean parasite densities in the two groups of infected patients were 30,150 per mm^3 and 8,009 mm^3 respectively, (Table 3.3) and the difference was statistically significant ($t = 2.98$, $p < 0.01$). Similarly, the mean temperature value of patients who responded to chloroquine therapy (*in vivo*) was lower, but not significantly, than the mean value in patients who

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TABLE 3.2

Pattern of *in vitro* responses of isolates (n=33) of *P. falciparum* to chloroquine (Calabar)

CHLOROQUINE RESPONSE (MIC - pmol/well)	NUMBER OF ISOLATES (% OF SUCCESSFUL ISOLATES)
NS	6
S (1)	-
S (2)	0
S (4)	6(22.2)
S (5.7)	5(18.5)
R (8)	6(22.2)
R (16)	5(18.5)
R (32)	1(3.7)
R (>32)	4(14.8)

MIC = Minimum inhibitory concentration

N/S = Culture not successful

S = Sensitive to chloroquine *in vitro*

R = Resistant to chloroquine *in vitro*

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TABLE 3.3

Clinical (temp.) and parasitologic (GMPD) responses of subjects from Calabar infected with chloroquine resistant and chloroquine sensitive *P falciparum*: comparison of *in vitro* and *in vivo* chloroquine responses

Chloroquine responses	*In vitro* studies n = 27; 11 (S); 16 (R)		*In vivo* studies, n=28; 13(S); 15(R)	
	Mean axillary temp. (C)[a]	GMPD (per mm^3)[b]	Mean axillary temp. (^oC)[c]	GMPD (per mm^3)[d]
S	38.95 (1.0)	30.150 (13,708-66,529)*	38.78 (1.0)	10.960 (2,951-40,738)*
R	39.30 (1.0)	8.009 (3,548-17,782)*	39.38 (1.0)	18.034 (8,933-36,391)*

(a) t = 0.350, $p > 0.1$
(b) t = 2.980, $p < 0.01$
* = 95% confidence interval
(c) t = 1.636; $p > 0.01$
(d) t = 0.778; $p > 0.1$
S = Sensitive to chloroquine *in vitro*.
R = Resistant to chloroquine *in vitro*
GMPD = Geometric mean parasite density.

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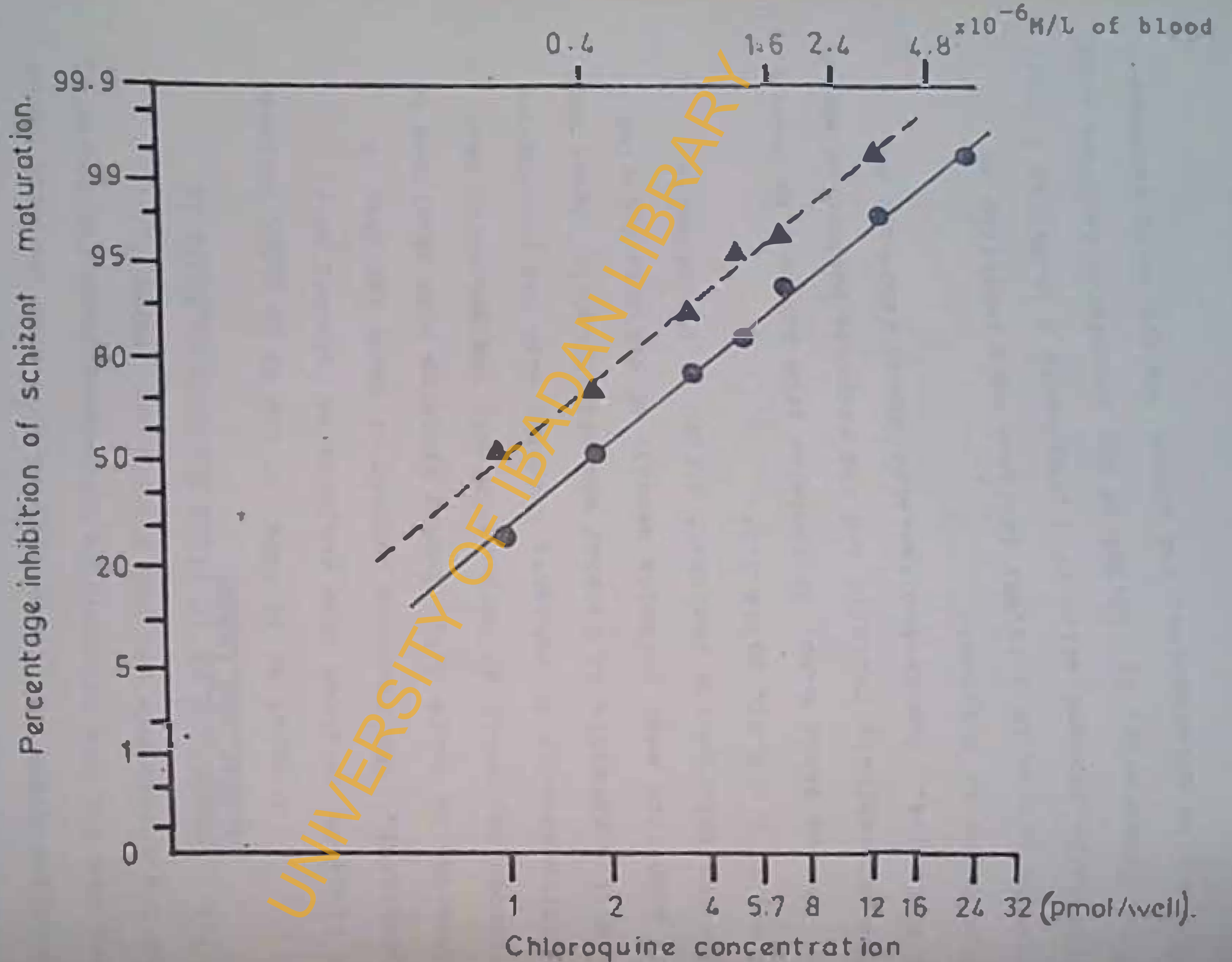

Fig. 3. 2 Inhibition of Schizont maturation in the *in vitro* chloroquine ... regression lines for two different

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exhibited chloroquine treatment failures. However, contrary to results obtained in the *in vitro* study, the geometric mean parasite density in the *in vivo* chloroquine sensitive group was lower (10,960/mm^3) than the mean value obtained for the chloroquine resistance group (18,034/mm^3), but the difference was not significant (table 3.3).

3.3.4. Comparison of *in vitro* Chloroquine tests in Calabar and Ibadan

A total of 49 (79%) of the 62 *in vitro* cultures of isolates obtained from subjects in Calabar, were successful. The response pattern of these isolates is presented in Table 3.4. Twenty isolates were sensitive to chloroquine, while 29 were resistant; the geometric mean parasite density in subjects infected with the chloroquine sensitive parasite in Calabar was significantly higher than the geometric mean parasite density of subjects infected with the chloroquine resistant strain of the parasite ($t = 3.9$, $p < 0.01$, table 3.5).

On the other hand, 34 isolates from subjects in Ibadan were successfully cultured and the response pattern is shown in Table 3.4. Twenty-two isolates showed responses to chloroquine as follows:-

Parasite growth in 2 (5.9%) isolates were inhibited at chloroquine concentration of 1 pmol/well; 3 (8.8%) at 2 pmol/well. Conversely, 12 (35.3%) of the successful isolates were resistant to chloroquine, and showed the following response

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TABLE 3.4

Comparison of patterns of *in vitro* responses of *P. falciparum* isolates from Calabar (n = 62) and Ibadan (n = 40)

Chloroquine response (MIC - pmol/well)		Number of isolates (% of successful isolates) CALABAR	IBADAN
NS.		13	6
S	(1)	-	2 (5.9)
S	(2)	-	3 (8.8)
S	(4)	11 (22.4)	15 (44.1)
S	(5.7)	9 (18.4)	2 (5.9)
R	(8)	8 (14.3)	3 (8.8)
R	(16)	9 (18.4)	2 (5.9)
R	(32)	7 (14.3)	2 (5.9)
R	(>32)	6 (12.2)	5 (14.7)

MIC = Minimum inhibitory concentration

N/S = Culture, not successful

S = Sensitive to chloroquine *in vitro*

R = Resistant to chloroquine *in vitro*

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TABLE 3.5

Comparison of Geometric mean parasite densities (GMPD) in subjects infected with Chloroquine sensitive and chloroquine resistant *P. falciparum* in Calabar and Ibadan

	CALABAR	IBADAN
CHLOROQUINE	GMPD[a] (per mm^3)	GMPD[b] (per mm^3)
RESPONSE	n = 49; 20(S) 29 (R)	n = 34; 22(S) 12 (R)
S	24,203(21878 -* 26303)	18,942(7,128 -* 50,234
R	6,440 (5888 -* 7031)	6,072(2805 -* 13,122)

a, t = 23.90, $P < 0.001$

b, t = 2.19, $P < 0.05$

* = 95% Confidence interval

R = Resistant to Chloroquine *in vitro*

S = Sensitive to Chloroquine *in vitro*

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TABLE 3.5

Comparison of Geometric mean parasite densities (GMPD) in subjects infected with Chloroquine sensitive and chloroquine resistant *P. falciparum* in Calabar and Ibadan

CHLOROQUINE RESPONSE	CALABAR GMPD[a] (per mm^3) n = 49;20(s) 29 (R)	IBADAN GMPD[b] (per mm^3) n = 34;22(s) 12 (R)
S	24,203(21878 ± 26303)	18,942(7,128 ± 50,234
R	6,440 (5888 ± 7031)	6,072(2805 ± 13,122)

a, t = 23.90, $P < 0.001$
b, t = 2.19, $P < 0.05$
± = 95% confidence interval
R = Resistant to Chloroquine *in vitro*
S = Sensitive to Chloroquine *in vitro*

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patterns:- 3 (8.8%) were inhibited at chloroquine concentration of 8 pmol per well; 2 (5.9%) at 16 pmol/well, 2 (5.9%) at 32 pmol/well and 5 (14.7%) at > 32 pmol/well. The geometric mean parasite density in subjects infected with chloroquine sensitive strains of the parasite was significantly higher than the mean value for subjects infected with the resistant strain ($t = 2.1895$, $p < 0.05$), (Table 3.5). The cumulative EC 99 of isolates in Ibadan was 2.4 mole/L of blood (12 pmol/well (Fig. 3.2).

3.4. Discussion

The pattern of *in vivo* and *in vitro* chloroquine resistance of *P. falciparum* has been investigated in some areas of Africa where the resistant strains of the parasite species have emerged. Teklehaimanot (1986) reported that 6 out of 7 patients who were infected with chloroquine resistant *P. falciparum* strains also showed *in vivo* resistance response to C25 treatment. Lemnge and Inamboa (1988) also noticed a high correlation between *in vivo* and *in vitro* evaluation of chloroquine (CQ) sensitivity of *P. falciparum* in Zambian children.

In the present study, a high correlation has also been observed between the *in vivo* and *in vitro* CQ response of *P. falciparum*. Isolates from children, who showed *in vivo* chloroquine resistance were mainly found to be resistant *in vitro*. However a 5-month-old baby and a five-year-old child who responded to C25 treatment, provided isolates which

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showed CQ resistance in vitro (MIC > 32 and 8 pmol/well respectively). Lemnge and Inamboa (1988) observed that most isolates from children at Kalene region of Zambia had MICs of 16 pmol/well, but no corresponding in vivo chloroquine resistance response was detected. Spencer et al (1983a) in Kenya had also made a similar observation in children aged between 6 and 24 months and suggested that any such findings in adults could be due to individual immune mechanisms that may be playing a role in modifying the expression of drug resistance to the malaria parasite in vivo. A plausible explanation of the present findings could be that the 5-month-old baby may still be carrying the passively acquired maternal antimalarial antibodies which may be playing a modifying role as suggested by Spencer (1983a).

It was also reported by Lamnge and Inamboa (1988) that 2 of 4 children who exhibited chloroquine therapy failures, provided isolates that were sensitive to chloroquine in vitro (MIC: = 2 and 4pmol/well respectively). Charlet et al (1985) had made a similar observation. In the present study it was similarly observed that two children were chloroquine therapy failures, while their isolates were sensitive to the drug in vitro. Walker et al (1983) found that CQ absorption was more variable in malaria infected Nigerian children than in non-infected Nigerian adults and suggested that such might reflect a mild association with malaria. It would therefore appear that the two children in the

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present study, who happened to be siblings, exhibited a significant gastrointestinal disturbance which was not detected during the study, and which may have influenced their absorption of chloroquine. This postulation could however not be confirmed in the absence of a pharmacokinetic data which were not available during the study. An alternative hypothesis may have to do with individual red cell type. Slomianny et al (1984) had shown that when a normally chloroquine sensitive rodent Plasmodium grows in anaemic mice (10 - 30% reticulocytes) parasites present biological characteristics of chloroquine resistant homologous strain. Moreover, the action of chloroquine *in vivo*, on *P. berghei* forms growing in reticulocytes, is markedly less effective than on the forms parasitizing mature red blood cells (Dei-Cass et al 1984). Since both the *in vivo* and *in vitro* studies of the present report were carried out on similar red blood cells, it is not likely that the disparity in chloroquine response between the two studies was due to the effect of individual red cell type.

The observation in this study of a high level of chloroquine resistance in Calabar both *in vivo* (53.6%) and *in vitro* (59.2%), as well as a high cumulative EC 99 of 4.6×10^{-6} M/L of blood (23 pmol/well) agrees with an earlier report of a 63% prevalence level observed in Oban, Cross River State in south East Nigeria (Ekanem et al, 1990).

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No schizont maturation inhibition at chloroquine concentration less than 4 pmol/well was observed in isolates from children in Calabar (Table 3.4).

Although the *in vivo* study was not carried out in Ibadan, the *in vitro* results showed that there was a much lower chloroquine resistance prevalence level in that population compared to Calabar. The *in vitro* study in Ibadan also showed that schizont maturation of some isolates of *P. falicparum* in the area could still be inhibited at chloroquine concentrations of 1 pmol/well (2 cases) and 2 pmol/well (3 cases) (Table 3.4). The cumulative EC_{99} of 2.4×10^{-6} M/L of blood in Ibadan was also much lower than what was observed in Calabar (4.6×10^{-6} M/L). It was observed that the mean parasite densities were significantly higher in the chloroquine sensitive *P. falciparum*-infected subjects than in those infected with the CRPf strain. This observation was made in all the *in vitro* studies carried out. However, the reverse was the case in the *in vivo* study conducted in Calabar. It is not very clear why the parasite densities between the two groups of infected subjects based on *in vivo* chloroquine response was different from those of the *in vitro* response. It is postulated however that the *in vivo* parasite density pattern may have been distorted by the very high parasite densities in those children whose isolates were characteristically not successfully cultured *in vitro*. The significantly lower mean parasite densities

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in individuals infected with chloroquine resistant *P. falciparum* in the two populations studied, was an interesting observation. LeBrass *et al* (1986) had noticed that chloroquine resistant malarial attacks were not severe clinically. Ekanem *et al* (1990) also observed that children from Oban, Cross River State of Nigeria, who were infected predominantly with CRPf showed milder clinical signs and symptoms than their chloroquine sensitive infected counterparts from Igbo-Ora, Western Nigeria. From these earlier reports and the results of the present study, it would therefore appear that CRPf malaria is associated with less pathogenicity. If this is true, then it would contrast the observations of Ikpatt *et al* (1990) in Calabar and of Miller (1989) in Thailand that cerebral malaria increases with the emergence of CRPf. The results of the present study which show relatively lower parasite density in CRPf malaria needs further investigations.

Individual preponderance in modifying the expression of chloroquine resistance seems to be a common phenomenon. The appearance of chloroquine resistant malaria in various populations has been sporadic (Brasseur *et al*, 1988). Lemnge and Inamboa (1988) had observed an appreciable difference in individual expression of chloroquine responses between two tribal communities of Zambia. In Nepal, inhabitants still express chloroquine sensitive malaria in the midst of apparent human migration from chloroquine resistant

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malaria infested neighbouring Asian countries (WHO, 1987a). In Nigeria, while the prevalence of chloroquine resistant malaria is high in the populations of the Eastern States including Calabar, the West is recording relatively low resistance prevalence levels as revealed by the studies of Ekanem *et al* (1990); an observation supported by the present study. It may therefore be tempting to speculate that chloroquine resistant *P. falciparum* in Calabar may reflect an incursion from the neighbouring Cameroun Republic, one of the countries in Africa where chloroquine resistant malaria was initially reported (Sansonetti *et al*, 1985). Ibadan is also appreciably close to the Republic of Benin, where a high prevalence level of CRPf malaria was reported by LeBrass *et al* (1986) yet the prevalence level in Ibadan is low.

The role of the mosquito vector in the transmission of Plasmodial species or strains may contribute to this sporadic nature of distribution of chloroquine resistant *P. falciparum*. In Nepal, continued prevalence of chloroquine sensitive *P. falciparum* is believed to be aided by the locally available chloroquine sensitive malaria transmitting *Anopheline* mosquito (WHO, 1987a). Wilkinson *et al* (1976) also suggested that chloroquine resistant *P. falciparum* may be better transmitted by certain vectors than by others. Entomological studies in Nigeria especially in the South Eastern regions are desirable for an understanding of the possible role of mosquito species in disseminating CRPf malaria in these regions.

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While the observed local difference in individual manifestation of chloroquine resistant malaria may be due to environmental and other host genetic factors, the present results of in vivo/in vitro pattern of responses by some of the children studied suggest specific or non-specific immunologic roles in individual modulation and expression of chloroquine-resistant malaria as suggested by Spencer et al (1983a). In the subsequent chapters attempts are made to assess the nature of immune responses that may possibly be elicited against the emerging Nigerian strain(s) of CRPf in susceptible individuals.

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CHAPTER 4

ASSESSMENT OF SEROREACTIVITIES AGAINST TOTAL BLOOD STAGE ANTIGENS BY ELISA AND STANDARD INDIRECT IMMUNOGLUORESCENT ASSAY (IFA)

4.1. INTRODUCTION

A good knowledge of the characteristics of host immune responses against locally prevalent antigens is necessary for a successful immune intervention in malaria control. Furthermore, to advance the progress towards vaccination in all populations in endemic areas, an assessment of seroreactivities in these populations against the various malaria antigens is desirable. This need is made more imperative by the appearance of changing strains of malaria parasites, including the emergence of *P. falciparum* strains resistant to chloroquine, in various malaria endemic regions, including Nigeria.

Nigeria is the most populous malaria endemic African country and therefore stands to benefit immensely from any malaria control programme. In spite of this position, no appreciable seroepidemiological study has been done in Nigerian populations using prospective vaccine candidate proteins as antigens. Williams *et al* (1987) however had previously conducted a study to assess the prevalence of anti-circumsporozoite (CS) and anti-ring infected erythrocyte antigen (RESA) antibodies in some Nigerians. In particular, no

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serological studies have been carried out in any Nigerian population using the total blood stage (ELISA) method, prior to or following the emergence of chloroquine resistant *P. falciparum* in the country.

In this chapter, the seroreactivities against blood stage antigens using the ELISA and standard IFA methods were evaluated in Nigerian children infected with chloroquine resistant and chloroquine sensitive *P. falciparum*. This was with a view to determining whether or not there is any difference in the antibody responses elicited following infections by the two strains of the parasite species.

4.2. Materials and Methods

4.2.1. Study Areas

This study was conducted in Calabar and Ibadan. The geographical locations of these cities have already been described in 3.2.1. The sera used were obtained from 40 malaria patients and 50 age/sex matched apparently healthy controls from Ibadan and 62 patients and 30 age/sex matched controls from Calabar. These have also been fully described in Chapter 3, sections 2.1 and 2.2. The serum samples for the ELISA and the IFA studies were either used fresh or stored at - 20°C until required for use.

4.2.2. Antibody Conjugates

Two types of antibody conjugates were used for the various analyses:-

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i. Rabbit anti-human IgG-Conjugate for ELISA

This antibody conjugated to horse radish peroxidase was supplied by the National Institute for Immunology, New Dehli, India, and was used in all the ELISA tests, at a concentration of between 1/10,000 and 1/20,000 in 2.5% defatted milk in Phosphate Buffered Saline (PBS, pH 7.2).

ii. Rabbit anti-human IgG conjugated to fluoresceine isothiocyanate. This was used in the standard immunofluorescent antibody assays for malaria antibody evaluation, and was purchased from Wellcome Foundation Limited, Darfford, England.

4.2.3. Phosphate Buffered Saline (PBS 0.01M pH 7.2)

Stock solution A: 62.404gms sodium di-hydrogen orthophosphate ($NaH_2PO_4.2H_2O$, M.W. 156.01) was dissolved in distilled water and made up to 2L

Stock solution B 56.8gms of disodium hydrogen orthophosphate (Na_2HPO_4, M.W. 141.97) was dissolved in distilled water and made up to 2L

Sodium chloride: (1.5M):87.75gms of sodium chloride (NaCl, M.W. = 58.44) was dissolved and made up to 1L in distilled water

14mls solution A

36mls solution B and

100mls NaCl,

were mixed and made up to 1L distilled water. With accurate weighing the pH of this final

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solution was always 7.2.

4.2.4. Citrate Buffer (0.1M, pH5)

Citrate Buffer, 0.1M, pH5 was used in diluting the substrate 1,2 phenylenediamine hydrochloride (OPD) for use in all the ELISA analysis performed. It was prepared as follows:

Solution A: 4.202gm citric acid was dissolved in 200mls distilled water

Solution B: 5.88gm trisodium citrate was dissolved in 200mls of distilled water

Solution C: 103mls solution A, 147mls Solution B, and 250mls of distilled water were added together and mixed.
The pH of this solution was adjusted to 5 if necessary.

4.2.5. Substrate Solution

10mg OPD was dissolved in 25mls of citrate buffer, 0.1M, pH 5. It was prepared shortly before required and kept away from light. Just before use, 25 ul of 10% Hydrogen peroxide (H_2O_2) was added.

4.2.6. ELISA for seroreactivity against total malaria blood stage antigens

The coated plates used for this assay were supplied by Dr. R. Dayal-Drager and Ms. C. Decrind, W.H.O. Geneva, and were stored at 4°C until ready for use. The

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coating had been done using sonicates of asynchronous blood stage infected A+ human red blood cells (5×10^{-4} cells per well) obtained from *in vitro* cultures of *P. falciparum*. Control plates had also been coated with the same amount of uninfected A+ human red blood cells.

To determine the total malarial blood stage sero-reactivities, the coated plates were used as directed by the suppliers (Dayal-Drager and Decrind, 1990). Test and control wells were preliminarily treated with a 1% solution of H_2O_2 for 1 hour and washed 3 times with 0.05% Tween 20 in PBS, pH 7.2. Doubling dilutions from 1/200 - 1/12,800 of each sample was made in Tween 20, containing 2.5% defatted milk, and applied to the wells in rows, using the inverse application technique. All sample applications were made in duplicates for both the test and control plates. This was allowed to react for 1 hour at room temperature. Thereafter, the wells were washed 3 times with Tween 20 - PBS solution. Bound antibody was reacted for 1 hour with anti-human IgG conjugated to horse radish peroxidase. After washing 3 times in PBS - Tween 20, and once in citrate phosphate buffer (CPB), 0.1M, pH5, 1,2 phenylenediamine hydrochloride (OPD) substrate (SIGMA, U.S.A.) was added at a concentration of 0.4mg/ml in CPB containing 0.01% H_2O_2. At the end of 20 minutes reaction time the colour development was stopped with 2.5M H_2SO_4. The intensity of the resulting violet colour change was determined spectrophotometrically

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at a wave length of 492nm, using ELISA plate reader, BIOTEK EL 307C.

All OD492 values of the control wells were substracted from OD492 values of the corresponding antigen wells. Eleven negative controls were included in each test and contro plate. These negative sera were obtained from students (mean age 23 ± 3 years) arriving the University of Ibadan, Nigeria, from Swaziland, a non-malaria endemic South African country. Their blood films were also made and confirmed negative for malaria parasites. Those included in the study had not visited any malaria endemic region before. Results were calculated as recommended by Andersen et al (1983). The mean OD492 values of the seronegative controls were multiplied by 2 and used as the cut-off point. With the plates used in the present study, the mean OD492 value of the seronegative sera was 0.059. A value of 2 x 0.059 was thus used as the cut-off point. The samples which gave OD492 value less than 0.118, at a serum dilution of 1/200 were considered seronegative.

4.2.7. Indirect Immunofluorescent Antibody Assay (IFA)

The immunofluorescent antibody assay for the detection and quantitation of malaria antibody was performed using the method of Hall et al (1978). A continuous culture line (W2) of *P. falciparum* resistant to chloroquine was used as antigen source throughout the study. Cultured *P. falciparum* was harvested when parasitaemia was between 3%

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and 5% with a late trophozoite/schizont population of 40%.

To prepare the antigen, parasite culture was spun down at 400 x g for 5 minutes and the supernatant fluid aspirated. The cells were further washed with RPMI 1640 medium without serum, before a 1% suspension was finally made in the same incomplete medium. Ten ul of the parasitized cell/medium mixture was applied to each of 12 wells engraved on a microscope glass slide. The mixture was continuously mixed during the application to ensure uniform dispersion of parasitized cells. The cell suspension was spread evenly around each well with the aid of the pipette tit and the slides were then left to dry at room temperature for about 1 hour, before being packaged in an absorbent tissue. The slides were thereafter placed in a slide box containing a small package of dessicant and stored at -70°C until required for use.

For use, packaged slides were allowed to thaw at 4°C for 30 minutes and to equilibrate at room temperature for another 30 minutes. Each test serum sample was diluted serially from 1/10 to 1/10,240 in PBS, pH 7.2, for application on to the antigen slides. Each antigen slide to be used was flooded with acetone for 3 minutes, and then a drop of each serial dilution of a test serum was placed on the respective wells, using the inverse sampling technique, i.e. the application of each sample on a slide started with the

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highest dilution of 1/10,240. Slides were placed in a moist chamber for 30 minutes and were thereafter thoroughly but gently washed for 5 minutes in 3 changes of PBS, pH 7.2. Each of the wells was then flooded with a 1/40 dilution (in PBS, pH 7.2) of fluorsecein labelled anti-IgG antibody, and the slides were again incubated in a moist chamber for 30 minutes. Slides were washed again in 3 changes of PBS, pH 7.2, before being mounted using 90% glycerol in PBS. Examinations of slides were done under an Olympus phase contrast immunofluorescence microscope, IMT - 2, with an eye piece 10 x 20 and a high powered objective (x 100). The serum dilution preceeding the one in which the antibody content was so low that no fluorescence was visible was considered as the antibody titre of the sample. A sample with an antibody titre that was below 1/20 dilution was considered negative.

4.3. RESULTS

4.3.1. Seroreactivities:

(i) ELISA

Based on the cut-off value of 0.118, 48 (77.4%) patients and 25 (83%) control subjects in Calabar were seropositive, while 34 (85%) patients and 34 (68%) controls in Ibadan were seropositive for total blood stage antigen of *P. falciparum* by ELISA (Table 4.1). At serum dilution of 1/200 the range of OD492 was 0.009 - 1.136 for the patient population and 0.006 - 0.690 in the control subjects

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TABLE 4.1

Aggregated IgG-specific anti-P falciparum seropositivity by age, detected by whole blood stage - ELISA and - IFA from Calabar and Ibadan in patients (P) and controls (C).

Method	Site	Age Group (years)	<1		1 - 4		>4 - 9		>9 - 13		TOTAL	
		Subject	P	C	P	C	P	C	P	C	P	C
ELISA	CALABAR	n	4	2	29	12	22	14	7	2	62	30
		Positive (+)	2	2	21	8	18	13	7	2	48	25
		Negative (-)	2	0	8	4	4	1	0	0	14	5
		Positive (%)	50	100	72	67	82	93	100	100	77.4	83.0
	IBADAN	Subjects	P	C	P	C	P	C	P	C	P	C
		n	2	2	13	23	15	15	10	10	40	50
		Positive (+)	2	0	10	10	12	14	10	10	34	34
		Negative (-)	0	2	3	13	3	1	0	0	6	16
		Positive (%)	100	0	77	43	80	93	100	100	85	68
IFA	CALABAR	Subjects	P	C	P	C	P	C	P	C	P	C
		n	4	2	29	12	22	14	7	2	62	30
		Positive (+)	4	2	28	11	22	14	7	2	61	29
		Negative (-ve)	0	0	1	1	0	0	0	0	1	1
		Positive (%)	100	100	97	92	100	100	100	100	98.4	97
	IBADAN	Subject	P	C	P	C	P	C	P	C	P	C
		n	2	2	13	23	15	15	10	10	40	50
		Positive (+)	2	1	12	21	15	14	10	10	39	46
		Negative (-ve)	0	1	1	2	0	1	0	0	1	4
		Positive (%)	100	50	92	91	100	93	100	100	97.5	92

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in Calabar. The OD492 ranges in Ibadan were 0.018 - 1.109, and 0.003 - 0.701 in the patients and control groups respectively. The antibody titre range for patients was 0 - 1/2,800 and 0 - 1/3,200 for controls in both study populations.

In Calabar, samples from subjects infected with the resistant strain of *P. falciparum* had significantly higher ($P < 0.01$) antibody seroreactivities (mean OD492) than those infected with the sensitive strain. Similarly, in Ibadan the mean OD492 value was significantly higher ($P < 0.05$) in the CRPf-infected subjects than in those infected with the sensitive strain (Table 4.2). Generally, the mean OD492 values increased with age within both the patient groups infected with the resistant strain and those infected with the sensitive strain. This pattern of reactivity was observed in subjects from the two populations studied (Table 4.2).

Fig. 4.1 shows the distribution of anti-total blood stage antibody reactivities (OD492) in patients when compared to controls in both localities, while the distribution of antibody titres (reciprocal) are presented in Fig. 4.2. There was no correlation between blood-stage antibody titre and parasite density in both populations studied.

(ii) IFA:

The cut-off titre for the IFA was taken as 1/20 (Hall *et al* (1978), and all the negative control sera from

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TABLE 4.2

Seroreactivities against whole blood stage antigens by age, in individuals infected with CRPF, or CSPF in Calabar and in Ibadan.

	Age range (Years)	n	Sera from patients infected with CRPF: Mean ELISA-OD492 (SD)	IFA-RGMT (95% C.I)	GMPD-/mm³ (95% C.I.)	n	Sera from patients infected with CSPF: Mean ELISA-OD 492 (SD)	IFA-RGMT (95% C.I.)	GMPD-/mm³ (95% C.I.)
CALABAR	< 1	3	0.32[illegible](0.224)	101 (22-457)	3,626 (479-27,542)	1	0.31	80	37,500
	1 - 4	14	0.[illegible]0(0.341)	89 (78-132)	11,984 (10,471-14,289)	5	0.151(0.135)	113 (19-138)	21,318 (15,488-29,512)
	> 4 - 9	9	0.634(0.250)	218	4,515 (3,407-5,888)	10	0.261(0.262)	452 (320-621)	27,098 (23442-30,902)
	> 9 - 13	3	0.[illegible](0.244)	[illegible] (407-25,620)	1,828 ([illegible]-7,586)	1	1.121	2,500	13,600
	Total	29	0.563* (.332)	177** (168-213)	6,440 (5,888-7,011)	[illegible]	0.346* (0.280)	259** (223-304)	24,228 (21078-26,300)
IBADAN	< 1	1	0.110	160	575	1	0.209	40	5,532
	1 - 4	5	0.353 (0.288)	92 (56-149)	9,270 (1,829-46,773)	7	0.233 (0.114)	72 ([illegible]-115)	31,832 (23,496-42,658)
	> 4 - 9	3	0.327 (0.236)	254 (25-2512)	6,086 (3,802-9,550)	11	0.283 (0.180)	206 ([illegible])	71,589 ([illegible]2,647-24,491)
	< 9 - 13	3	0.755 (0.300)	1,016 (240-4266)	14,147 (5,102-38,905)	3	0.158 (0.111)	1,612 (908-2,[illegible])	[illegible] ([illegible]-23,291)
	TOTAL	12	0.428* (0.337)	268** (174-294)	6,072 (2,805-7,122)	22	0.225* (0.145)	189** (162-219)	18,842 (7,128-50,231)

*t = 3.400, P 0.01; **t = 0.794, P 0.1 (Calabar)
*t = 2.046, P 0.05; **t = 0.022, P 0.1 (Ibadan)
SD = standard deviation; CI = 95% confidence interval
GMPD = Reciprocal of geometric mean parasite density
CRPf = chloroquine resistant P. falciparum
CSPf = chloroquine sensitive P. falciparum
RGMT = Reciprocal of geometric meant titre

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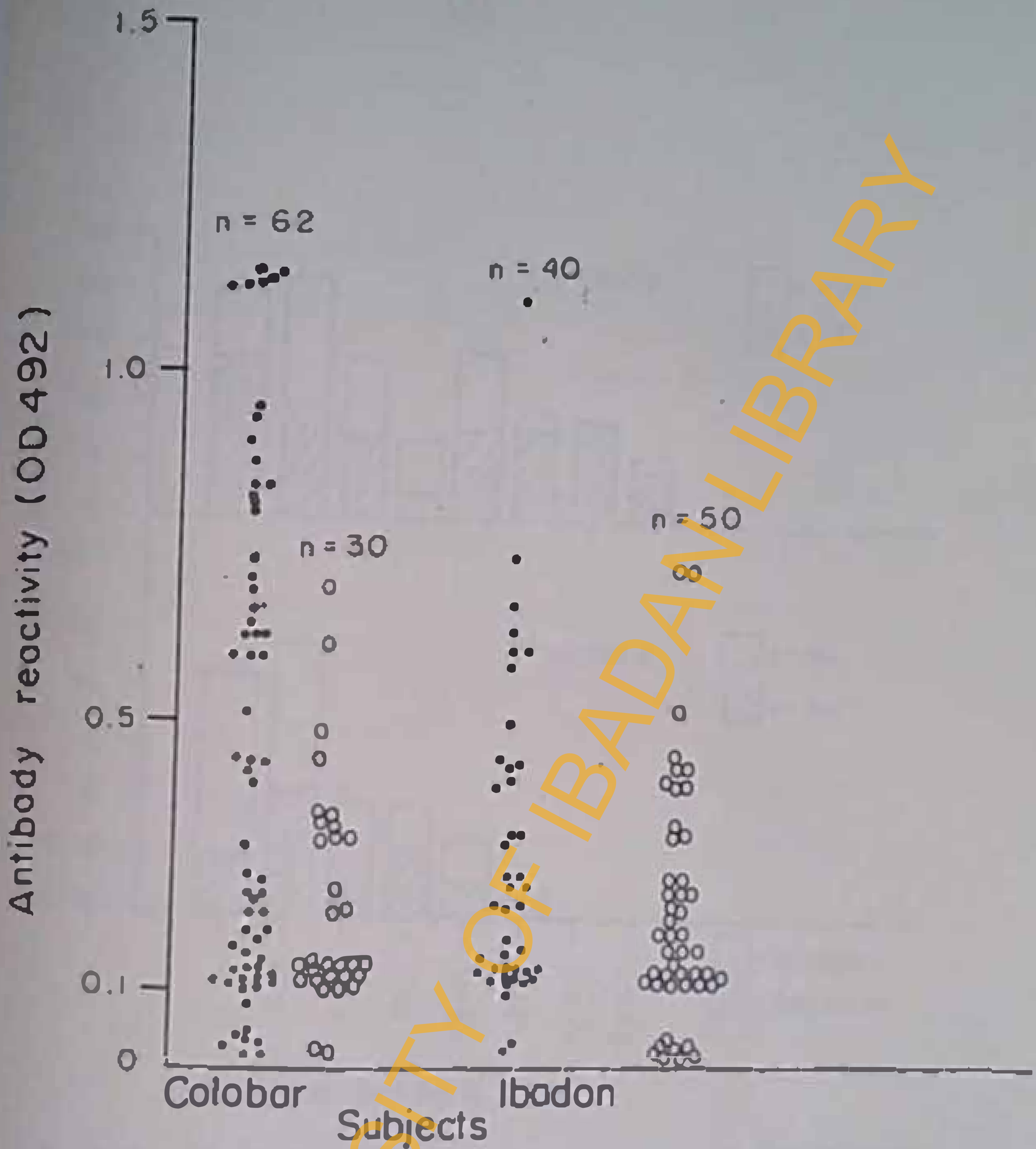

Fig. 4.1 The distribution of anti-total blood stage antibody reactivities by ELISA, in patients (•), and controls (∘) from Calabar and Ibadan.

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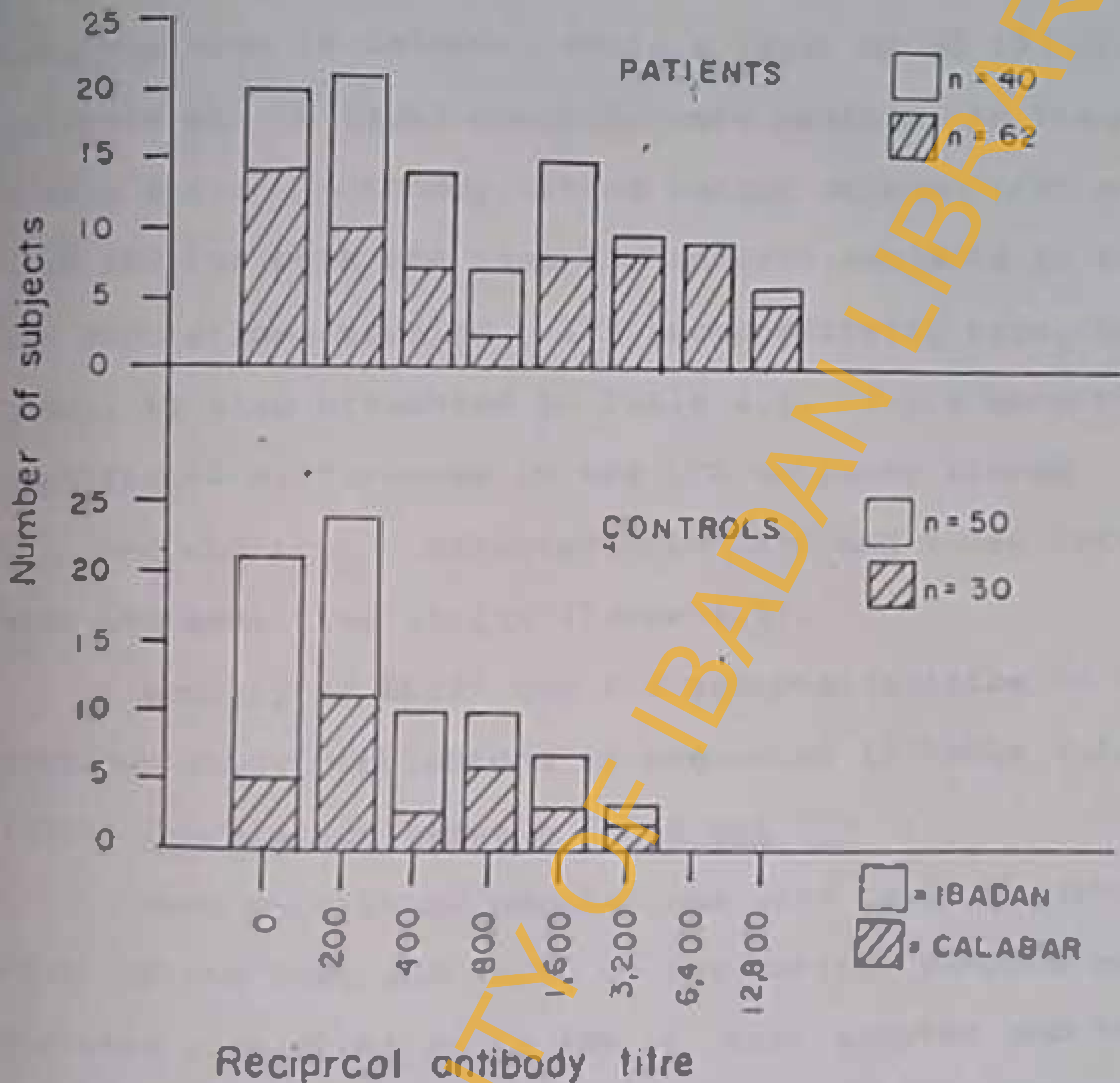

Fig. 4.2 : (Top) Frequency distribution of reciprocal anti-total blood stage parasite antibody titres in 102 P. falciparum infected Nigerian children from Calabar. Titre at zero means OD492 0.118 at 1/200 serum dilution. The distribution is apparently bi-peaked, indicating that the tested population consisted of two groups with varying response patterns.

(Bottom): Frequency distribution in 80 sex/matched, apparently healthy controls. Only the antibody distribution in Calabar remains bi-peaked compared to that of Ibadan.

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the non-immune individuals had titres less than 1/20. A total of 61 (98.4%) patients and 29 (97%) controls were positive in Calabar, while a total of 40 (97.5%) patients and 50 (92%) controls were positive in Ibadan (Table 4.1). Antibody titres ranged between 1/20 and 1/10,280 for both the test and control subjects in the two populations studied. IFA seropositivity rate, by age group, is also presented in Table 4.1. There were no significant differences in the IFA antibody titres of individuals infected with CRPf and those infected with the sensitive strain (Table 4.2).

A summary of ELISA and IFA seropositivities in the combined study populations is presented in Table 4.3.

(iii) Correlation between ELISA and IFA

When both study populations were taken together, 80.4% of the test and 73.8% of the control samples were positive with ELISA, while 98% of test samples and 93.7% of the controls were positive with IFA (Table 4.3). As shown in Table 4.4, both serologic test methods simultaneously gave positive results in 80 (78%) of the total 102 test samples and in 56 (70%) of the 80 sera from control subjects. Also, both techniques simultaneously gave negative results in none of the test samples, but in 2 (2.5%) of the controls. Table 4.4 also shows that the

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TABLE 4.3

SUMMARY OF SEROPOSITIVIES IN ALL SUBJECTS STUDIED IN BOTH LOCALITIES

	ELISA		IFA	
	PATIENTS n = 102	CONTROLS n = 80	PATIENTS n = 102	CONTROLS n = 80
number +ve	82	59	100	75
number -ve	20	21	2	5
% positive	80.4	73.8	98.0	93.75

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TABLE 4.4

IgG SPECIFIC P. FALCIPARUM ANTIBODIES IN PATIENTS, n = 102 (P) AND CONTROLS, n = 80 (C) IN CALABAR/ IBADAN: RESULTS OF IFA AND ELISA BY AGE GROUPS

	AGE GROUP (YEARS)	NUMBER EXAMINED	PERCENTAGE SERA			
			ELISA − IFA +	ELISA + IFA −	ELISA − IFA −	ELISA + IFA +
PATIENTS (P)	< 1	6	33.3 (2)	0 (0)	0 (0)	66.7 (4)
	1 - 4	42	26.2(11)	4.8(2)	0 (0)	71.4(30)
	> 4 - 9	37	18.9 (7)	0 (0)	0 (0)	81.0(30)
	> 9 - 13	17	0 (0)	0 (0)	0 (0)	100 (17)
	TOTAL	102	19.6(20)	1.96(2)	0 (0)	78 (80)
CONTROLS (C)	< 1	4	25 (1)	0 (0)	25 (1)	50 (2)
	1 - 4	35	45.7(16)	5.7(2)	2.86(1)	45.7(16)
	> 4 - 9	29	6.9 (2)	3.4(1)	0 (0)	89.7(26)
	> 9 - 13	12	0 (0)	0(0)	0 (0)	100 (12)
	TOTAL	80	23.75(19)	3.75(3)	2.5 (2)	70 (56)

Number (n) of patients in parenthesis

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TABLE 4.5

IgG-ANTI-P. FALCIPARUM ANTIBODIES IN PATIENTS n(102) AND CONTROLS n(80) IN CALABAR/IBADAN: CORRELATION BETWEEN IFA AND ELISA TITRES.

	IFA TITRE (Reciprocal)	ELISA TITRE (OD_{492})				TOTAL
		<0.118*	0.118-0.400	0.401-0.700	> 0.700	
PATIENTS	< 20*	0	0	2	0	2
	20-160	9	24	10	7	50
	320-1280	10	14	9	8	41
	2560-10240	1	3	3	2	9
	TOTAL	20	41	24	17	102
CONTROLS	< 20*	2	3	0	0	5
	20-160	11	20	6	2	39
	320-1280	8	18	2	0	38
	2560-10240	0	7	1	0	8
	TOTAL	21	48	9	2	80

*Regarded as sero-negative

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ELISA was positive alone in 2 (1.96%) of the test and 3 (3.75%) of the control samples. Thus, the false negative rate by IFA (IFA$^-$/ELISA$^+$) was 1.96% (2) for the test samples and 3.75% (3) for the controls. Similarly the false negative rate by ELISA (ELISA$^-$/IFA$^+$) was 19.6% (20) for the test and 23.75% (19) for the control samples.

ELISA$^+$/IFA$^+$ rate increased with age from 66.7% in the age group < 1 year to 100% in the group above 9 years in the malaria infected subjects. In the control group there was a fall in the ELISA$^+$/IFA$^+$ rate from 50% in those aged less than 1 year to 45.7% in the age group 1 - 4 years. Thereafter there was a rise in the ELISA$^+$/IFA$^+$ rate, with age.

The best correlation of infection to serologic response was in the older age group (Tablees 4.1 & 4.2). High IFA titres were generally not associated with increased reactivity by ELISA (Table 4.5).

4.4. DISCUSSION

Evaluation of antibody to total blood stage malaria antigen by ELISA and by standard IFA technique present inconsistent results especially in sera from children (Spencer *et al*, 1979). The relatively high proportions of false negative results obtained with these tests in this study, is consistent with the results of

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other serologic surveys (Collins and Skinner 1972; Collins, Warren and Skinner 1971; Warren *et al* 1976; Gleason *et al* 1971; Campbell, Martinez and Collins 1980; Spencer *et al* 1981). The occurence of this level of false negative results does not however impair the usefulness of serology as an epidemiologic tool, particularly if false positives are not a factor (Spencer *et al* 1981). In the present study, serologic end points for both test methods were based on comparison with the end points of sera from people who have never been exposed to malaria infections. The IFA end point determination was in addition, based on a previous recommendation (Hall *et al*, 1978). The end points chosen therefore give negligible number of false positive results. These levels of sensitivity are quite adequate to measure differences between population groups or temporal changes within populations resulting from apparent changes in local antigenic challenges. Infected individuals with no detectable antibodies based on the results of the present test methods would therefore reflect individual inability to mount humoral immune response against the malaria antigen used in the test. Such individual variation in immune response against some malaria antigenic epitopes has been noted and attributed to genetic restriction (Troye-Blomberg *et al*, 1990; Brabin *et al*, 1989; Bjorkman *et al*, 1990).

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Although the pattern of seroreactivities of the two methods was similar within each study group, discrepancies were observed in 21.56% of the test and in 27.40% of the control subjects (Table 4.4). Spencer et al (1979) observed a similar phenomenon for whole parasite malaria antibodies in comparative ELISA/IFA studies conducted in various geographical locations. Out of 261 sera from individuals in Vietnam, 5.4% were $IFA^{+}/ELISA^{-}$ and 17.6% were $IFA^{-}/ELISA^{+}$; out of 351 sera from individuals in Hondura, 6.6% were $IFA^{+}/ELISA^{-}$ and 22.8% were $IFA^{-}/ELISA^{+}$. The authors attributed these discrepancies to the fact that different antibodies were measured by the two serological methods, an explanation that may also apply to the results of the present study. As expected, the best correlation of infection to serologic response was in the older age groups, with 100% $ELISA^{+}/IFA^{+}$ rate in those >9 - 13 years, both in the patient and control groups (Table 4.4). In an earlier report by Spencer et al (1981), a similarly higher correlation in the oldest age bracket of 15 - 19 years was observed, with 87.9% rate in the ELISA and 92% in the IFA method. This increased antibody prevalence rate observed in the older age group supports earlier suggestions by others, that the degree of previous exposure to malaria infection is a contributing factor to seropositivity against malaria antigens (Perlmann et al, 1984).

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The value of the ELISA detectable antibody to blood-stage (total) malaria antigen in protective immunity has not been adequately investigated. Wahlgren *et al* (1986b) found no clear-cut relationship to the development of immunity, when the overall antibody activities to blood stage parasite antigens were determined by ELISA. However those authors found a pronounced rise in IgM, IgG1 and IgG2 antibody activities at ages when partial immunity was already present. Deloron *et al* (1987) also observed that most of the plasma, containing antibodies to ring-infected erythrocyte surfacce antigen, also had antibodies against blood stage *P. falciparum* antigen as detected by ELISA. Anti-blood stage antibodies evaluated by ELISA represents antibodies to total blood stage antigens (Wahlgren *et al* 1986b). Such antigens supposedly include antigens detectable by the standard IFA and the ring-infected erythrocyte surface antigens (RESA),(Deloron *et al*, 1987). Antibodies to the latter has been found to prevent merozoite invasion (Wahlin *et al*, 1984) and to correlate positively with clinical immunity (Wahlgren *et al*, 1986b; Peterson *et al*, 1990). In this work, both the prevalence rates and the titres of IgG-specific antibody to total blood stage antigen (ELISA) was observed to increase with age, after the first year of life, with a sharp rise within the oldest age bracket (≥ 9 - 13 years). Substantial seronegative results were also obtained within both the acute malaria

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and the apparently healthy control groups. This pattern of seroreactivity partly agrees with previous reports on the epidemiology of anti-RESA antibodies in children living in malaria endemic areas (Deloron et al, 1990). The observation in this work, of a small number of patients at the two extremes of childhood where antibody concentrations (by ELISA) were also high, probably reflects adequate immunity conferred by anti-blood stage parasite antibody. These extremes are infancy and children above the ages of 9 years. The high mean anti-total blood-stage antibody reactivity observed in early childhood also agrees with the findings of Williams et al (1987) who reported a remarkable incidence of antibody to RESA in cord blood of Nigerians. It is likely therefore that in the present study, the ELISA method, while detecting antibodies to total blood stage antigens also detects significant amount of antibody against RESA, amongst others.

A very significant observation in this project was the higher ELISA seroreactivity in acute malaria patients who were CRPf infected. Sy et al (1990) studying the in vivo growth inhibition of P. falciparum from different regions of the philippines had observed a wide divergence of the ability of immune sera to inhibit the growth of geographically distinct parasites. They concluded that parasite variability could account for the diversity in immune response to malaria infections. Although individual genetic

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factors, age and varying degrees of individual exposures to infection may not be excluded, the present results suggest that CRPf may stimulate higher total blood stage IgG specific antibody production under conditions of natural exposure to malaria infections. The associated mechanism for this higher antibody stimulation by the CRPf strains is not immediately clear. This however may reflect an antigen-dependent restriction of antibody production occuring at the level of antigenic epitopes as suggested by Yount et al (1968).

This suggestion is supported by the recent finding that CRPf has a membrane protein (M.Wt = 155 - 170 KD) which is absent in the chloroquine-sensitive (CSPf) strins of the parasite. Furthermore, it may reflect the duration of infection coupled with the degree of previous exposure to the chloroquine resistant parasite strain. A longer duration of malaria infection, as evidenced by the presence of gametocytes was shown to be associated with higher seropositivity and reactivities regardless of age (Spencer et al 1981). CRPf may have a tendency for longer duration in infected individuals, since it has been shown to be associated with milder clinical signs and symptoms (LeBrass et al 1986; Ekanem et al, 1990). This difference in seroreactivities between the two groups of infected patients (i.e. CRPf and CSPf infected) was not observed in results obtained using the IFA method.

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In conclusion, the present findings support the speculations in Nigeria that CRPf malaria had probably been in existence much earlier in the South Eastern parts (Eke et al, 1979; Ezedinachi et al, 1988), than in the Western parts of the country (Ekanem et al 1990). This assumption is further supported by the fact that in Calabar, the antibody titre (ELISA) in the CRPf-infected children was significantly higher than in the chloroquine-sensitive P. falciparum-infected subjects ($P < 0.01$), when compared to a similar difference in antibody titres between these two groups of infected patients in Ibadan, ($P < 0.05$). The observations in this study therefore suggest that the total blood stage antigen against which the IgG-antibody is produced, may embody a marker for the CRPf strain of P. falciparum. The nature of this antigen has not been previously identified (Wahlgren et al, 1986b). Studies are therefore needed, in malaria endemic areas, to ascertain that the varying individual seropositivities seen in various geographical areas are not only due to individual immune response variations, but could also be due to the nature of the infecting P. falciparum strains locally available. The present result further supports the findings of Ye et al (1989) that CRPf has a high molecular weight protein marker, on its membrane, which is absent in the CSPf. The two strains of P. falciparum may be showing immunological

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differences as postulated by Sy *et al* (1990), and this underscores the need for the characterisation of the antigenic components of the CRPf, alongside those of the chloroquine sensitive strains of the parasite species in Nigeria.

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CHAPTER 5

EVALUATION OF TOTAL SERUM IgM, IgG AND SUBCLASSES, COMPLEMENT Bf, C3, C4 PROTEINS AND THE CLASSICAL COMPLEMENT HAEMOLYTIC ACTIVITIES

5.1. INTRODUCTION

The results of the study reported under this chapter are based on the analysis of serum and plasma samples collected from children in two different geographical populations of Nigeria, Ibadan and Calabar, which are known to vary in chloroquine response to P. falciparum (Ekanem et al, 1990). These investigations aim at assesing the nature of humoral immune responses that may be associated with the emerging Nigerian strain(s) of CRPf. The results may also explain the varying clinical consequences in the two study populations, which has already been noticed in these two geographical areas (Ekanem et al, 1990).

5.2. Materials and Methods

5.2.1. Subjects and Samples

The sera and plasma samples were obtained from 40 malaria patients and 50 age/sex matched apparently healthy control subjects in Ibadan, and from 62 patients and 30 controls from Calabar. These have already been described in chapter 3 section 2.2.

Fresh EDTA plasma samples were used for complement haemolytic titrations employing tube and gel methods. These

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samples were separated immediately after collection, at 4°C and used within 1 hour of collection or stored at -70°C and used within one month. For immunodiffusion assays, serum samples were used fresh or after storage at -20°C.

5.2.2. Antisera

Monospecific antisera against human complement proteins Bf, C3 and C4, as well as antisera against human IgM and IgG were used, and were purchased from Atlantic Antibodies (Maina, USA) and the Nordic Immunological Laboratories, BV (Tilburg, The Netherlands) respectively, Monoclonal antibodies against IgG 1, -2, -3, and -4 were obtained from "The Binding Sites", Birmingham, U.K.

5.2.3. Standard Proteins

The standard protein solution used for the immunochemical quantitation of serum Bf, C3, C4, IgM and IgG (total) were obtained from Behring Institute, West Germany, while the standard proteins for IgG subclasses were provided in aliquots by Dr. Raykundalia, Department of Immunology, University of Birmingham, U.K. The aliquots were stored at 4°C until required for use.

5.2.4. Mancini immunodiffusion technique

Serum concentrations of the immunoglobulins G and M as well as the concentrations of complement proteins Bf, C3 and C4 were determined by the single radial immunodiffusion method of Mancini, as modified by Fahey and Mckelvey (1965).

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An additional modification to this method was made for the quantitation of IgG subclasses as described by Salimonu et al (1982).

(i) Assay of Bf, C3, C4, IgM and Total IgG

Equal volumes of optimally diluted antiserum and 3% noble agar (Difco Inc., Detroit, U.S.A.) at 56°C were mixed thoroughly and poured onto agar-coated (10 x 10cm) plates. Wells of 2mm diameter, 1cm from each other were cut with a metal puncher attached to a vacuum pump. Each well was filled with 5 ul of the test or the corresponding standard sera, using a 5 ul Behring Dispenser (Behring Institute, Western Germany). The glass plates were then placed in humid boxes and incubated at 4°C for 3 hours for IgG, as recommended by Salimonu et al (1978), and for 18 hours for the other proteins. Diameter measurements of the rings formed at right angles to each other were made using Behring immunodiffusion plate reader model Osram 64425 (Behring Institute, West Germany). The protein standard was set up in each plate at 25, 50, 75 and 100 percent dilution. A log plot of concentration against ring diameter of the standard protein was made, and the diameter of each test sample was read off on a standard curve to obtain the serum protein concentration.

(ii) Immunodiffusion assays for IgG subclasses

The method for the assay is as described by Salimonu et al (1982) with minor modifications. In brief, a volume

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of an optimal dilution of appropriate antisera (which had been found to be monospecific by immunoelectrophoresis) was mixed with an equal volume of 3% agar at 56°C. The mixture was poured onto agar-coated plates. A series of wells (diameter = 2mm) was cut in the agar plates and filled with 5 ul of test or standard serum. The diameters of the precipitin rings were read after the incubation of the IgG plates for 24 hours at room temperature. The ring diameter was measured to the nearest 0.1mm, using also the Osram 64425 (Behring Institute, Western Germany) immunodiffusion plate reader.

Calibration

From each of the IgG subclass standard solutions, calibration curve concentrations were made as presented in table 5.1.

TABLE 5.1

IgG Subclass Calibration Standard Dilutions

Concentration (%)	100	70	40	10
Standard Serum (ul)	100	70	40	10
Dilution buffer-PBS (ul)	0	30	60	90

The actual IgG subclass concentrations in standards are given in table 5.2.

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TABLE 5.2

IgG subclass concentration in calibration standard solutions

Calibration %	Concentrations (g/l) IgG1	IgG2	IgG3	IgG4
100	1.0	0.60	0.20	0.20
70	0.7	0.42	0.14	0.14
40	0.4	0.24	0.08	0.08
10	0.1	0.06	0.02	0.02

Samples to be tested were diluted in buffer in order to bring the IgG subclass concentration into the test range of the plates. The dilutions of samples (test and controls) are presented in table 5.3.

TABLE 5.3

Sample dilutions for IgG Subclass assays

	IgG1	IgG2	IgG3	IgG4
Test sample (ul)	5	8	10	100
Dilution buffer - PBS - (ul)	95	92	90	0

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Any test sample that gave a ring diameter^2 of more than 8.5mm was diluted further, and the test repeated. If less than 5.0mm, the test sample was diluted less or applied neat and the test repeated also.

The square of the diameter of the immunoprecipitate rings of the calibrators in mm^2 on the ordinate versus the actual IgG subclass concentration in g/l on the abscissa, using linear graph paper, was plotted. A line of best fit was drawn to the four points. The relative IgG subclass concentration of the test sample was read, by interpolation of the square of diameter of their immunoprecipitin rings on standard curves. This relative concentration was transformed to the actual IgG subclass level by multiplying with the dilution factor of the test sample.

5.2.5. Sheep Erythrocytes

Whole sheep blood was collected into an equal volume of Alsever's solution and stored at 4°C. Only one sheep was bled throughout the study, at 2 weekly intervals, and was kept at the Virology Department of the University of Ibadan.

The sheep erythrocytes in Alsever's solution were allowed to stabilize for 4 days before use, and were used within 4 weeks of collection.

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5.2.6. **Alsever's Solution:**

Composition:

Dextrose	-	20.5g
Citric acid	-	0.55g
Sodium citrate	-	8.0g
Sodium chloride	-	4.2g

These were dissolved and made up to 1 litre with distilled water. The solution was then mixed and autoclaved at 15 lb/in^2 for 15 mins, and the pH adjusted to 6.19. For use, equal volume of whole blood and Alsever's solution were mixed and allowed to stabilize for 4 days at 4°C, before use.

5.2.7. **Complement Haemolytic Assay:**

(i) Complement buffers

The classical pathway complement buffers were prepared as follows:

(a) Mannitol Veronal Buffered Saline (Stock)

Sodium barbitone	1.02g
Mannitol	51.74g
IM Hcl	3.5mls

Distilled water was added to 700 ml mark and the pH adjusted to 7.4 before finally making up the volume to 1L.

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(b) Veronal buffer (VB), pH 7.2

One veronal buffer tablet containing 0.3mM Ca^{2+} and 1.0 mM Mg^{2+} was dissolved in 100ml of warm distilled water.

(c) Stock Metal Solution

$MgCl.6H_2O$ and $CaCl_2.2H_2O$ were thoroughly decicated, dissolved and made up to 100ml in distilled water as follows:

IM $MgCl_2.6H20$ - 20.33g

0.3M $CaCl_2.2H20$ - 4.41g

(d) Gelatin Mannitol Veronal Buffered Saline (MVBS++)

This was the complement working buffer containing both Mg and Ca ions in concentrations of 0.5mM and 0.15mM respectively, and 0.1% gelatin. One litre was made up each day as follows:

Stock mannitol buffered saline - 600mls

Veronal buffer - 400mls

Stock metal solution - 0.5mls

Gelatin - 1g.

(e) Veronal buffered saline (5 x Stock)

NaCl - 41.20g

Sodium barbitone - 5.09g

These were dissolved in 700mls of distilled water, the pH adjusted to 7.4 with IM Hcl and the volume finally made up to 1L.

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(f) Veronal buffer - EDTA

This buffer did not contain Mg or Ca ions and was the medium in which cells were sensitized throughout the study. It was made up as follows:

5 x Stock Veronal buffered saline	-	20mls
0.2M EDTA	-	5mls
Distilled water to	-	100mls
Gelatin	-	0.1g.

(ii) Classical complement haemolytic assay by tube titration:

Complement functional activity via the classical pathway was assayed in fresh EDTA blood plasma, using optimally antibody (haemolysin)-sensitized SRBC as indicator cells, in a mannitol veronal based buffer, pH 7.2, containing 0.5mM and 0.15mM of Mg and Ca ions respectively. The method is as described by Mayer (1961), but with slight modifications (Umotong, 1983). Briefly, fresh serum dilutions ranging between 1/10 and 1/80 were made in 7 tubes and each allowed to react with 0.5ml of 1% sensitized sheep red blood cells at $37^{o}C$ for 1 hour, in a total reaction volume of 2.5mls. The degree of complement mediated lysis was determined by reading OD_{541} of the supernatant haemoglobin released. The OD_{541} of test was read against that of zero control tube containing buffer and sensitized cells, but no serum, and

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was compared with 100% lysis tube containing sensitized cells and distilled water only. The percent lysis in each test tube was calculated and plotted against the reciprocals of the serum dilutions. This yielded a sigmoid curve (Fig. 5.1) in compliance with Von Krogh's equation. The CH_{50} was read off as the reciprocal of serum dilution at which there was 50% lysis of the indicator cells.

(iii) Classical Complement Haemolytic Assay by Gel Haemolysis

Classical complement pathway function was also assayed by radial diffuion, using a modification of the technique of Thompson and Rowe (1967). Individual plates were prepared by adding 0.2ml packed SRBC to 10ml of 1% molten agar gel in $MVBS^{++}$ at $56^{o}C$ with rapid stirring, and then pouring the mixture unto a level glass slide (10 x 10mm). To use, wells, 2.0mm in diameter were punched at approximately 1-cm intervals, and filled with 5 ul of plasma samples. Each plate was covered, and allowed to stand at room temperature for 30 minutes and then inverted and left at $4^{o}C$, in a moist chamber overnight. Subsequent incubation at $37^{o}C$ for 1 hour resulted in the formation of circular zones of haemolysis. The diameters of the rings of lysis about each well were measured to the nearest 0.1mm, using Behring immunodiffusion plate reader.

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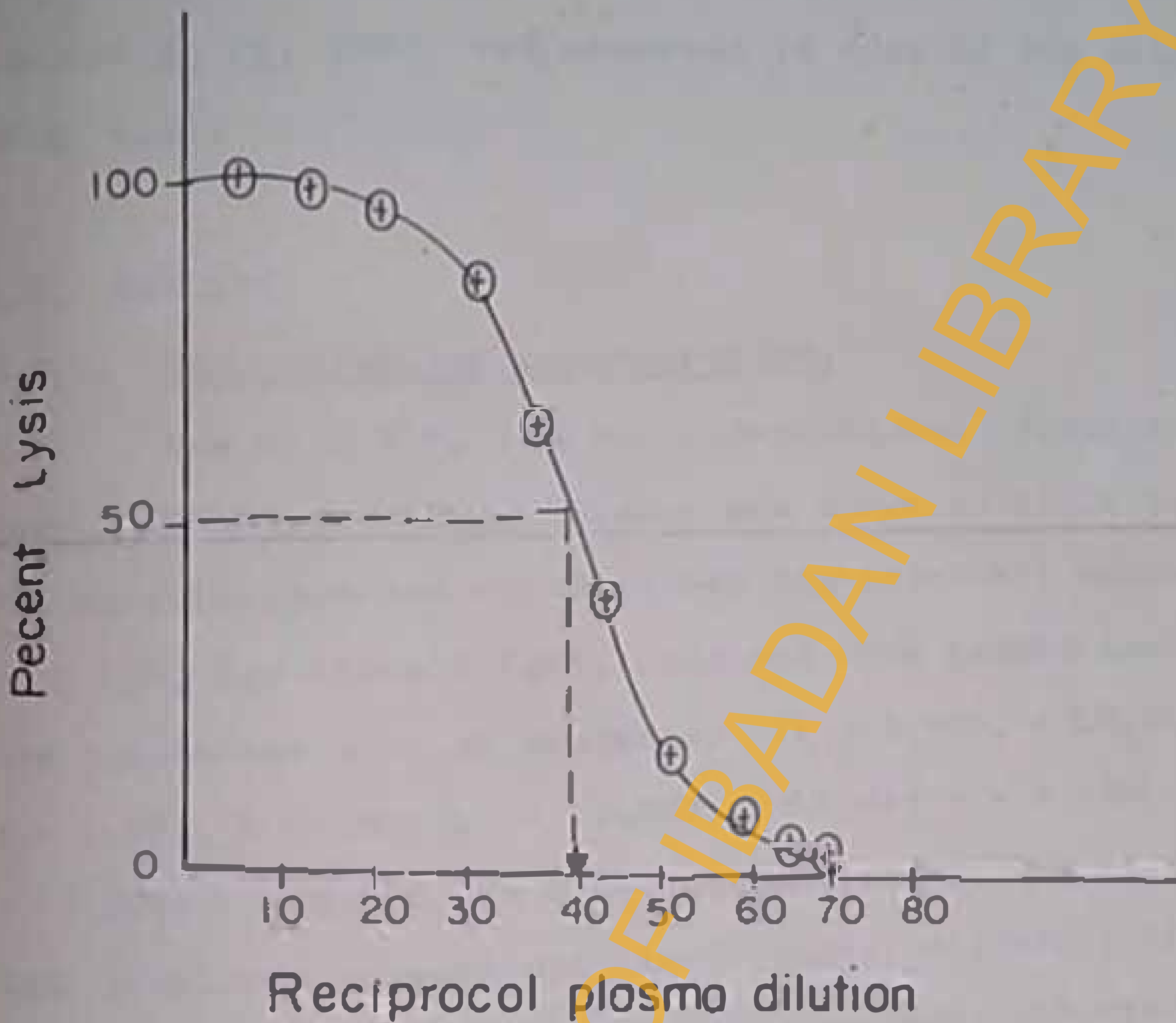

Fig. 5.1 Complement hoemolytic curve for CH_{50} determination. The arrow on the obscisa indicates the reciprocal dilution at which 50 % lysis of sensitized rbc occurs (CH_{50})

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a pattern of haemolysis called "target lysis" (Gewurz *et al*, 1982) was observed in most of the wells (Fig. 5.2).

5.3. RESULTS

5.3.1. Immunoglobulin concentrations

The mean IgM, IgG and IgG-subclasses 1,2,3 and 4 levels of children from Calabar are shown in table 5.5. The malaria-infected children had significantly higher mean IgM, IgG (total) IgG1, IgG3 and IgG4 levels than the uninfected control subjects. ($t = 3.001$, $P < 0.01$; $t = 3.273$, $P < 0.01$; $t = 2.867$, $P < 0.01$; $t = 4.419$, $P < 0.001$; $t = 2.000$, $P < 0.05$) respectively. The mean IgG2 level was however not significantly different from that of the controls. Furthermore, patients infected with the CRPf had higher mean levels of IgG and IgG subclasses than those infected with the chloroquine sensitive strain(s). These differences however were significant only in respect of IgG3 ($t = 6.616$, $P < 0.001$).

On the other hand, patients from Ibadan had significantly higher mean levels of all the immunoglobulins than the uninfected controls. The significant levels of these differences were as follows: IgM, $t = 3.537$, $P < 0.001$; IgG (total) $t = 3.393$, $P < 0.01$; IgG1, $t = 3.296$, $P < 0.01$; IgG2, $t = 2.543$, $P < 0.02$; IgG3, $t = 2.859$, $P < 0.01$; IgG4, $t = 2.667$, $P < 0.01$), (table 5.6). Similarly in Ibadan the

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Fig. 5.2: Complement mediated haemolysis of sensitized sheep erythrocytes in gels, showing ring formation. Each ring represents one sample. Most of the samples exhibit "target" phenomenon.

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TABLE 5.4

Mean serum levels of Immunoglobulins, malaria IgG-specific antibody titres and seroreactivities: Comparison between the two groups of *P.falciparum* infected patients (CRPf - and CSPf - infected) in Calabar

Immunological Profiles Mean (g/L)	CRPf infected (n = 29)	CSPf - infected (n = 20)	t	P	Total Patients (n = 62)**	Non infected (n = 30)	t	P
IgM	1.83 (0.83)	1.85 (0.61)	0.097	>0.1	1.95 (0.82	1.58 (0.36)	3.001	<0.01 (S)
IgG (total)	20.89 (5.39)	19.09 (0.09)	1.800	>0.05	21.55 (6.81)	16.35 (7.30)	3.273	<0.01 (S)
IgG1	13.75 (3.75)	13.06 (5.01)	0.523	>0.1	13.46 (3.72)	10.61 (4.79)	2.867	<0.01 (S)
IgG2	4.88 (1.30)	4.78 (1.87)	0.202	>0.1	4.84 (1.37)	4.37 (1.94)	1.191	>0.1
IgG3	0.74 (0.21)	0.39 (0.16)	6.616	<0.001 (S)	0.59 (0.24)	0.40 (0.166)	4.419	<0.001 (S)
IgG4	0.19 (0.15)	0.18 (0.07)	0.313	>0.1	0.18 (0.11)	0.14 (0.08)	2.000	<0.05 (S)
Anti-whole parasite anti-body, ELISA (ODA 92)	0.56 (0.34)	0.25 (0.29)	3.204	<0.01 (S)	0.43 (0.36)	0.24 (0.17)	3.036	<0.001 (S)
Anti-Cs anti-body (ODa92)	0.14 (0.15)	0.14 (0.13)	-	-	0.165 (0.16)	0.160 (0.23)	0.107	>0.1
Anti-malaria antibody-IFA (RGMT)	177 (158-195)*	259 (22-304)*	0.794	>0.1	201 (136-294)*	123 (74-204)*	1.471	>0.1

Standard deviation (SD) in parentheses   * = 95% Confidence interval   S = Significant

RGMT = Reciprocal of geometric mean titre   CS = Circumsporozoite

**Patients infected with CRPf, CSPf plus those whose isolate cultures were not successful.

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TABLE 5.5

Mean serum levels of Immunoglobulins, malaria IgG-specific antibody titres and seroreactivities: Comparison between the two groups of P.falciparum infected patients (CRPF- & CSPf - infected) in Ibadan.

Immunological Profile	CRPf-infected	CSPf-infected			All the infected children studied	Apparently healthy (non-infected)		
Mean (g/L)	n = 12	n = 22	t	p	n = 40**	n = 50	t	p
IgM	2.5(0.82)	2.16(1.04)	1.049	>0.1	2.25(0.97)	1.67(0.42)	3.537	<0.001(S)
IgG(total)	20.01(7.97)	18.57(6.95)	0.526	>0.1	20.4(6.91)	14.18(7.66)	3.393	<0.01 (S)
IgG1	13.32(5.24)	12.19(4.73)	0.342	>0.1	13.61(4.49)	9.33(4.93)	3.296	<0.01 (S)
IgG2	4.2(2.07)	4.35(1.39)	0.165	>0.1	4.45(1.43)	3.56(1.92)	2.543	<0.02 (S)
IgG3	0.56(0.29)	0.40(0.22)	1.668	>0.05	0.53(0.23)	0.43(0.23)	2.859	<0.01 (S)
IgG4	0.18(0.09)	0.17(0.08)	0.040	>0.1	0.17(0.08)	0.13(0.06)	2.667	<0.01 (S)
Anti-whole Parasite Antibody-ELISA (OD492)	0.43(0.32)	0.23(0.15)	2.046	<0.05(S)	0.30(0.23)	0.21(0.166)	2.093	<0.05(S)
Anti-CS Antibody (OD 492)	0.135(0.285)	0.205(0.244)	0.722	>0.1	0.20(0.27)	0.133(0.14)	1.432	>0.1
Anti-malaria antibody-IFA(RGMT)	266(174-294)*	189(162-219)*	0.022	>0.1	237(130-414)*	202(108-378)*	0.388	>0.1

Standard deviation SD in parenthesis   * = 95% Confidence interval

RGMT = Reciprocal of geometric mean titre   CS = Circumsporozoite

S = Significant .

**Patients infected with CRPf, CSPf plus those whose isolate cultures were not successful.

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mean IgG and IgG subclass levels were higher in CRPf-associated infections except that the mean IgG3 level was not significantly higher (t = 1.668, $P > 0.05$) as was the case in Calabar.

Fig. 5.3 shows IgG3 distribution in patients as compared with controls in both Calabar and Ibadan. On comparison of the two geographical populations studied, none of the mean values of the immunoglobulins evaluated showed any significant differences (Table 5.7).

5.3.2. Relationship between immunoglobulin levels and IgG-specific malaria antibodies

The mean IgG3 levels were plotted against each of the IgG-specific anti-parasite antibodies (anti-total blood-stage antibody, and anti-circumsporozoite (SC) antibody), and the results from the two study populations are as follows, (Fig. 5.4): IgG3 levels showed the same pattern of distribution with IgG-specific total blood stage antibody reactivity, but not with IgG-specific anti-circumsporozoite antibody reactivity. The same patterns were observed in patients from both Calabar and Ibadan.

There were also positive correlations between IgG3 and blood stage antibody reactivities in patients from Calabar (r = 0.455) and Ibadan (r = 0.160), (Figs. 5.5 and 5.6). However, only the correlation in subjects from

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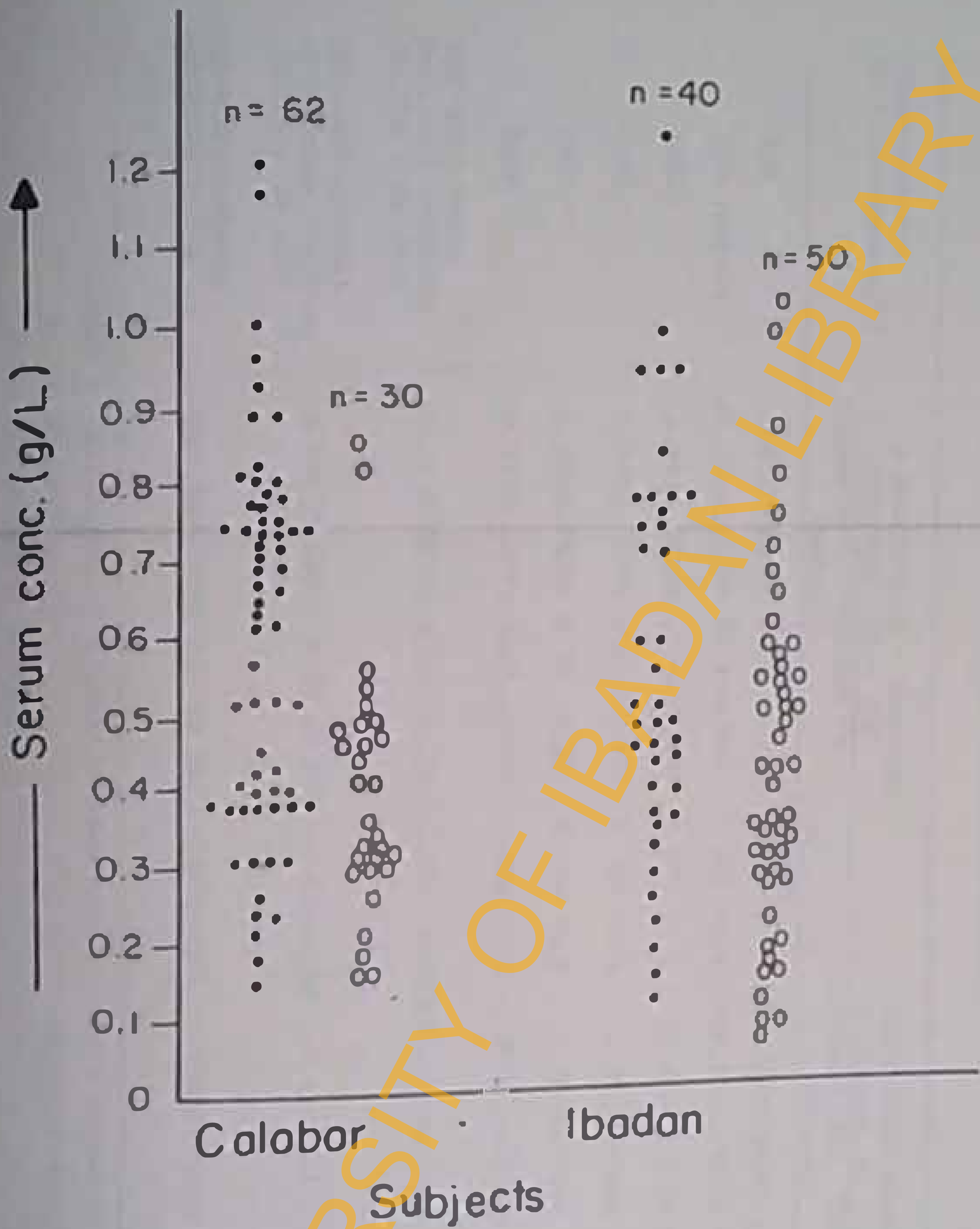

Fig. 5.3 Distribution of IgG3 concentrations in malaria patients (•) and controls (∘) in subjects from Calabar and Ibadan.

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TABLE 5.6

Comparison of humoral immune parameters in Sera from children in Calabar and Ibadan

Immunological Profiles-mean (g/L)	P.FALCIPERUM - INFECTED CHILDREN, df = 100 Comparison, (Calabar Vs Ibadan	t	P	APPARENTLY NORMAL CHILDREN, df = 78 Comparison, (Calabar Vs Ibadan)	t	P
IgM	1.95 (0.82) Vs 2.25 (0.95)	1.642	>0.1	1.58 (0.36) Vs 1.67 (0.42)	1.023	>0.1
IgG-(total)	21.55 (6.81) Vs 20.4 (6.91)	1.543	>0.1	16.35 (7.30) Vs 14.18 (7.66)	0.736	>0.1
IgG1	13.46 (3.72) Vs 13.61 (4.49)	0.897	>0.1	10.61 (4.79) Vs 9.33 (4.93)	1.145	>0.1
IgG2	4.84 (1.37) Vs 4.45 (1.43)	1.368	>0.1	4.37 (1.94) Vs 3.56 (1.92)	1.816	>0.01
IgG3	0.59 (0.24) Vs 0.53 (0.23)	1.266	>0.1	0.40 (0.17) Vs 0.43 (0.23)	0.668	>0.1
IgG4	0.18 (0.11) Vs 0.17 (0.08)	0.058	>0.1	0.14 (0.08) Vs 0.13 (0.06)	0.592	>0.1
IgG-anti-whole parasite antibody (ELISA - OD 492)	0.43 (0.36) Vs 0.30 (0.23)	2.222	<0.05 (S)	0.24 (0.17) Vs 0.21 (0.166)	0.772	>0.1
IgG-anti-circum-sporozoite antibody (ELISA - OD 492)	0.165 (0.16) Vs 0.20 (0.27)	0.742	>0.10	0.160 (0.23) Vs 0.133 (0.14)	0.582	>0.1
IgG-anti-malaria antibody - IFA (RGMT)	210 (0.675)* Vs 237 (0.78)*	0.667	>0.1	123 (0.58)* Vs 202 (0.98)*	1.262	>0.1

RGMT = Reciprocal of geometric mean titre
S = Significant
* = Log standard deviation.

df = degree of freedom
Standard deviation (SD) in parenthesis

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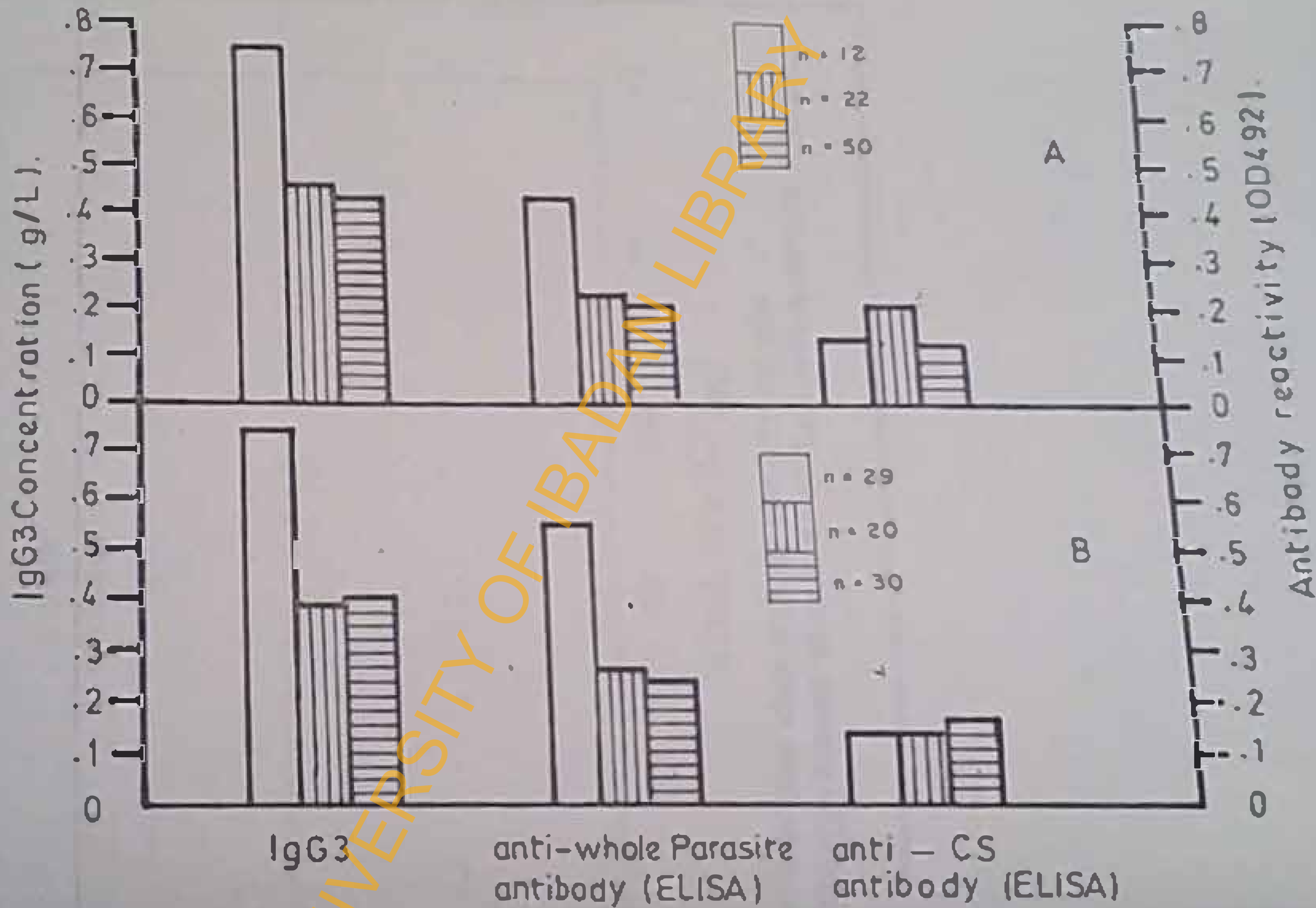

FIG 5.4 Comparison of anti-blood stage and anti-circumsporozoite (cs) antibodies with IgG3 levels in CRPf – infected [ ], CSPf – infected [ ] and uninfected controls [ ] in Ibadan A ... B ...

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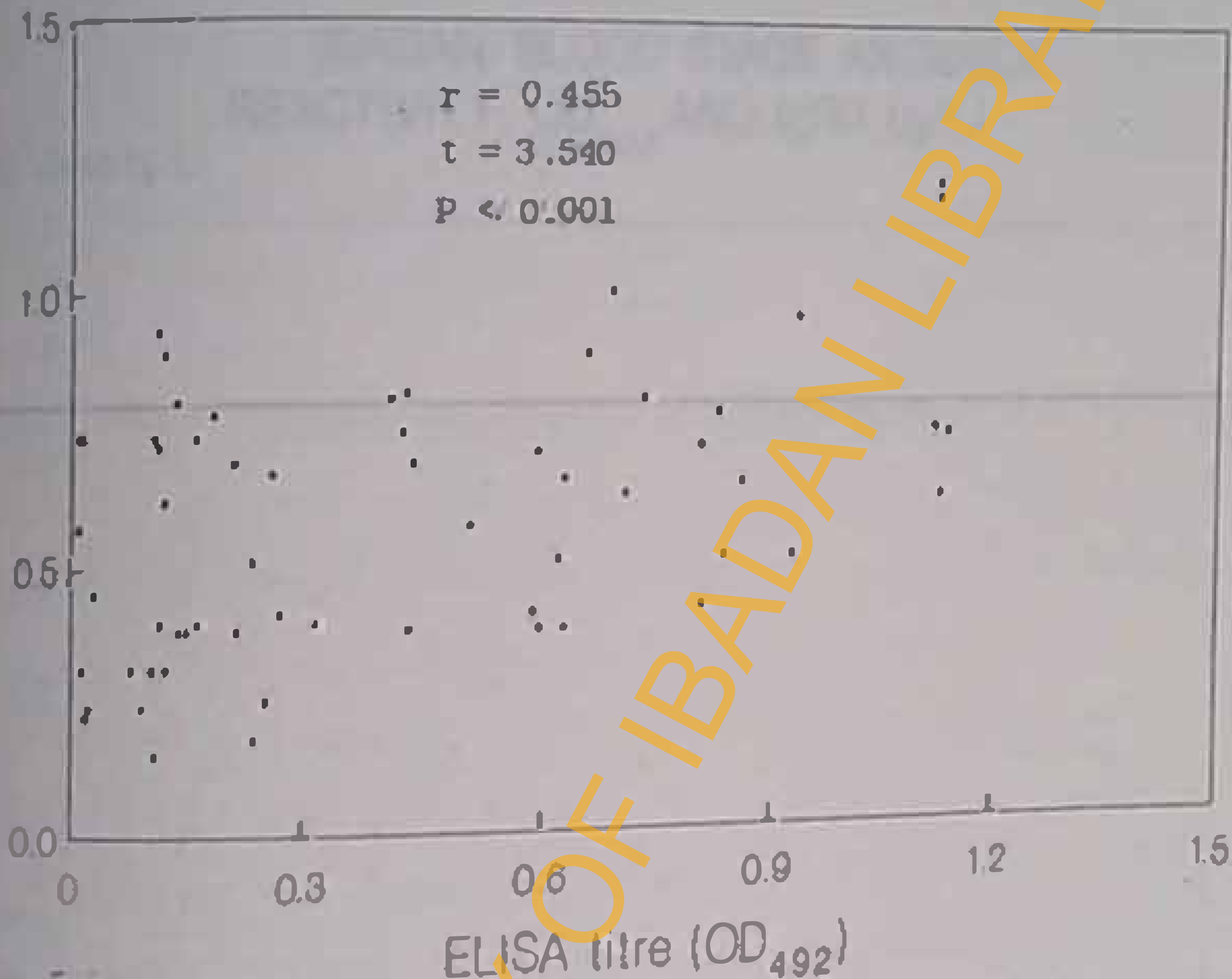

Fig. 5.5
Correlation between blood stage antibody reactivity and IgG3 levels in *P. Falciparum* infected children, n = 60. Each point represents 1 sample

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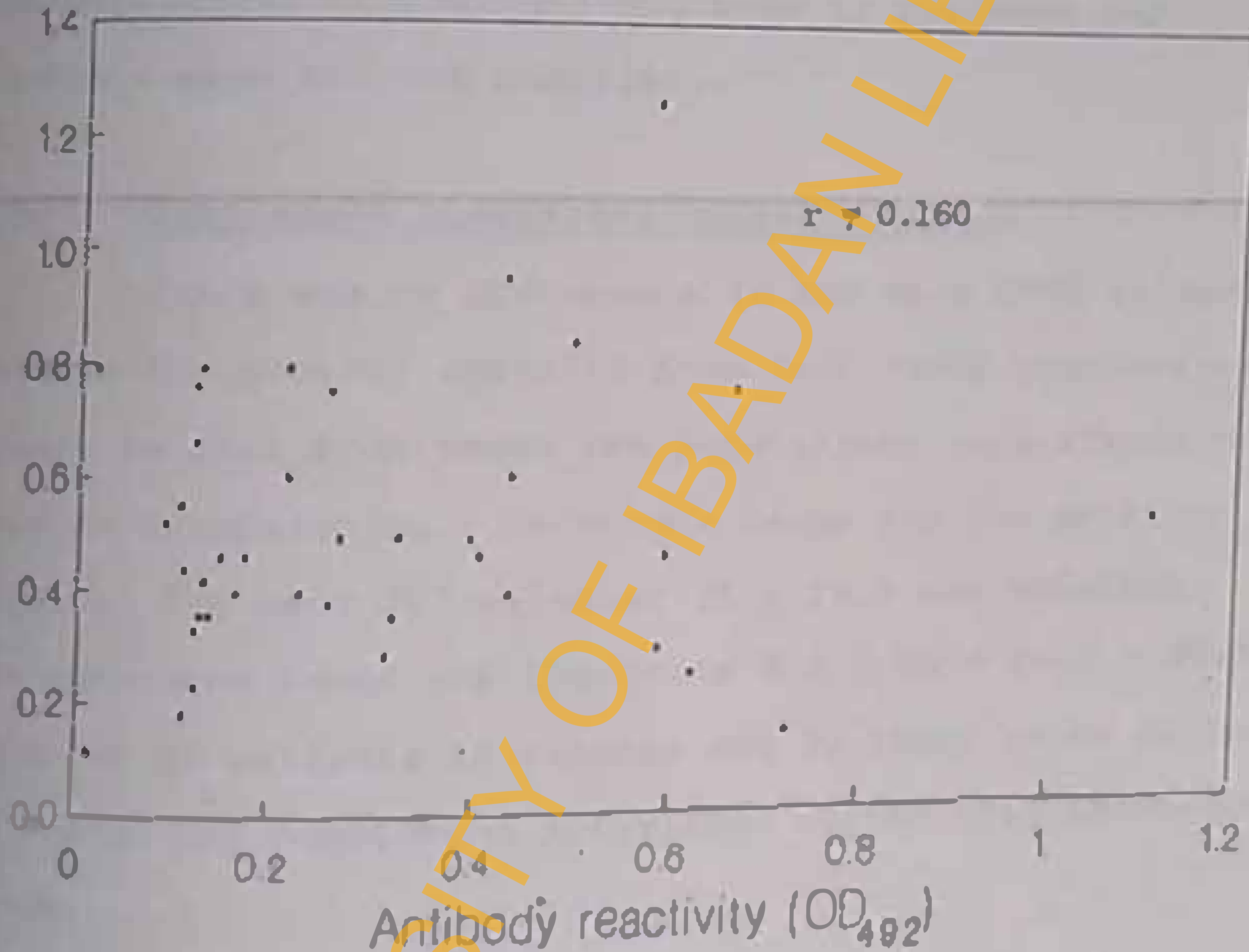

Fig. 5.6 Relationship between total blood stage antibody reactivity and IgG3 levels in *P. falciparum* infected children in Ibadan.

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Calabar was significant ($t = 3.450$, $P < 0.001$). A similar analysis in controls from both populations did not show any correlation. Regression analysis of anti-total blood-stage antibody reactivities against other immunoglobulin concentrations, both in patients and controls also did not correlate.

5.3.3. Complement haemolytic activity (CH50)

There was no difference in the mean CH50 values between the control subjects from both study populations. Plasma samples from these two populations were therefore used in establishing a reference range for the patient groups. The mean ($\bar{x}$) value of 35 ± 15.8 was obtained. The reference range was therefore $\bar{x} \pm 2$ SD = 19.2 - 50.8. 47 (75%) of 62 patients in Calabar and 26 (65%) of 40 patients in Ibadan had complement activities within this reference range.

5.3.4. Antigenic levels of complement proteins C3, C4 and Bf:

The mean C3 and C4 levels, as well as CH50 titres were lower in patients than in the control subjects in both Ibadan and Calabar. The differences were significant except for the C3 (Table 5.8 and 5.9). On the other hand, mean Bf concentration was significantly higher in the

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TABLE 5.7

Mean plasma Complement levels: comparison between the infected patients (CRPf and CSPf - infected) in Calabar

MEAN COMPLEMENT VALUES	CRPf - INFECTED (n = 29)	CSPf - INFECTED (n = 20)	t	P	ALL THE INFECTED CHILDREN STUDIED (n = 62)**	APPARENTLY HEALTHY (NON-INFECTED) CHILDREN (n = 30)	t
C3 (g/L	0.75(0.20)	0.73(0.18)	0.365	> 0.1	0.74(0.18)	0.78(0.24)	0.81
C4 (g/L)	0.37(0.22)	0.30(0.16)	1.289	> 0.1	0.35(0.27)	0.59(0.23)	4.4
Bf (g/L)	0.34(0.15)	0.40(0.19)	1.181	> 0.1	0.35(0.21)	0.23(0.11)	3.6
CH50(Unit/ml)	22.5(6.2)	20.9(6.5)	0.865	> 0.1	23.9 (6.2)	35.1 (7.1)	7.

CRPf - Chloroquine resistant _P. falciparum_

CSPf - Chloroquine sensitive _P. falciparum_

* - Statistically significant

Standard deviation (SD) in parenthesis

** - Patients infected with CRPf, CSPf plus those whose isolate cultures were not successful.

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TABLE 5.7

Mean plasma Complement levels: comparison between the infected patients (CRPf and CSPf - infected) in Calabar

MEAN COMPLEMENT VALUES	CRPf - INFECTED (n = 29)	CSPf - INFECTED (n = 20)	t	p	ALL THE INFECTED CHILDREN STUDIED (n = 62)**	APPARENTLY HEALTHY (NON-INFECTED) CHILDREN (n = 30)	t
C3 (g/L	0.75(0.20)	0.73(0.18)	0.365	> 0.1	0.74(0.18)	0.78(0.24)	0.810
C4 (g/L)	0.37(0.22)	0.30(0.16)	1.289	> 0.1	0.35(0.27)	0.59(0.23)	4.428
Bf (g/L)	0.34(0.15)	0.40(0.19)	1.181	> 0.1	0.35(0.21)	0.23(0.11)	3.636
CH50(Unit/ml)	22.5(6.2)	20.9(6.5)	0.865	> 0.1	23.9 (6.2)	35.1 (7.1)	7.253

CRPf - Chloroquine resistant *P. falciparum*

CSPf - Chloroquine sensitive *P. falciparum*

* - Statistically significant

Standard deviation (SD) in parenthesis

** - Patients infected with CRPf, CSPf plus those whose isolate cultures were not successful.

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TABLE 5.7

Mean plasma Complement levels: comparison between the infected patients (CRPf and CSPf - infected) in Calabar

[M]EAN [COM]PLEMENT [V]ALUES	CRPf - INFECTED (n = 29)	CSPF - INFECTED (n = 20)	t	P	ALL THE INFECTED CHILDREN STUDIED (n = 62)**	APPARENTLY HEALTHY (NON-INFECTED) CHILDREN (n = 30)	t	P
(g/L	0.75(0.20)	0.73(0.18)	0.365	> 0.1	0.74(0.18)	0.78(0.24)	0.810	>
(g/L)	0.37(0.22)	0.30(0.16)	1.289	> 0.1	0.35(0.27)	0.59(0.23)	4.428	<
(g/L)	0.34(0.15)	0.40(0.19)	1.181	> 0.1	0.35(0.21)	0.23(0.11)	3.636	<
0(Unit/ml)	22.5(6.2)	20.9(6.5)	0.865	> 0.1	23.9 (6.2)	35.1 (7.1)	7.253	<

CRPf - Chloroquine resistant *P. falciparum*
CSPf - Chloroquine sensitive *P. falciparum*
* - Statistically significant
Standard deviation (SD) in parenthesis
** - Patients infected with CRPf, CSPf plus those whose isolate cultures were not successful.

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TABLE 5.8

Mean Plasma complement Levels: Comparison between the two groups of *P.falciparum* infected patients (CRPF - & CSPf - infected) in Ibadan.

...n ...plement ...ue	CRPF-infected (n=12)	CSPf-infected (n=22)	t	p	All the infected children studied (n =40)**	Apparently healthy (non-infected) controls (n = 50)	t	p
(g/L)	0.76 (0.23)	0.73 (0.21)	0.75	>0.1	0.72 (0.23)	0.79 (0.19)	1.555	>0.1
(g/L)	0.28 (0.11)	0.31 (0.08)	0.837	>0.1	0.30 (0.09)	0.54 (0.18)	8.276	<0.001*
(g/L)	0.38 (0.17)	0.34 (0.07)	0.800	>0.1	0.35 (0.11)	0.25 (0.11)	4.300	<0.001*
...0 (Unit/ml)	21.3 (7.0)	19.0 (4.7)	1.020	>0.1	20 (5.6)	35.9 (8.7)	10.490	<0.001*

CRPF = Chloroquine resistant *P. falciparum*

CSPf = Chloroquine sensitive *P.falciparum*

* = Statistically significant

Standard deviation (SD) in parenthesis.

** = Patients infected with CRPf, CSPf plus those whose isolate cultures were not successful.

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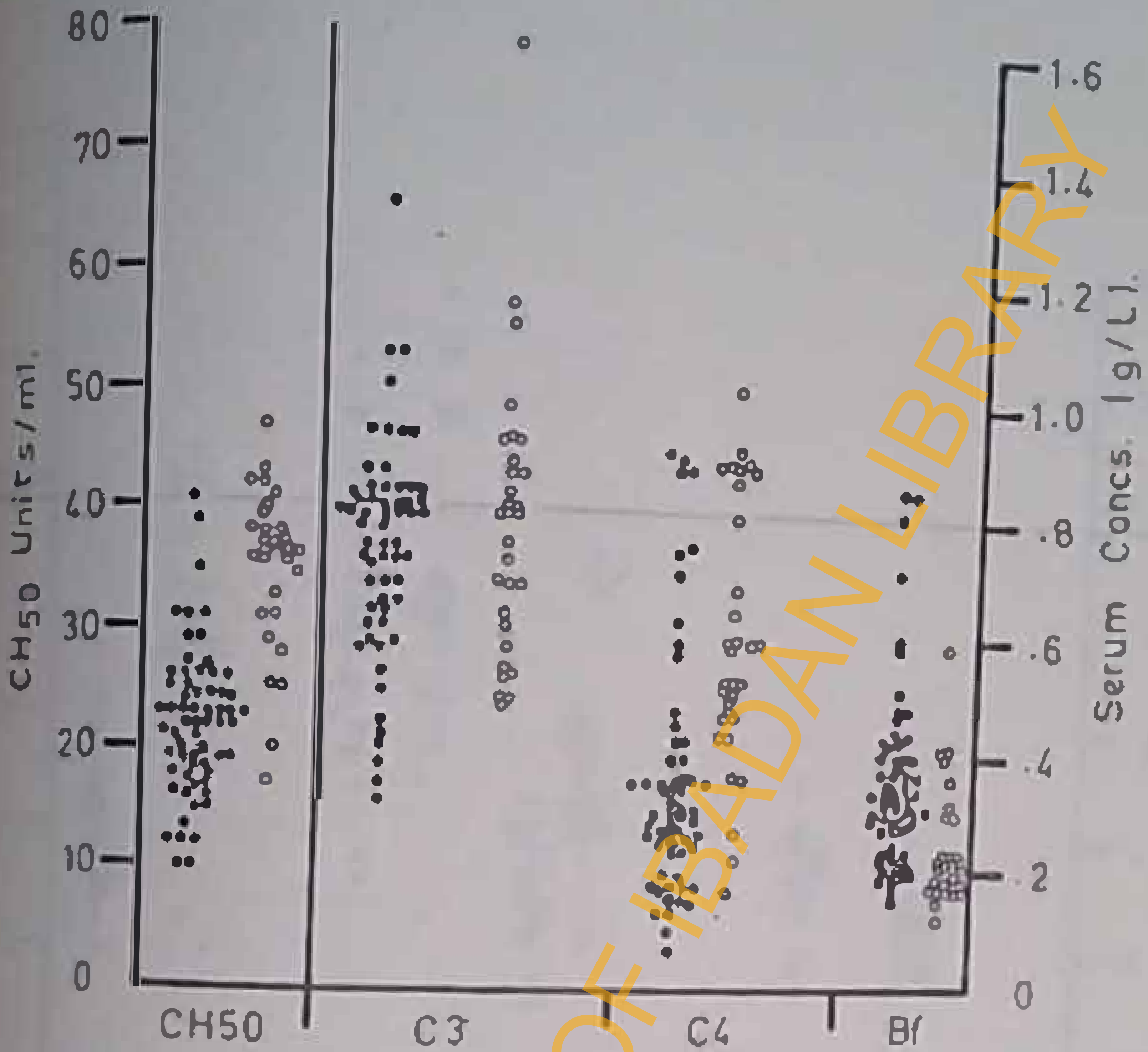

Fig. 5.7: Distribution of complement haemolytic titres (CH50) and C3, C4, Bf serum concentrations in P. falciporum infected children (●) from Calabar; n = 62 and sex/age-matched controls (O) n = 30.

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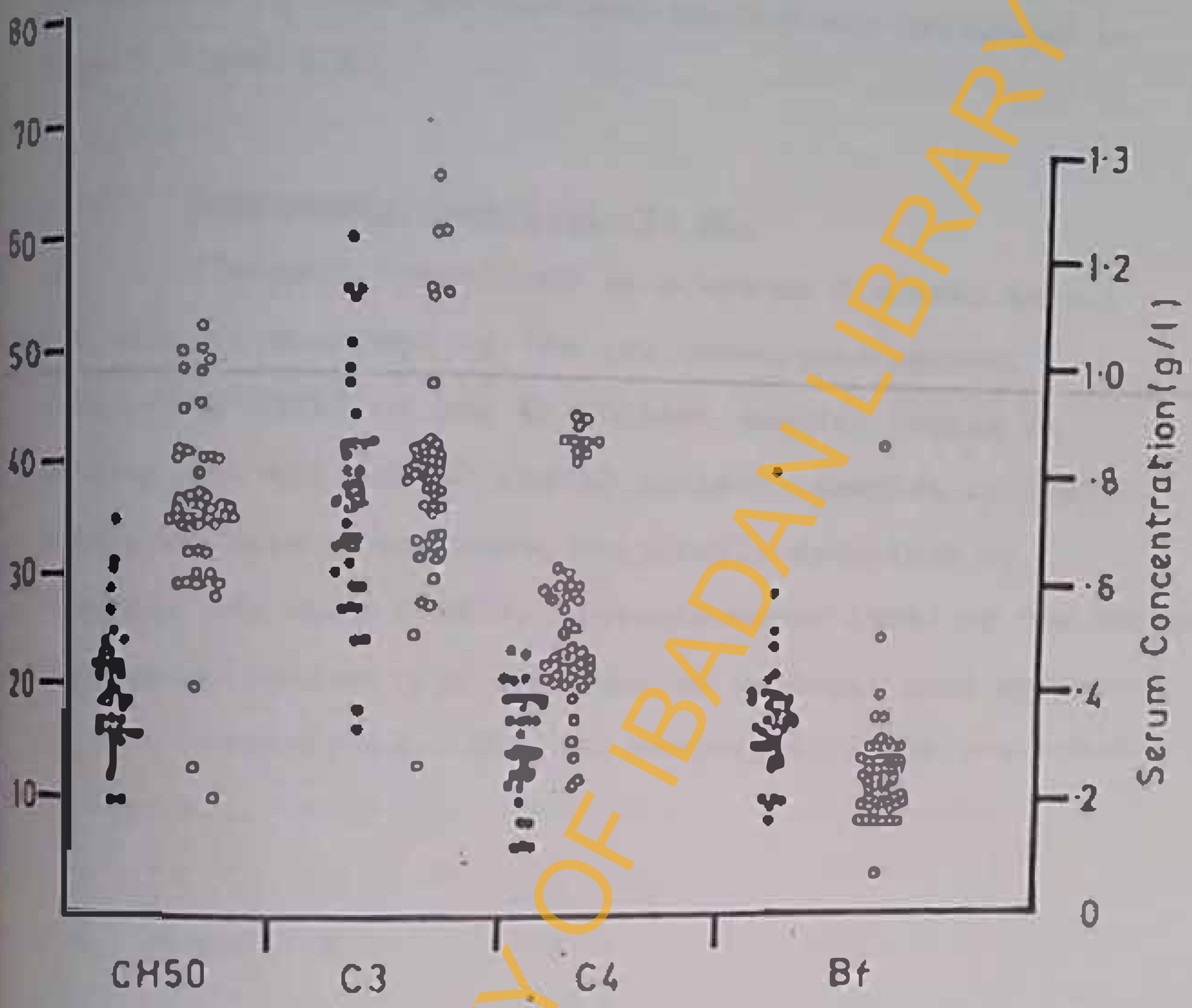

Fig.58 Distribution of Complement haemolytic titres (CH50 Unit/ml) and C3, C4, & Bf Serum concentrations (g/L) in *P. falciparum* infected children from Ibadan, n = 40, (●) and sex/age matched controls, n = 50 (O)

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control subjects ($p < 0.01$). The distribution of these parameters in both populations studied are presented in Fig. 5.7 and 5.8.

5.3.5. Complement haemolysis in gel

"Target" haemolyses were common features in all the samples analysed by the gel haemolysis method. Fifty-nine (95%) of the 62 patient samples tested in Calabar and all (100%) the 40 patient samples in Ibadan exhibited this phenomenon, originally described by Thompson and Rowe (1967). Seventy-three (91%) of the entire 80 samples collected from the control subjects also showed 'target' haemolysis. The gel haemolytic rings are shown in Fig. 5.1.

5.4. DISCUSSION

There is sufficient evidence to support the fact that serum gammaglobulins, particularly IgG and some of its subclasses are usually more elevated in the populations of tropical countries than in Caucasians (Lichtman, 1967; Morell et al, 1971; Apampa et al 1980). This state of hypergammaglobulinaemia in tropical countries has been attributed to the effect of predominant parasitic infections,

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particularly malaria (Salimonu et al (1982).

Racial differences in immunoglobulin levels do exist (Apampa et al 1980) and there are also differences even within a geographical area as has been reported in two New Guinean ethnic populations (Voller, 1982). In Nigeria, two independent reports, Wemambu (1984) and Ogbimi and Omu (1989) had shown higher levels of immunoglobulins in Benin population than in Ibadan. These and other similar findings emphasize the considerable variability in immunological responses between individuals, especially in antibody production (Ogbimi and Omu 1989). Although individual genetic influence may be a predominant factor in determining immune responses against parasitic infections, variation in parasite antigenic components may also play a significant role (Sy et al, 1990).

The results of the present study show that P. falciparum-infected children had higher IgM, IgG and most of the IgG subclasses. Lower levels of complement proteins C3, C4 and of the classical pathway haemolytic titre (CH50) were also observed in the infected subjects. These observations confirm earlier reports of elevated IgM and IgG (McGregor, 1972; Salimonu et al, 1982), as well as lower CH50, C3 and C4 in malaria patients (Greenwood and Brueton, 1974; Gupta et al 1982; Kidwai et al, 1986).

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The present result appears to indicate that complement activation in malaria is via the classical pathway. Some previous reports had indicated that complement is consumed during malaria infections (Greenwood and Brueton, 1974; Kiwai *et al* 1986), although the associated mechanisms are still not known (Williams *et al*, 1973).

The role of the alternate complement pathway in malaria has not been adequately investigated, and although the functional activity of this pathway was not studied in this work, the significantly higher mean level of Bf observed in patients in comparison to controls, seems to reflect this protein only as an acute phase component.

Generally, infected children both in Calabar and Ibadan had higher mean IgG levels than the controls. These differences were also significant in both populations ($p < 0.01$). However, a noteworthy observation was the significant elevation of IgG3 in patients infected with CRPf when compared to those infected with the chloroquine sensitive strain. A regression analysis of IgG3 levels against IgG-specific antibodies from both populations showed positive correlation which was significant in Calabar ($r = 0.455$, $t = 3.540$, $P < 0.001$).

It would appear from these findings that IgG3 production is associated with the emerging strain(s) of CRPf. The antigenic component(s) that may possibly be responsible

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for the enhanced production of this immunoglobulin G subclass may thus be detectable by ELISA method for total blood stage antibody. The apparent association of this immunoglobulin isotype with CRPf malaria that appears evident from this study is difficult to explain in relation to the milder clinical manifestation of CRPf infection reported earlier on by LeBrass et al (1986) and Ekanem et al (1990). A lower mean parasite density found in subjects infected with CRPf compared with those infected with the sensitive strain, which was observed in this study, is also difficult to explain. However, the possible role of IgG3 in CRPf malaria may not be connected with its role as an effective complement activator. This is because the levels of hypocomplementaemia in both CRPf- and CSPf-infected children observed in this study were not significantly different. This view is supported by earlier reports that complement-mediated parasite clearance is not a major offensive in protection against malaria (McGregor, 1972; Diggs et al, 1972). On the other hand, antibody-dependent cell-mediated reactions involving mononuclear cells or granulocytes are believed to be important for protection in malaria (Wahlgren et al 1986b). Such reactions are primarily mediated by IgG1 and IgG3 (Spiegelberg, 1974). Moreover, IgG3 and IgG1 are the only

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immunoglobulin isotypes capable of activating neutrophils, macrophages, large granular lymphocytes (LGL) natural killer (NK) cells and T-lymphocytes (Jefferis and Kumarantre, 1990). It is therefore conceivable that CRPf infection, in association with increased IgG3 may induce an enhanced phagocytic function thereby reducing parasite load more effectively. This hypothesis is in line with the findings of Facer (1980), which postulated an IgG-mediated opsonization of sensitized red blood cells. The mean values of almost all the humoral immune parameters evaluated in this work were comparable in both populations studied. It was also observed that the pattern of distribution of IgG subclass levels in both malaria and non-infected subjects has changed from that presented in a previous report by Salimonu et al (1982) for Ibadan. Generally, there has been an increase in IgG3 and IgG4 concentrations from what was last reported by these authors. IgG2 concentration, however, remained unchanged. These changes in pattern of IgG isotype distribution may be due to the effects of antigenic changes with time, within the environment. The evaluation of immunoglobulin in Calabar has never been carried out prior to the present study and so no comparisons can be made. Therefore the present results will serve as base line data for subsequent

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evaluations in Calabar.

This study has demonstrated that haemolysis in gel, as an *in vitro* technique for the assessment of complement activity may be adapted to provide a reliable semi-quantitative index of total plasma complement activity in our local populations. It may be particularly useful as a rapid screening test. However, because of the problem of standardization inherent in this assay method, it should be used as a basis for selection or exclusion of samples for further study, rather than as a means of determining quantitative values, equivalent to those obtained by tube titration method.

Another significant aspect of this study was the observation of a "target phenomenon" in a high percentage of patients and control subjects. Target phenomenon was first described by Thompson and Rowe (1967), when they observed that plasma from a few Caucasians suffering from a variety of diseases (16 out of 1,200) showed inner zones of unlysed cells, along with an outer ring of complete lysis, when diffusing through an agarose plate containing optimally sensitized sheep red cells. These workers did not demonstrate target phenomenon in plasma from 40 healthy individuals tested at the same time. They therefore

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suggested that the target phenomenon was due to the patient's IgG that doubly coated the already sensitized sheep red blood cells,thus preventing them from lysing. In the present study,the majority of both patients and controls exhibited "target phenomenon". It is postulated from these findings that target phenomenon may be associated with parasitic infections which abound in tropical environments at both clinical and subclinical levels. Thus our apparently healthy controls may have been harbouring other kinds of infections albeit subclinically. Further studies aimed at establishing a possible relationship between parasitic infections, particularly malaria, and "target phenomenon" are recommended.

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CHAPTER 6

SEROREACTIVITY AGAINST SYNTHETIC PEPTIDE (NANP)40 OF CIRCUMSPOROZOITE PROTEIN OF PLASMODIUM FALCIPARUM

6.1 INTRODUCTION

Antibody reactivities against circumsporozoite (CS) protein of _Plasmodium falciparum_ were determined by enzyme linked immunosorbent assay (ELISA) technique using the synthetic peptide (NANP)40, representing the repeating sequences from CS protein of the parasite, as antigen. This technique has previously been used by some investigators, firstly to determine whether or not measurement of antisporozoite antibody levels can be used as an indicator of the level of malaria transmission in a study community (Campbell _et al_, 1987). Secondly, it has been employed to investigate the possible relationship between sporozoite antibodies and protection against malaria infection (Snow _et al_, 1989). Since individual genetic influence has also been noted as a determining factor in immune response against CS protein (Del-Guidice 1987), the present study was also aimed at assessing anti-CS seroreactivities in the two populations of Nigerians under study. It was envisaged that the result may indicate a possible individual or regional variation in immune response against this protein in

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Nigeria. The knowledge may help to explain the apparent variation in CRPf prevalence levels, as well as the different clinical outcomes of malaria infections, noticed in the two Nigerian populations (Ekanem et al, 1990), represented in this study.

6.2 MATERIALS AND METHODS

6.2.1 Study Areas, Subjects and Samples

The study areas, subjects and samples are as described for the previous investigations reported in the preceding chapter.

6.2.2. Enzymelinked Immunosorbent Assay (ELISA) for Antibody

To determine seroreactivities against circum-sporozoite (CS) protein, ELISA antigen (NANP)40 precoated plates, obtained from Dayal-Drager and Decrind (1990) were used. Samples were diluted 1/200 in PBS-Tween 20, containing 2.5% defatted milk. 100 ul of each diluted serum was applied both to a test well and to a corresponding well on a control ELISA plate pre-coated with PBS only. All samples were tested in duplicates. The rest of the procedure was carried out as reported in Chapter 4, section 2.4 for anti-total blood stage antibody assays.

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With the plates used, the mean OD492 values of the seronegative sera obtained from non-immune students from Swaziland, was 0.075. A value of 2 x 0.075 = 0.150 was used as the cut-off point.

6.3 RESULTS

6.3.1 In vitro Response test and Parasitology

The results of *in vitro* response test and parasitology are similar to those reported in Chapter 4, Section 3.1.

6.3.2. ELISA Seroreactivities

Based on the cut-off point of 0.150, 27 (43.5%) of 62 patients and 9 (30%) of 30 control subjects in Calabar were seropositive. Similarly 18 (45%) of the 40 patients and 15 (30%) of the 50 controls in Ibadan were positive against (NANP)40, (Table 6.1). OD492 at serum dilution of 1/200 ranged between 0 and 0.636 for the patient population, and between 0 and 1.077 for the control subjects in Calabar. The OD492 range in Ibadan was between 0.009 and 1.017 and between 0.004 and 0.526 for the patient and control groups respectively. The mean seroreactivity and positivity rates for both populations increased with age in both the patients and control groups. However, there were no significant

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differences in antibody reactivities between the CRPf - infected and the CRSf - infected individuals (table 6.2).

6.3.3. Relationship Between anti-(NANP)40 Antibody and Malaria Infection

The overal positivity rates in patients was higher than in the non-infected controls, both in Calabar and in Ibadan, (43.5% Vs 30% and 45% Vs 30% respectively). However, these differences were not statistically significant. Moreover, the mean seroreactivities in patients and controls were similar in both study populations (Table 6.1). The pattern of distribution of anti-CS antibody reactivities in both populations is presented in Fig. 6.1

6.3.4. Relationship between anti-CS Antibody and Blood Stage Antibodies

The changes in anti-CS reactivities in relation to anti-total blood stage antibody reactivities at serum dilutions of 1/200 did not show any statistically significant correlation for both patient and control groups in both populations studied. Similarly, there were no statistically significant correlations between anti-CS (ELISA) reactivity and blood-stage antibody (IFA) in both patient and control groups from the two populations.

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TABLE 6.1

Antibodies against circumsporozoite repetitive peptide $(NANP)_{40}$ in 102 *P. falciparum*-infected subjects, and 80 age/sex matched controls from Ibadan and Calabar

Age (years)	Mean OD_{492} (% Positivity)							
	CALABAR				IBADAN			
	n	PATIENTS	n	CONTROLS	n	PATIENTS	n	CONTROLS
< 1	4	0.052±0.048(0)	2	0.027±0.006(0)	2	0.019±0.018(0)	2	0.030±0.009(0)
1 - 4	28	0.154±0.144(39.3)	12	0.175±0.189(41)	13	0.072±0.086(23)	23	0.014±0.043(0)
> 4 - 9	23	0.197±0.187(56.5)	14	0.102±0.112(35.7)	15	0.188±0.23(53.3)	15	0.168±0.14(40)
> 9 - 13	7	0.175±0.112(57)	2	0.591±0.682(50)	10	0.42 ±0.40(70)	9	0.306±0.135(88.9)
Totals	62	0.165±0.160(43.5)*	30	0.010±0.23(30)*	40	0.20 ±0.27(45)**	50	0.133±0.140(30)**

$^*\chi^2 = 1.600$, $p > 0.1$ $\quad$ $^{**}\chi^2 = 2.[illegible]$, $p > 0.1$

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TABLE 6.2

Mean anti-CS antibody reactivities (OD 492) in children infected with CRPf or CSPf in Ibadan and Calabar

Locality	CRPf-infected children n = 29	CSPf-infected children n = 20	t	P
CALABAR	0.14 (0.15)	0.14 (0.13)	-	-
IBADAN	0.135 (0.285)	0.205(0.244)	0.722	> 0.1

CS = Circumsporozoite
CRPf = Chloroquine resistant *P. falciparum*
CSPf = Cloroquine sensitive *P. falciparum*

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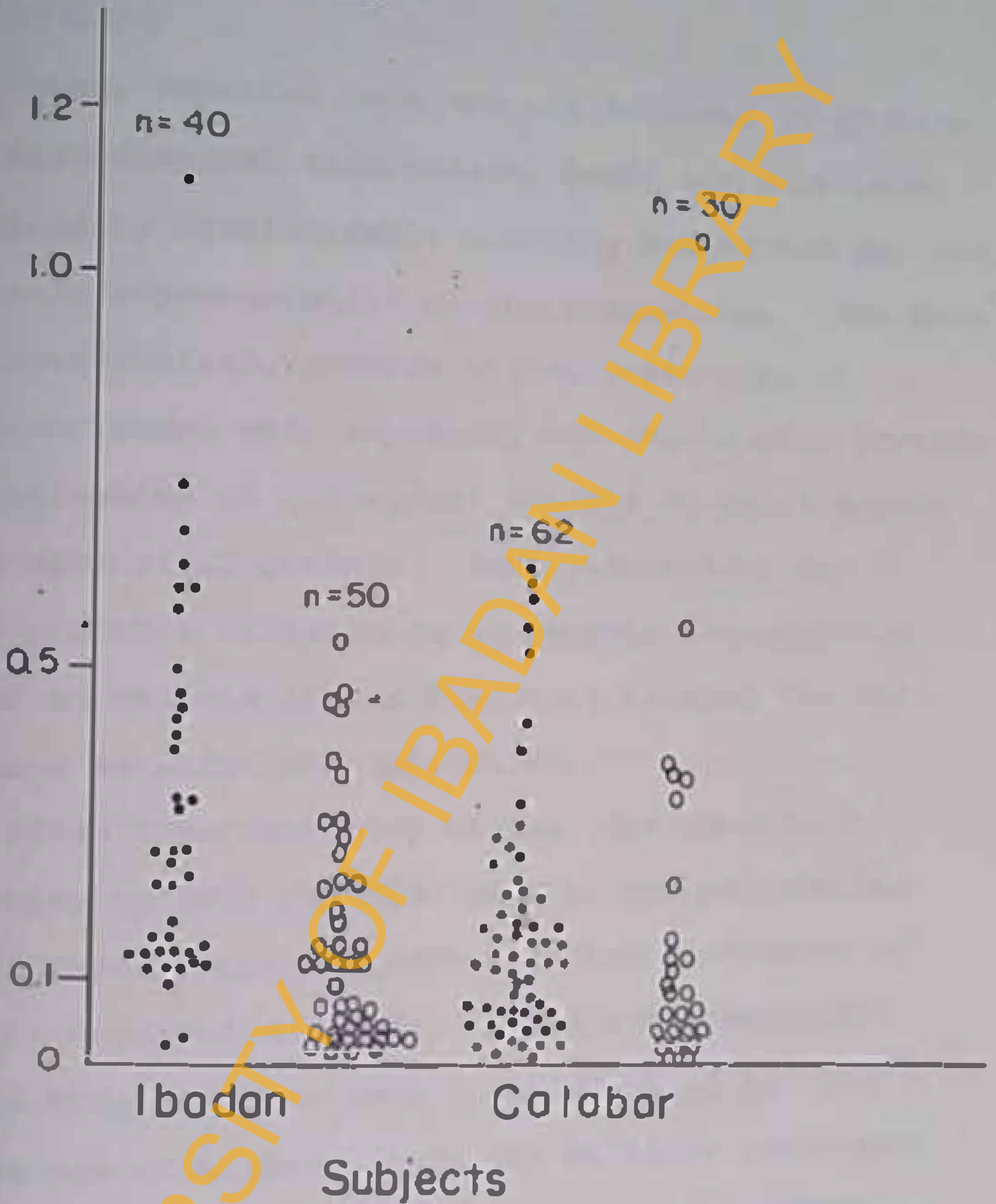

Fig. 6.1 Distribution of anti-cs $(NANP)_{40}$ antibody reactivities in patients (•) and controls (∘) in Calabar and Ibadan.

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6.4. DISCUSSION

The study reported here was not intended to provide a seroepidemiological information, hence the sera were not obtained by statistically sampling method and may not be precisely representative of the communities. The data should, nevertheless, provide a good indication of transmission rates with exposure, and should also provide a good indication of individual ability to mount immune response against CS protein. Such information may indicate possible differences in malaria transmission rates and in malaria immune responses between the two populations selected for this study.

Children under one year of age were generally seronegative against (NANP)40 both in the patient and control groups. This suggests a minimal influence of maternally acquired antibody. A similar observation and suggestion had been made by Campbell et al, 1987). After the age of 4 years, both the antibody prevalence rates and the levels of reactivity to this synthetic peptide increased with age. A similar trend has previously been reported in various other geographical areas (Hoffman et al 1986); Campbell, et al 1987; Deloron et al, 1987). As suggested by Pang et al (1988), this age-related increase, probably reflects a cumulative exposure to

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P. falciparum parasites, rather than to protection. With regard to the present study, this postulation also stems from the fact that both the overall mean prevalence rate and level of reactivity to (NANP)40 did not differ significantly between the infected and the non-infected groups in the two populations studied. Webster et al (1987) showed that CS antibody levels rise and fall rapidly in response to natural P. falciparum infections in previously infected Thai adults. Presumably, the present pattern, as seen in the controls may have resulted from sporozoite innoculations which did not lead to the development of blood stage infections as suggested by Pang et al (1988), and Wijesundera, (1990). The lack of correlation between anti-CS antibodies and anti-blood stage antibodies in this study, further supports the probable association of age-related increase in antibody level and cumulative exposure to parasite infection. Thus attempts to measure the protective role of CS antibody in a natural setting require frequent antibody determinations and malaria smears (Pang et al, 1988).

Another important observation in the current study was the relatively lower prevalence rates of anti-CS antibody and levels of its reactivities in the 1 - 4 years age bracket, in subjects studied in Ibadan, as compared

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to the corresponding age bracket in Calabar, both in the patient and control groups. The positivity rate was relatively higher in Ibadan in comparison to Calabar, in the oldest age bracket (> 9 - 13 years). However the overall mean positivity rates and the levels of reactivity compared well in the two populations. It is not immediately clear why subjects aged between 1 and 4 years in Ibadan showed this relatively lower anti-(NANP)40 antibody prevalence rate and reactivity, despite the fact that this group of patients from the two study populations showed identical parasite densities as seen in Chapter 4, (Table 4.2). In a previous study in 3 Kenyan villages conducted by Campbell et al (1987) using a synthetic CS peptide, it was shown that the village differed in the ages at which antibody first appeared. Thus, in one village, only 12% of the children had antibody by the age of 5, while in the other two villages 60% and 73% had antibody by 4 years of age. It is tempting to speculate that the high anti-(NANP)40 antibody prevalence rate and reactivities in the age bracket (1 - 4 years) in Calabar appears to have no protective advantage against CRPf infection. This is because children below the age of 3 yesrs from the Calabar group had high prevalence of CRPf malaria as assessed by the *in vivo* method. An *in vivo* study was not conducted in

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Ibadan, but the present *in vitro* observation indicates that the prevalence rate of CRPf is still very low.

This study has also shown that anti-(NANP)40 has little, if any, effect on protection from infection, as shown by the apparently identical seropositivity rate and levels of reactivity between the infected and non-infected groups in the two populations studied. The protective effect of anti-CS antibody has already been refuted by Hoffman *et al* (1987) in Kenya; Pang *et al*(1988) and Webster, (1988) both in Thailand; Burkot *et al* (1989) in Papua New Guinea, and March *et al* (1986) in The Gambia. Since older children in both Ibadan and Calabar had similar seropositivity rates and reactivity levels, the apparent difference in immune response between the two study populations at 1 - 4 age bracket may not be due to genetic influence, a factor known to be important in malaria immunity (Rosenberg and Wirtz, 1990, Del-Guidice *et al* 1987). Furthermore it is known that immune responses against malaria is at its rudimentary stage in ages below 4 years (Greenwood *et al* 1987).

From the results of the present study therefore, it could be adduced that *P. falciparum* transmission rates in the two study populations are not significantly different, and that the two populations also do not appear to vary significantly in immune response against (NANP)40.

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CHAPTER 7

GENERAL DISCUSSION AND CONCLUSION

7.1 INTRODUCTION

The global increase during the past 15 years of *Plasmodium falciparum* malaria, the most lethal of the human malarias has highlighted the need for an effective vaccine against the disease (Anders *et al*, 1990). The emergence of chloroquine resistant *P. falciparum* (CRPf) malaria, first in South America and South East Asia, and more recently in Africa including Nigeria (Kean, 1979) has confounded the malaria problem. As a prerequisite to an application of a malaria vaccine directed against this parasite species, a number of preliminary studies are imperative. These include:

(i) the monitoring of drug sensitivity of *P. falciparum* in areas where malaria is endemic.

(ii) studies leading to a good understanding of the characteristics of host immune responses against locally prevalent antigens.

(iii) the assessment of the possible age and time-related variations in individual or regional immune status in respect of

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immunological parameters likely associated with malaria immunity.

(iv) the assessment of possible protective values of defined P. falciparum epitopes in populations that are potential targets of a putative malaria vaccine.

Studies on immunological responses against malaria in different populations have shown that immune response variation against this parasite has a strain specific component (James et al 1932; Jeffery, 1966; Volley, 1970; Hommel, 1986; Sy et al, 1990). Individual genetic variation in malaria immunity also appears to be a common feature in malaria endemic areas studied (Bjorkman et al, 1990). In mice, the response to some defined P. falciparum protein is controlled by immune response genes (Good et al, 1988) and it is postulated that the major histocompatibility complex (MHC) restriction on T-Cell response to these epitopes may also occur in humans (Wirtz and Rosenberg, 1990). Thus it has been suggested that if such a genetic regulation exists in humans, then it is conceivable that some individuals will respond better to the P. falciparum vaccine than others (Del Giudice et al, 1987).

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7.2 DISCUSSION

An assessment of humoral immune response to malaria was undertaken in two selected Nigerian populations. In Calabar, where CRPf incidence had been speculated much earlier, a high *in vitro* prevalence rate of 59.2% in children was recorded. A simultaneous *in vivo* prevalence of 53.6% was also recorded in the same children thereby giving a high *in vivo/in vitro* correlation in this population. Due to the modulating influences of host immunity in malaria chemotherapy (Spencer *et al* 1983b) a high *in vivo/ in vitro* correlation is expected only in the relatively less immune individuals. Immunity of infants against malaria depends on maternally transferred components. Children seldom acquire significant active immunity against malaria in endemic areas, (Greenwood *et al* 1987). It is also possible that individuals vary in the amount of immunity acquired at childhood. Whereas in an Indian study (Gautam *et al* 1980) the age of greatest susceptibility to malaria is between one and three years, with a decline thereafter, the victims of the disease in Calabar are largely those aged between one and ten years (Ikpat *et al*, 1990). This would suggest that while children in other tropical and sub-tropical regions develop acquired immunity to malaria at a much tender age, a Nigerian child achieves

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significant immunity only after 10 years (Ikpat et al, 1990). In the light of this, the high correlation between in vitro and in vivo studies in children from Calabar recorded in the present study is therefore not unexpected. Moreover, the relatively mild clinical manifestation of CRPf malaria in children from Oban, near Calabar, observed by Ekanem et al (1990), may indicate that the CRPf strain(s) is less pathogenic in nature and may not be due to the effect of individual immunity.

Except in respect of IgG-specific anti-total blood stage antibody reactivity where mean OD492 value was signifcantly higher in patients from Calabar than in Ibadan, ($P<0.05$), possibly due to the high prevalence of CRPf in Calabar, the results of the present study did not indicate any regional variation in all other immunological parameters evaluated, both in patients and in controls. If the differences in the intensity, prevalence rate and clinical manifestations of CRPf malaria observed between children in the two populations studied are due to immunological differences, then such may reside at the cellular level. The role of cellular mechanism in malaria immunity has been assessed by various workers (Weid and Long, 1988; Troye Blomberg et al, 1990). In another recent report,

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Peyron et al (1990) suggested that tumour necrosis factor, secreted by activated macrophages and lymphocytes, may be associated with the development of resistance against malaria. Further work should therefore continue to evaluate possible cellular immune differences that may exist between these two populations, Calabar and Ibadan, with different prevalence levels of CRPf malaria.

During this study, humoral immune response differences were observed, due probably to the immunological differences between the two parasite strains (CRPf and the chloroquine sensitive strains). The mean IgG3 levels were found to be higher in the CRPf-infected individuals than in those infected with the chloroquine sensitive strain of the parasite, both in Calabar and in Ibadan. However, only the difference in Calabar was statistically significant ($t=6.616$; $P<0.001$). Also, the mean IgG-specific anti-total blood-stage antibody reactivities were higher in subjects with CRPf malaria, when compared to those infected with the sensitive strain. This difference was significant in infected subjects from both Calabar ($P<0.01$) and Ibadan ($P<0.05$). IgG3 levels and total blood-stage antibody reactivities in patients from Calabar also correlated positively ($r = 0.455$; $t = 3.450$; $P<0.001$) but the correlation was not significant, in Ibadan.

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Sy et al (1990) studying the in vitro growth inhibition of P. falciparum from different regions of the Philippines observed a wide divergence in the ability of immune sera to inhibit the growth of geographically distinct parasites, and concluded that parasite variability could account for the diversity in immune response to malaria infections. Thus the observation of high IgG3 levels and IgG-specific total anti-blood stage antibody reactivities associated with CRPf infections in both populations, would appear to support the findings of Ye et al (1989), that CRPf has a membrane protein (MWt = 155 - 170 KD) which is lacking in the chloroquine sensitive parasite. The present results may therefore indicate the possibility that increased anti-total blood stage antibody or IgG3-specific malaria antibody or both, could serve as a marker for CRPf malaria infection. This possibility may thus circumvent the need for in vitro field tests for chloroquine responses of P. falciparum, a method that is cumbersome, especially in field studies. It is also arguable that a probable IgG3-specific malaria antibody associated with CRPf infection, may account for the less severe clinical manifestations of malaria observed earlier on by Ekanem et al (1990) in CRPf-infected subjects, as compared to those infected predominantly with the sensitive strain. Antibody-dependent cell-mediated reactions involving mononuclear

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cells or granulocytes are believed to be important for protection in malaria (Wahlgren et al 1986b). Such reactions are primarily mediated by IgG1 and IgG3 (Spiegelberg, 1974). Since these two sub-classes of antibodies are the only immunoglobulin isotypes capable of activating a wide range of specific and non-specific cellular mechanisms (Jefferis and Kumaratne, 1990) it is probable that CRPf infection, presumably associated with increased IgG3 production, may induce an enhanced phagocytic function thereby reducing parasite load more effectively. This may curtail the rate and amount of parasite toxins released from infected red blood cells and thereby present the mild symptomatology observed in CRPf malaria. If this is true, then it would contrast the observation of Ikpat et al (1990) that the incidence of cerebral malaria appears to increase with the emergence of CRPf malaria in Calabar. More studies are therefore needed to elucidate more clearly the clinical implications of the emerging CRPf strain(s) with regard to the occurence of cerebral malaria.

In this report, no clear-cut relationships to the development of clinical immunity were found when the blood stage antibody reactivities to the total parasite antigens were determined by ELISA. A similar observation was made by

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Wahlgren et al (1986b). Furthermore, antibody against circumsporozoite antigen did not show a clear-cut evidence of protection against P. falciparum infections. Although a number of authors believe that anti-CS antibody is associated with protection (Del-Guidice et al 1987; Esposito et al 1988; Snow et al, 1989), some others believe otherwise (Pang et al, 1988; Webster et al 1988; Burkot et al 1989). Nevertheless, the present results clearly show that the two Nigerian populations studied are adequately responsive to both anti-total blood stage antigen and circumsporozoite antigen. It is known that antibodies against the former also embody antibodies directed against the ring-infected erythrocyte surface antigen (RESA), (Deleron et al 1987) which has been found to prevent merozoite invasion (Wahlin et al 1984) and to correlate positively with clinical immunity (Wahlgren et al, 1986b). It is therefore inferred that the two populations studied are also responsive against RESA, the most putative malaria vaccine candidate (Perlmann et al 1989). This postulation is supported by the work of Williams et al, (1987) who found a significant anti-RESA antibody reactivities in the cord blood of Nigerians.

7.3. SUMMARY AND CONCLUDING REMARKS

Chloroquine resistant P. falciparum (CRPf) prevalence level as evaluated by in vitro and in vivo methods in

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Calabar was found to be 59.2% and 53.6% respectively, giving a high correlation between the two methods in a relatively less immune children population in this area. No *in vitro* schizont maturation inhibition was recorded in chloroquine concentrations less than 4 pmol per well. Probit analysis of log/dose response test showed an EC 99 (effective concentration of chloroquine at which 99% of schizont maturation is inhibited) of 4.6×10^{-6}M per litre of blood (23 pmol chloroquine per well).

On the other hand, 35.3% prevalence level of CRPf was recorded in a similar age group in Ibadan, using the *in vitro* evaluation method. Schizont maturation inhibition was observed in low chloroquine concentrations of 1 and 2 pmol per well as well. Probit regression showed a cumulative EC 99 of 2.4 x 10M/L (12 pmol/well).

A divergence of parasite densities was observed between children infected with the CRPf and those infected with the sensitive strain, the latter group harbouring a significantly higher mean parasite density than the former, in both Calabar and Ibadan ($t = 3.9000$; $P < 0.001$ and $t = 2.190$, $P < 0.05$ respectively).

Immunological analysis for the levels of IgM, IgG and its 4 subclasses, complement proteins Bf, C3, C4 and the classical pathway haemolytic activity (CH50) in serum or plasma

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samples collected from patients in these two geographical populations as well as in age/matched controls, did not show any significant variations. IgG-specific reactivities against intracellular blood stage antigen by IFA, and circumsporozoite antigen by ELISA,were also not significantly different between the two populations. However, the mean anti-total blood stage antibody reactivity was significantly higher in patients from Calabar than Ibadan. Prevalence rates of anti-blood stage antibody reactivities by ELISA and IFA were recorded in all age groups studied, both in Ibadan and Calabar. The seroreactivities also increased with age in the two populations. Anti-CS antibody did not appear early in age, in the two populations, both in patients and in controls. There were however differences in the ages at which anti-CS antibody first appeared, between the children in Calabar and those in Ibadan. Thus, anti-CS antibody did not appear in children aged between 1 and 4 years in Ibadan, unlike in the similar age group in Calabar. Nevertheless, children from both populations showed identical mean anti-CS antibody reactivities. Anti-total blood stage antibody and IgG3 were both significantly increased in CRPf-infected children in Calabar (t=3.204, P<0.01 and t=3.400 P<0.01 respectively). In Ibadan, CRPf-infected children also had increased mean

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values of these two parameters, but only the increase in anti-total blood stage antibody reactivity was significant ($t=2.046$, $P<0.05$).

The mean complement protein C4 and the classical pathway haemolytic titre (CH_{50}) were significantly reduced in the patient groups studied, as compared to the controls ($t=4.420$, $P<0.001$ and $t=7.253$, $P<0.001$ for C4 and CH_{50} respectively in Calabar; $t=8.276$; $P<0.001$ and $t=10.490$; $P<0.001$ for C4 and CH_{50} respectively in Ibadan). The mean C3 level was reduced in the two patient populations but not significantly. Bf of the alternative pathway, on the other hand, was significantly increased in the controls from Calabar and Ibadan ($t=3.636$, $P<0.001$ and $t=4.300$, $P<0.001$ respectively). There was no divergence in the profiles of these complement proteins between the CRPf-infected children and those infected with the sensitive strain(s) of the parasite, in the two populations.

It is concluded that CRPf prevalence level in Calabar is significantly high and confirms an earlier report by Ekanem et al (1990) in the neighbouring Oban community. The prevalence rate in Ibadan has rapidly increased from 7.1% in 1986 (Salako and Aderounmu 1987) to 35.3%, as revealed by the present study, although the level is

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much lower than in Calabar. These developments call for immediate control measures, in order to stem down any increases in the pathological sequalae usually associated with malaria e.g. cerebral malaria, particularly in Calabar. A suggestion is also made for further analysis of the anti-IgG-specific anti-total blood stage antibody, suspected to be of the IgG3 isotype. A confirmation of the association of increased IgG3 production with CRPf is needed, as this will contribute to the better understanding of the pathogenesis of CRPf malaria.

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APPENDIX

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Correlation between in vivo and in vitro response of chloroquine-resistant *Plasmodium falciparum* in Calabar, South-Eastern Nigeria

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Since chloroquine-resistant *Plasmodium falciparum* (CRPF) has emerged in Nigeria, we monitored the susceptibility of the parasite strain to a standard chloroquine (C25) dose in our Children's Emergency Unit. Chloroquine (CQ) is the drug of choice for malaria chemotherapy in Nigeria. The WHO 7-day in vivo evaluation and Rieckmann's microtitre technique (in vitro test) were used. 33 children of mean age 4.9 years were enrolled in the study. 27 (81.8%) of the in vitro cultures were successful. 16 (59.3%) of the successful isolates still showed schizogony at CQ concentration of 5.7 pmol/well and above. 28 (84.8%) of the children completed the in vivo study. 15 (53.6%) were parasitaemic on day 7 and/or day 14 and were regarded as parasitologic failures. The isolates from 14 of these children showed corresponding in vitro resistance of CQ concentrations equal to or above 5.7 pmol/well. The proportion of RIII (= 13.3%) appears to have increased as compared to 5.9% recorded in 1987. We conclude that there appears to be a good correlation between in vivo evaluation of parasitologic failures (53.6%) and in vitro resistance (59.3%). It thus appears that CRPF is definitely increasing in South Eastern Nigeria. This can be expected not only to complicate malaria chemotherapy in the Children's Emergency Unit of the University of Calabar Teaching Hospital, but will contribute immensely to the deterioration of malaria therapy and control in Nigeria.

Key words: Malaria resistance; Chloroquine; *Plasmodium falciparum*, chloroquine resistant

Introduction

In most developing tropical countries of the world chloroquine is the drug of choice against malaria infection, which causes high mortality in children as well as non-immune visitors and severe morbidity in people of all ages, including semi-immune adults. *Plasmodium falciparum*, the most lethal of human plasmodial species, started to become resistant to CQ in the late 1950's. However, this new strain appeared in East and Central Africa about 10–15 years later (Sanconetti et al., 1985). From there the spread presumably continued into West African Countries, including Nigeria.

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where it has probably had the greatest impact in the South-Eastern States of the country.

In Kenya, the first case of chloroquine-resistant *P. falciparum* (CRPF) malaria in Africa, was reported in a non-immune visitor (Fogh et al., 1979). However, the development was not manifested in the indigenous population until 1981. However, in 1982, a sudden development of chloroquine resistance was recorded in the indigenous infant population (Spencer et al., 1983). In 1986, a 46.5% prevalence of chloroquine resistance in malaria was recorded in indigenous Cameroonian infants, following an earlier report of a few cases in non-immune visitors (Sanconetti et al., 1985; Lebrass et al., 1986). In Ibadan, Western Nigeria, continuous surveillance showed a prevalence of only 7.1% in 1986 (Salako and Fadeke-Aderounmu, 1987). In Owerri, Eastern Nigeria, Eke reported a suspected case of CRPF in 1979, and in 1987 the Centre for Diseases Control (CDC) reported CRPF in a non-immune visitor to Enugu (Jackson et al., 1987).

The geographical distribution of CRPF has already been described (Lemngo and Inaboa, 1988; Brasseur et al., 1988). Subsequently, we carried out a malaria therapy survey in Oban, South-Eastern Nigeria, and confirmed an in vivo CRPF rate of 63.6% (Ekanem et al., 1990). In another survey, in Agbani and Jato-Aka, both in South Eastern Nigeria, an in vivo CRPF rate of 41.2% and the high resistance level RIII was found in 5.9% of cases (Ezedinachi et al., 1988). However, in a similar study in Igbo Ora, Western Nigeria, no CRPF was found (Ekanem et al., 1990). This present study is a part of the national malaria therapy surveillance scheme.

Materials and Methods

This study took place between August and October 1989, at the University of Calabar Teaching Hospital. A total of 806 children were screened for *P. falciparum*. 33 children were enrolled in the study. They were examined by physicians, and those found to be too sick to take oral medication, or to have had previous chloroquine medication (positive Dill Glazko urine test) were excluded from the study. Informed consent for participation was obtained from the parents or guardians of the children.

In vivo studies

The WHO extended 14-day follow-up in vivo test (WHO, 1973) was used. The children were treated with chloroquine (C25) syrup and/or tablets. The C25 was supplied by the National Malaria and Vector Control Division of the Federal Ministry of Health, Lagos. This C25 was pretested for potency. The children were treated with 25 mg chloroquine/kg body wt. on 3 consecutive days, and were followed up for at least 14 days. Thick and thin blood smears were stained using Giemsa and Leishman stains respectively, on the first day (day 0), and then on days 3, 7, and 14. Therapeutic response to 4-aminoquinoline was assessed using the classification of the WHO (WHO 1973).

In vitro studies

The Rieckmann microtitre technique (Rieckmann et al., 1978) was used to determine the in vitro susceptibility of *P. falciparum* isolates to chloroquine (CQ) using WHO

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test kits. After incubation, thick blood films were stained with Giemsa, and the number of schizonts per 200 asexual parasites was determined.

Isolates in which 10% of the parasites developed to schizonts in the control well were considered successful. Minimum inhibitory concentration (MIC) was defined as the lowest concentration of the drug at which no schizonts were observed. The presence of schizonts in wells with more than 5.7 pmol/well was considered to indicate CQ resistance (Timmermann et al., 1982). The concentration of CQ showing 50% (EC_{50}), 95% (EC_{95}) and 99% (EC_{99}) inhibition of schizonts was estimated by probit analysis of log dose/responses (WHO, 1982; Warsame et al., 1988).

Results

In vivo evaluation

28 (84.8%) of the children completed the study. In 13 (46.6%) of them the parasitaemia was cleared and they remained free of parasites during the 14-day observation period. They were thus classified as showing an S-response (complete cure).

In 15 (53.6%) of the children, either the day 0 parasite load was not reduced by at least 75% on day 2, or there was a resurgence of parasites on day 7 and/or 14. These were regarded as parasitological failures (Table 2). The resistance levels of these were RI (5 cases), RII (6 cases) and RIII (4 cases). (Fig. 1).

The clinical response was good except in the RIII group, where the day 2 mean temperature rose above the day 0 mean temperature. The mean temperature of the RII group also rose considerably on day 14 (Fig. 2).

In vitro test — the pattern of in vitro susceptibility of P. falciparum to chloroquine

27 (81.7%) of the 33 cultures were successful. 11 (40.7%) of the isolates were sensitive at chloroquine concentrations of 5.7 pmol per well. 16 (59.3%) of the isolates showed schizont maturation in CQ concentrations of more than 5.7 pmol per well, (6 isolates at 8 pmol/well; 5 at 16 pmol/well and 5 at 32 pmol/well) (Tables 1 and 3), and were regarded as in vitro resistant cases.

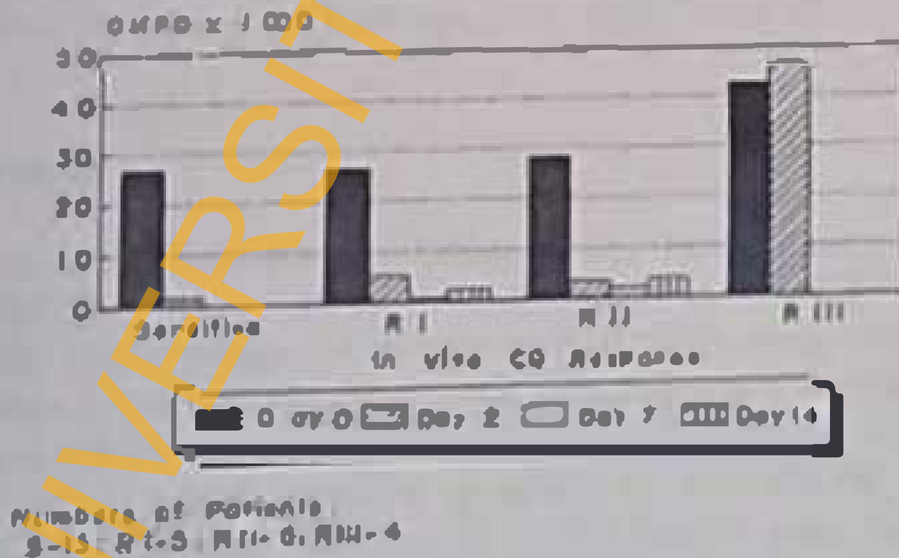

Fig. 1. Geometric mean parasite densities versus in vivo chloroquine response, by day of study, Calabar, 1989.

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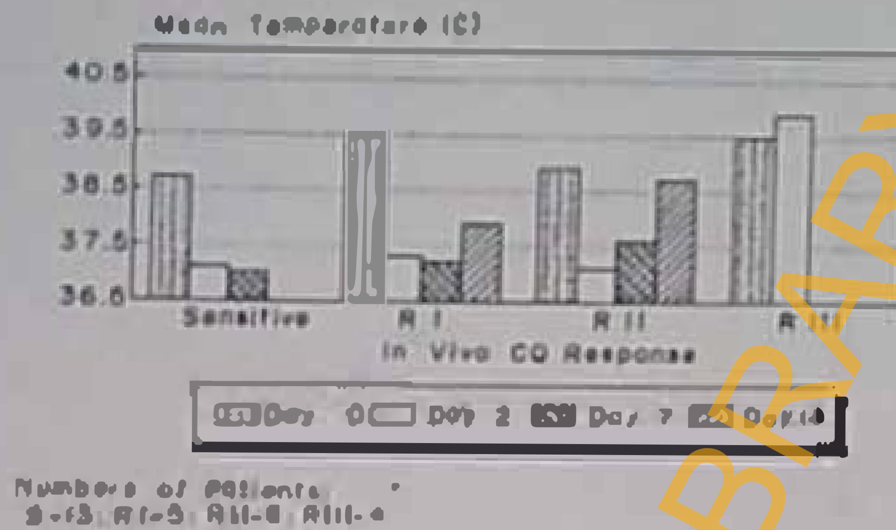

Fig. 2. Mean axillary temperatures versus in vivo chloroquine response, by day of study, Calabar, 1989.

TABLE 1

Summary of in vivo and in vitro *P. falciparum* susceptibility to chloroquine in Calabar, Nigeria

Assessment method	Total number (n)	Number successful (%)	Number sensitive to CQ (%)	Number resistant to CQ (%)
In vivo	33	28 (84.8)	13 (46.4)	15 (53.6)
In vitro	33	27 (81.8)	11 (40.7)	16 (59.3)

The cumulative EC_{99} (effective CQ concentration at which 99% of the parasite growth was inhibited) of isolates was 4.6×10^{-8} m/l (23 pmol per well) (Fig. 3).

Comparison between in vivo and in vitro chloroquine-resistant P. falciparum

In vivo: 15 (53.6%) of the children had CRPF (RI–RIII). In vitro: 16 (59.3%) of the isolates showed resistance at 8 to 32 pmol per well. 12 (80%) isolates from the 15 in vivo resistant strains were also resistant in vitro (i.e. 4, $R = 8$, 4, $R = 16$ and 4, $R \geq 32$ pmol per well) although 2 isolates from the strains showing low grade in vivo resistance, i.e. (RI strains) were just sensitive in vitro (MIC = 5.7 pmol/well). All the isolates from the strains showing high grade in vivo resistance (RII and RIII) showed in vitro resistance at $R \geq 8$ pmol per well (Table 2).

Discussion

In our earlier field study in Oban, 65 km from Calabar (where the present study was carried out), we confirmed the existence of in vivo CRPF (Ekanem et al., 1990). The present study compared in vivo and in vitro tests, and showed a good correlation between in vivo and in vitro levels of resistance. Previous studies in Africa (Teklehaimanot, 1986) reported a good correlation between in vivo and in vitro tests. In 6 out of 7 patients with parasites showing in vitro CRPF, in vivo resistance was also observed. However, Walker et al. (1983) from Ibadan, Nigeria found that CQ absorption was more variable in infected children than in non-infected volunteers. It is therefore useful to support the in vivo result with in vitro tests.

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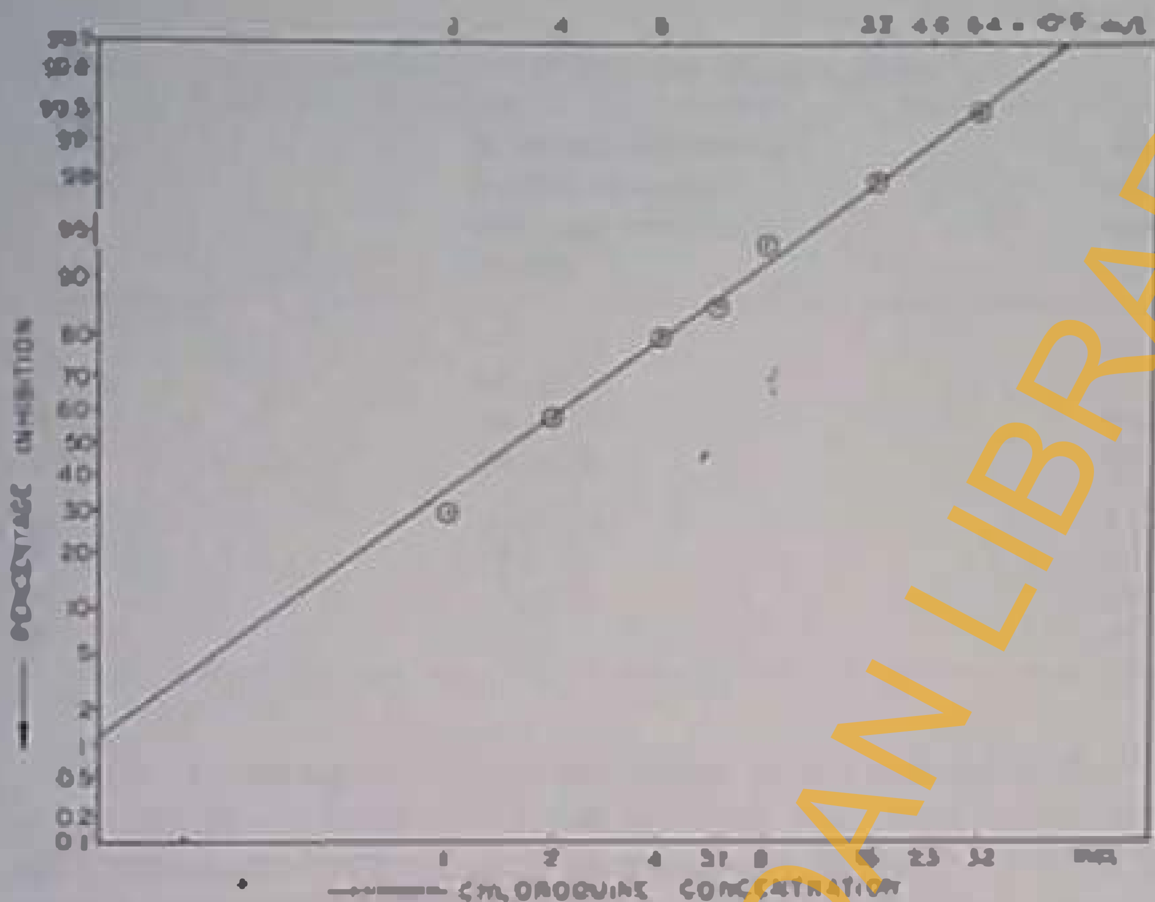

Fig. 3. Probit analysis of log dose/response.

Percentage inhibition (y axis)	30	58.2	80	86.1	94.3	98.1	99.5
Chloroquine conc. (x axis)	1	2	4	5.7	8	16	32

Cumulative EC_{50} is at 23 (pmol) ± 4.6 × 10[illegible] mol/l for the 27 successful isolates from children.

TABLE 2

In vivo chloroquine failures compared to corresponding in vitro responses

Day	In vivo failures			Corresponding in vitro responses (MIC[b])
	n	Mean age (yrs)	GMPD[a] per mm³	
2	4	2	39058	R(16) — 1 isolate R(32) — 2 isolates N/S[c] — 1 isolate
7	6	4.6	1650.6	R(8) — 1 isolate R(16) — 3 isolates R(32) — 2 isolates
14	5	4.8	717.9	R(8) — 3 isolates S(5, 7) — 2 isolates
Total	15	—	—	R — 12

[a]GMPD = mean geometric parasite density.

[b]MIC = minimum concentration of CQ in pmol/well.

[c]N/S = isolate culture, not successful.

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TABLE 3

Pattern of in vitro susceptibility of *P. falciparum* to chloroquine

CQ conc. (pmol)	% schizont maturation (arithmetic mean) compared with control (n = 27)	Isolates showing maximum inhibition of schizont maturation (n = 27)	
1	100 (27)	—	Sensitive
2	100 (27)	—	
4	74.1 (20)	7 (25.9%)	
5.7	59.3 (16)	4 (14.8%)	
8	37.0 (10)	6 (22.2%)	Resistant
16	18.5 (5)	5 (18.5%)	
32	11.1 (3)	2 (7.4%)	
32	—	3 (11.1%)	

Some interesting individual variations were observed in this study. Isolates from a five month-old baby and a five year-old child were found to show high grade in vitro resistance, but both children had a complete cure in vivo. Spencer et al. (1983) made similar observations in children aged between 6 and 24 months. The five month-old baby in this study may still have had passive immunity acquired from her mother, which played a modifying role in the immune response. Similarly, two children did not respond to C25 in vivo, but the isolates from them were sensitive in vitro. Incidentally, these two children are siblings. We did not detect any gastro-intestinal disturbances in these children during the study which might have explained a possible deficiency in CQ absorption in them (Walker et al., 1983).

In 1986/87 clinicians in Calabar had begun to experience increasing difficulties with malaria chemotherapy using C25, which led us to carry out the field study which showed an in vivo CRPF rate of 63%. (Ekanem et al., 1990). The present study, showing in vivo and in vitro CRPF levels of 53.6% and 59.3% respectively, 13.3% RIII, as well as a high cumulative EC_{99} of 4.6×10^{-6} M (23 pmol/well) (Fig. 3), demonstrates a high degree of resistance and appears to explain the difficulties in malaria treatment with CQ in Calabar. Although environmental and host genetic factors may play a role in the variation of *P. falciparum* susceptibility to CQ, other factors like specific and/or non-specific immunologic reactions may also play a role. A study designed to assess the nature of the immune responses elicited against the emerging Nigerian strains of CRPF in Nigerians is desirable. Such a study may help to explain further the apparently worsening malaria situation in Nigeria, especially in the South-East.

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