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For Dr K. Saakwa-Mante.

With many thanks for all your help,

and best wishes

from

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IMMUNOLOGICAL INVESTIGATION OF MALARIA IN GHANA

VOLUME I IMMUNOEPIDEMIOLOGY OF MALARIA IN GHANA

VOLUME II IMMUNOPATHOLOGICAL SEQUELAE OF MALARIA

by

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VOLUME I

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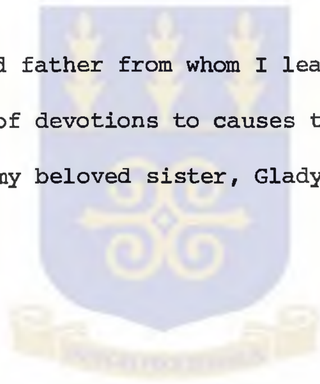

Dedicated to my mother and father from whom I learnt that life worth living is a series of devotions to causes that one believes in, and to the memory of my beloved sister, Gladys Alice Ama Kawkyewa.

"No man is an Island, entire of Itself; every man is a piece of the Continent, a part of the main; if a clod be washed away by the sea, Europe is the less, as well as if a promontory were, as well as if a manor of thy friends or of thine own were; any man's death diminishes me, because I am involved in Mankind; And therefore never send to know for whome the bell tolls; It tolls for thee."

JOHN DONNE - MEDITATION XVII

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ABSTRACT

A comprehensive immunological survey of malaria in rural and urban populations in Ghana is reported. Sera from Ghanaians resident in Ghana and Ghanaian students resident in the United Kingdom and Germany, pregnant women, blood donors and from patients with acute malaria, nephrotic syndrome, tropical splenomegaly, Burkitt's lymphoma, schistosomiasis, onchocerciasis and tuberculosis were included. Control sera were obtained from healthy adult Caucasians, Caucasians with connective tissue and parasitic diseases other than malaria, non-immune Caucasians with primary malaria infections and Nigerians. Serum from monkeys (*Macaca mulatta*) infected with *P.knowlesi* were also tested.

Infecting parasites were identified by examination of blood slides. Malarial antibodies were detected by enzyme immunoassay (ELISA) and immuno-fluorescence (IFA). Antibodies to *M.tuberculosis*, *V.cholerae*, *T.pallidum*, *E.histolytica*, *T.gondii*, *T.gambiense*, *T.rhodesiense*, *S.mansoni* and *T.canis* were determined by a 'rapid' IFA test. Onchocercal antibodies were determined by ELISA using *O.gutturosa* antigen. Antibodies to Epstein Barr Viral Capsid Antigen (VCA) and hepatitis B surface antigen (HBsAg) were measured by IFA and passive haemagglutination inhibition respectively. Serum levels of IgG, IgA, IgM, IgE, albumin, complement C3, C3 converted products, immune complexes and autoimmune antibodies were measured. All data were analysed by computer.

The value of ELISA in the diagnosis and epidemiology of malarial infection was demonstrated in this study and the results showed good correlation with those obtained by immunofluorescence. Both tests were employed in the studies of malaria in rural and urban populations. The analysis of

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parasitological and serological indices obtained for the 2 populations confirmed the endemicity of malaria in Ghana: Plasmodium falciparum was the predominant species; prevalence rates were higher in the rural than the urban population; antibody levels in both populations showed an age-related increase. The overall sero-epidemiological findings thus conformed to the classical concept of the acquisition of malarial immunity and suggested that a stable state of malaria prevails in Ghana.

This survey revealed a high incidence of autoantibodies, raised levels of soluble immune complexes and evidence of in vivo complement activation in malaria.

It has been suggested that in malaria, antigen-antibody complexes stimulate immune effector mechanisms. Products of these might interfere with the development of protective immunity and result in the initiation of immune complex diseases and its perpetuation.

The findings reported would support this hypothesis.

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Dr. G.D. Johnson introduced me to the specialized field of autoimmune serology, his patient tuition, and carefully orchestrated intellectual challenges have inspired another novice. I can only build on to this basic grounding, and I am deeply grateful.

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OBJECT OF STUDY

Malaria is endemic in Ghana, and is one of the principle causes of infant morbidity and mortality. It exacts a toll on human life and health grave enough to constitute a threat to economic and social development.

Populations, especially rural communities appear to be indifferent to diseases which are chronic and have been in the community for generations, but one cannot be complacent about malaria because it inflicts severe and widespread battering on the human body, disturbing many of the body's systems. It bombards the reticuloendothelial system, obstructs capillaries and consequently tissues are rendered anoxic; complement is activated, immune complexes are formed and deposited often with grave immunological disturbances, leading to a highly variable and complex clinical picture.

Resistance to malaria does develop under natural conditions but only after repeated natural infection over a period of years, and, even then, immunity may not be absolute in that parasitaemia continues in the absence of clinical symptoms. Resistance is species specific, and often modified by physiological, biochemical and environmental factors.

The diagnosis of malaria is normally by direct identification of the parasite by microscopy. This requires a certain expertise if it is to be adequate, it is tedious, and time consuming. Malaria elicits specific antibody responses detectable by serological techniques and these can be used in immunodiagnostic procedures to support clinical observations.

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Antibody presence does not necessarily indicate immunity, although functional immunity has been shown to be mainly related to the humoral responses. The measurement of these responses has become very important in recent years. In spite of much effort and a sizeable literature, there is still no simple, reproducible, and meaningful test of functional immunity in malaria. Nevertheless the study of malaria antibodies in a population can be used for seroepidemiological purposes.

This study sets out to review existing malarial immunodiagnostic tests, and evaluates the enzyme linked immunosorbent assay (ELISA) for sensitivity and specificity and as an immunodiagnostic and sero-epidemiological tool for use in malaria.

Recent advances made in malaria, at the cellular and immunological levels have been impressive. The following manifestations of immuno-pathology have been described, Nephropathies, Tropical Splenomegaly Syndrome, Cerebral Malaria, Anaemia, Autoimmunity and Immunosuppression. This study hopes to contribute to this new information on malaria, by the practical application of immunodiagnostic techniques to study the immune responses, both beneficial and adverse that malaria induces in man and their clinical and epidemiological significance. The population of Ghana in this respect lends itself as a natural experimental model.

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SECTION I : INTRODUCTION

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CHAPTER ONE

MALARIA - A GENERAL REVIEW

Malaria is a disease transmitted by the bite of anopheline mosquitoes and caused by protozoa of the genus plasmodium. There is some variation in malaria produced by the different plasmodia, but in all, the hallmark of the disease is the febrile paroxysm, preceded by shivering and followed by sweating often occurring at regular intervals. The disease, when acute, is accompanied by headaches and muscle pain; often severe. When it becomes chronic, splenomegaly and anaemia are common features (Wilcock's and Manson-Barr, 1972).

The genus plasmodium includes malaria of man and animals. Four species are known to infect man under natural conditions. These are:

- Plasmodium malariae (Leveran, 1881)
- Plasmodium vivax (Grassi and Feletti, 1891)
- Plasmodium falciparum (Welch, 1897)
- Plasmodium ovale (Stephens, 1922)

Plasmodium malariae is thought to be the oldest of the human species and must have been harboured by the common ancestor of the chimpanzee and man since it is present in both today. Plasmodium ovale and Plasmodium vivax evolved during the oligocene. Plasmodium falciparum on the other hand is specific to man and its virulence leads one to speculate on its more recent acquisition.

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A. IMMUNITY TO MALARIA

There is a well documented body of evidence that malarial infections stimulate immune responses both humoral and cellular which act specifically or non-specifically to limit or blunt the multiplication of parasites (Brown, 1969; 1971; and Brown, 1976). However the immunity that results is essentially resistance to superinfection, and depends on continued existence of the parasites within the host to maintain it. It takes years to achieve any measure of protective immunity and this develops only under natural conditions where malaria transmission is intense and where drug usage is infrequent. Immunity is thus manifested by diminishing parasite densities and absence of clinical symptoms (McGregor, 1965). Brown (1969) and Brown (1976) examined the general features of immunity to malaria, essential components being macrophages, humoral and cellular responses.

MACROPHAGES Macrophages of the lymphoreticulo endothelial system play a central role in control of malaria infections. Taliaferro (1941) recorded that these cells were responsible for phagocytosis, production of new phagocytes, and synthesis of antibodies which act specifically on the parasite to eliminate and limit parasitaemia. Phagocytosis in malaria is initially non-specific, nevertheless it has a protective effect (Taliaferro and Cannon, 1936; Taliaferro and Mulligan, 1937). This non-specific macrophage activation has been shown to be a component of a number of bacterial and protozoal infections (Jordan and Merigan, 1975). Clark et al (1975) reported that BCG is particularly good at stimulating this response, and the same has been reported for Corynebacterium parvum (Nussenzweig et al, 1967). Several workers show evidence that these

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activated macrophages may split the third component of complement (C3) into products that increase the red cell permeability to other toxic factors such as endotoxin and interferon (McGregor, 1972; Mauel, 1977; Allison and Clark, 1977; Clark, 1978). The early malariologists (Taliaferro and Cannon, 1936; Taliaferro and Mulligan, 1937; Garnham, 1938) noted that phagocytosis of parasites in the non-immune was at first sluggish, but increased markedly as immunity was acquired. Brown (1971) described further studies which indicate that the enhanced phagocytosis in malaria is probably opsonin mediated. It has also been shown that splenic macrophages always appeared more active than those of liver or bone marrow. Current immunological thought also accords macrophages a prominent role in the establishment of parasite immunity (Allison and Clark, 1977; Schorlemmer et al, 1976; Allison, 1978). Its biological properties make it eminently suitable for interacting with B and T lymphocytes and their products.

HUMORAL IMMUNITY Humoral immune responses to malaria infections are now well established. B-lymphocytes respond to stimulation by malarial antigens, by synthesizing increased amounts of immunoglobulins G, A and M (Tobie et al, 1966). These increases are observed soon after parasitaemia becomes patent and not before. The increase is greatest in IgG. Specific malarial antibody activity has been detected in the IgG, IgA and IgM fractions, Collins et al (1971) found that IgA and IgM were transient but IgG persisted longest. In malarious endemic areas IgG and IgM are especially elevated, and frequently serum IgM levels and malaria antibodies show strong correlation (Rowe et al, 1968; Targett, 1970; Voller et al, 1971b; Cornille Brogger et al, 1978). Greatly enhanced daily rates of immunoglobulin synthesis were also observed in

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malarial infections (Cohen and McGregor, 1963). However absorption studies showed that large proportions of the immunoglobulins are without specific malarial antibody function (Curtain et al, 1964; Cohen and Butcher, 1969). McGregor (1968) suggested that these absorption studies only utilized restricted range of parasite antigens and therefore difficult to ascertain the absolute proportion of immunoglobulins with total malaria activity.

Information on how antigen stimulates B-lymphocytes, what determines the immunoglobulin class of antibodies, and which B-lymphocytes synthesize in response to malarial antigen, is lacking. Age-related immunocompetence of host, and previous malarial experience may all be important factors. Voller (1974) noted that in general immunoglobulins and malarial antibody levels tend to increase with age in malarious areas. Non-immune adults have also been shown to develop extremely high levels of immunoglobulin and malarial antibodies after a single short initial infection. Voller (1974) also discussed the greater susceptibility of younger animals to malaria than the older members of the same species. McGregor et al (1970) showed good correlation between parasitaemia and IgM in children and parasitaemia with IgG in adults and suggested that IgM may have special significance in early life.

The type of antibodies and immunoglobulin class stimulated may also depend on the physicochemical nature of malarial antigens. In nature particulate antigens are known to stimulate predominantly IgM responses, and IgG appears in response to soluble antigen and toxins (Hobbs, 1970). The same may apply for malaria. In man, 30 distinct antigens have been identified in association with asexual erythrocytic stages of *P.falciparum*.

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They have been classified into three main groups L (labile), R (resistant) and S (stable) according to their ability to withstand heating, and considerable physicochemical differences have been observed (Wilson et al, 1969). Not all these antigens are plasmodial. Some are products of parasite metabolism, others represent altered components of host tissue but all are capable of evoking an antibody response in the human host.

The fact that all antigens currently detected in malarial infections are associated with the asexual erythrocytic stage, confirms the observation made by several workers that acquired immunity to malaria seems to be stimulated primarily by erythrocytic schizonts and liberated merozoites (Cohen and McGregor, 1963; McGregor, 1965), and is not effective against other stages of the parasite's life cycle. Garnham and Bray (1965) found that, in Simian malaria, the immunity was not effective against sporozoites, erythrocytic forms or the first generation of erythrocytic schizogony. Russel et al (1941) showed that antibodies to inactivated sporozoites did not extend to trophozoite induced infection. This lack of sporozoite acquired immunity may in part be due to the short exposure to immune competent cells and not due to lack of immunogenicity of sporozoite antigens (Fairley, 1947; Nussenzweig et al, 1972). Nardin et al (1978) on the contrary have reported high anti-sporozoite antibodies in Gambian adults and less in children. The implication of this observation will be fully worked out in time.

Immunity to the exoerythrocytic stage is not known. Brown (1976) suggest that although exoerythrocytic schizonts and their merozoites cause a considerable cellular disorganisation in the liver, it is possible that once in the parenchymal cells they are secure from immune attack. Even

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less is known of protective responses to gametocytes. Beck et al (1970) have shown that red cells containing gametocytes are antigenically different from red cells containing asexual parasites. It is possible that in chronic malarial infections, stage specific immunity operates, but its detection awaits the discovery of more sensitive and specific immunological test systems.

Thus it is agreed that a majority of the antibodies produced are in response to the asexual erythrocytic stage, and to their soluble and related products. These antibodies can be detected by the use of various serological techniques, resulting from the precipitation of soluble antigen, fluorescence, agglutination, and opsonisation of parasitized cells. Some of these antibodies afford protection and some are harmful. The presence of protective circulating antibodies was first shown by Sotiriades (1917). The classic demonstration by Cohen et al (1961) of passive transfer of large amounts of IgG from Gambian immune adults giving considerable reduction of parasitaemia in heavily infected Gambian children was firm proof. Further affirmation was provided by Edozien et al (1962), Cohen and McGregor (1963) and Briggs et al (1966). Rogers (1974) has shown good correlation between opsonising antibodies and resistance to infection. Cohen et al (1972) and Mitchell et al (1975) have demonstrated the presence of antimerozoite antibody which prevent red cell invasion by merozoites. The protective IgG observed by Cohen et al (1972) can be transmitted transplacentally and is responsible in part for the raised immunoglobulin levels and relative insusceptibility to infection of newborn babies in malarial endemic areas (Edozien et al, 1962; Cohen and McGregor, 1963; Briggs et al, 1966).

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The protection afforded by malaria antibodies is not complete since there is persistence of chronic infection after life long exposure, with periodic recrudescence and or relapses. This state of premonition in human malaria was studied by Bagster Wilson and Wilson (1937). Bray (1962) and Bruce-Chwatt (1963) reported that inoculation of individuals living in endemic aresa of P.falciparum could result in patent parasitaemia and clinical symptoms. Marchoux (1926) clearly stated the problem of relapses, that malaria is sometimes reborn after an apparent cure when the patient has quite forgotten his past miseries. He postulated three themes to explain these relapses. The first was the parthenogenesis of macro-gametocytes; this has been disproved with better understanding of the parasite's life cycle. The second, persistence of a blood infection was further advanced by Ross and Thomson (1910) and Corradetti (1965) still adheres to it. Marchoux's third theory, reactivation of an encysted form may correlate with the longevity of P.vivax and P.ovale in the liver as evidenced by secondary and tertiary exoerythrocytic schizogomy after years of primary infection. However there is as yet no evidence for exoerythro-cytic relapses in P.falciparum and P.malariae (Garnham, 1977). Marchoux's third theory of reactivated encysted forms responsible for true relapses in malaria is currently gaining favour (Markus, 1978; Krotoski et al, 1980). The encysted parasitic form has been ascribed the name 'hypnozoite' (Markus, 1978) and this term describes any dormant sporozoites or dormant sporozoite-like stages in the life cycles of plasmodium or other Haemosporidae. Krotoski et al (1980) provide proof of dormancy in late infections of P.cynomolgi in the liver of monkeys. These have yet to be demonstrated in human malaria. The persistence of P.falciparum infection

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after 3 years and P.malariae after 50 years (Garnham, 1977) is probably usually due to persistence, in low numbers of the parasite in the bloodstream. Plasmodium falciparum and P.malariae can thus cause chronic infections which apparently remain asymptomatic for years. When immunity wanes, parasites multiply profusely and a recrudescence takes place (Garnham, 1977). Garnham verifies the above by his observation of low grade parasitaemic phenomena in monkey with P.inui infection (a quartan type malaria) for 7 years. However Brown and Brown (1965) and Brown et al (1968) consider that the relapses and recrudescences in malarial infection can be attributed to parasitic antigenic variation promoted by malarial antibodies. However an understanding of the intimate interaction between antigen and immunocompetent cells and their related functions is a prerequisite to the understanding of antigenic variation, relapses, recrudescence and protective immunity.

CELLULAR IMMUNITY Whether or not T-lymphocytes play a role in man's immune response to malaria is unclear (McGregor, 1974). There is however plenty of evidence from experimental models that T cells respond to parasite antigens in malaria and in mice they are essential for recovery from malaria (Brown et al, 1968; Brown et al, 1970; Phillips et al, 1970; Clark and Allison, 1974; Playfair, 1978). Results of studies on T cell responses in man are conflicting. Blast transformation of peripheral lymphocytes from individuals recovered from malaria infection incubated in the presence of antigen has been reported (Kass et al, 1971; Wyler and Oppenheim, 1974). Osunkoya et al (1972) reported blastoid transformation in unstimulated cultures of peripheral leucocytes from P.falciparum infected children. This observation has not been confirmed (McGregor, 1974). Depression of T cell function in acute

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malaria has been reported by Osunkoya et al (1972). This was not confirmed by Greenwood (1972) and Greenwood et al (1977). Phillips et al (1970) show the presence of delayed hypersensitivity as measured by skin testing. Depletion of circulatory T and B lymphocytes has been recorded (Wyler, 1976; Ade-Serrano and Osunkoya, 1977). But Greenwood et al (1977) point out that the depletion of T and B lymphocytes without evidence of T cell suppression may be a mark of adaptation of cells rather than immunosuppression. Greenwood et al (1977) also found increases in K (killer) cell activity in peripheral blood of acute malaria patients. There is no documented evidence for direct cytotoxic activity of immune lymphocytes against parasites or parasite infected red cells. In experimental situations the responses are much more clearly defined and more specific. Clark and Allsion (1974) noted a progressive and sustained infection with P.yoelii in congenitally thymus-dependent nude mice. Striking T lymphocyte proliferation in spleen has been observed in P.yoelii infections (Jayawadina et al, 1975). Enhanced lymphocyte transformation and inhibition of macrophage migration with spleen cells from P.berghei infected rats has also been reported (Greenwood et al, 1971c). Passive transfer of immunity in mice is more effective with cells than serum (Playfair, 1978).

From all the above one can only speculate as to the role of T cells on humoral and cell-mediated responses. Malaria parasites present a complex and constantly changing antigenic stimulus, therefore it is unlikely that immune mechanisms either humoral or cellular operate independently. More importantly standardized methods of evaluating T cell functions are required before their role in malaria can be fully appreciated.

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B. PATHOGENESIS AND PATHOLOGY OF MALARIA

Malaria infection leads to invasion, alteration and destruction of red cells. They effect systemic and local circulatory changes, and immune phenomena which are all important in the pathophysiology of the disease. Malaria species differ significantly in their ability to invade red cells, P.vivax and P.ovale which cause benign tertian malaria attack only immature erythrocytes. Plasmodium malariae cause quartan malaria and infect only senescent red blood cells. Thus during infection with these species no more than one to two percent of all cells are infected at any one time. Plasmodium falciparum, malignant tertian malaria, invades red cells of any age resulting in extremely high levels of parasitaemia. The pathology of malaria therefore can be said to be essentially that of P.falciparum infections.

The red blood cells once parasitized are destroyed at the time of sporulation or in the presence of specific opsonising antibody. They are phagocytosed in the liver or spleen. Anaemia usually develops and may be severe especially in P.falciparum. This species also induces physical changes in parasitized cells resulting in intravascular agglutination and stasis. Paroxysms of fever coincide with sporulation and the destruction of red cells, but the actual cause of the fever remains obscure. Serum factors present during malaria infection may act synergistically with lactic acid and interfere with cell activity leading to cell damage. Pyrogens released from these damaged cells may be responsible for the fevers (Maegraith, 1966; Wilcocks and Manson-Barr, 1972; Woodruff, 1978).

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Acute malaria causes widespread deleterious effects in all the major organs. The immune system is no exception and the immunopathological effects of malaria have stimulated wide interest and research.

C. IMMUNOPATHOLOGY OF MALARIA

Various immunopathological mechanisms (humoral and cellular) have been implicated in the wide range of pathologies associated with malaria. However more progress has been made in the understanding of the humoral components of these mechanisms. The roles of parasite antigens, antigenic variation, immune complexes and activation of complement involved in these mechanisms have been reviewed (Brown, 1974; Wilson, 1974; Houba, 1975; WHO, 1975). The following are some of the immunopathological mechanisms described: Anaemia, Nephropathies, Tropical Splenomegaly Syndrome, Autoimmunity and Immunosuppression.

ANAEMIA Malarial anaemia is the result of an excessive destruction of red blood cells from several causes, the primary cause being red blood cell destruction when parasite schizogony occurs. Normal erythrocyte shape is biconcave and it is thought that changes in the shape of the erythrocyte induced by malarial infection will enhance the process of erythrophagocytosis (Conrad, 1967). However anaemia, more profound than can be explained by erythrocytic destruction by parasites or by erythrocytophagocytosis has been frequently seen in human and animal malaria (Zuckerman, 1964; Brown, 1969). Zuckerman (1964) suggested that such anaemia in excess of parasitaemia could not be explained by erythrophagocytosis alone, but possibly an autoimmune haemolysis or opsonization of uninfected red cells may be implicated. Greenwood et al (1978) have found no evidence for a haemolytic mechanism and they support the erythrophagocytic mechanism, but other reports would tend to support Zuckerman's (1964) suggestion (Topley et al, 1973; Facer et al, 1979; Woodruff et al, 1979).

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A positive Coombs test reaction in acute malaria suggesting possible autoimmune mechanism has been demonstrated (Zoutendyke and Gear, 1951; Demirag and Sozer, 1956; Barrett-Conor, 1967; Adner et al, 1968; Gilles et al, 1969; Topley et al, 1973; Facer et al, 1979). However negative Coombs reaction has been reported in other studies (Pirofsky, 1969; Rosenberg et al, 1973; Coombs, 1976; Ree, 1976; Greenwood et al, 1978). This lack of correlation between the two groups of workers may be attributed to the use of different antiglobulin reagents and lack of intra-laboratory standardization of immunological methodologies.

Antibodies to erythrocytic components have been shown to occur with considerable frequency in sera from persons and animals exposed to repeated malaria infection (Mayer and Heidelberger, 1946; Adeniyi-Jones, 1967; Kano et al, 1968). However none of these antibodies has yet been shown to be the cause of anaemia.

The effects of malaria on the complement system in both human and animal malaria has been widely studied. Depression of serum complement components, the increase of total complement activity and the location of complement on uninfected red cell surfaces has been noted (Cathiore, 1910; Vincent, 1910; Dulaney, 1948; Fogel et al, 1966; Cooper and Fogel, 1966; Rosenberg et al, 1973; Greenwood and Brueton, 1974;Ree, 1976; Krettli, 1976; Petchclai et al, 1977; Facer et al, 1979; Woodruff et al, 1979). Results from these studies confirm the role of complement mediated immune processes in the sequestration and subsequent destruction of red cells in malaria. Whether these processes are mediated through the classical or the alternative pathway requires further study.

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NEPHROPATHIES The complications of malaria involving the kidney are massive haemolysis, giving rise to haemoglobinuria and in the most severe cases renal failure; or proteinuria associated with acute and chronic infections which lead to glomerulonephritis with permanent sequelae (British Medical Journal, 1976). Immune mechanisms have been implicated in these nephropathies, and the two types of lesions with probable immunological origin are acute transient nephritis and chronic malaria nephrotic syndrome (Voller, 1974).

Transient nephritis has been reported in P.falciparum infections in man and Aotus monkey (Berger, 1967; Bhamarapravati et al, 1973; Voller, 1974; Powell and Meadow, 1971). It also occurs in models of acute malaria infections such as P.cyanomolgi (Ward and Conran, 1966) and also in rodent malaria (Ehrich and Voller, 1972; Boonpuncknavig et al, 1972; 1973). Clinical symptoms of acute glomerulonephritis within 3 weeks of infection together with response to antimalarial therapy and the disappearance of renal abnormalities in a wide majority of cases are the characteristic features of this disease. It is thought however, that transient nephropathies could progress to chronic malaria nephrotic syndrome (Voller, 1974).

The chronic malarial nephrotic syndrome is the best example of malaria immunopathology, and there is convincing evidence of an association between P.malariae infection and a nephrotic syndrome in Africans (Giglioli, 1930; 1962; Gilles and Hendrickse, 1963; Kibukamusoke et al, 1967). This association has been confirmed by experiments in Aotus monkeys with P.malariae and P.brasilianum infections (Voller et al, 1971a; Voller et al, 1973; Voller, 1974; Houba et al, 1976). This high incidence of P.malariae infection in association with chronic nephrotic syndrome

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has been interpreted as a causal relationship between the infection and nephrosis hence the well known classification of the condition as the 'Quartan malaria nephrotic syndrome.' The characteristic features are those of nephrotic syndrome, but do not respond to antimalarials and in those patients with poorly selective proteinuria, corticosteroids and immunosuppressive drugs are of little use (Adeniyi et al, 1970; Hendrickse et al, 1972; Houba, 1975). Dixon et al (1961) first studied the lesions of nephrotic syndrome with associated quartan malaria in Nigerian children. They found that histologically the lesions were compatible with immune-complex type lesions described in serum sickness (heavy deposits of immunoglobulin and complement alongside glomerular vessels). The suggestion was then made by Hendrickse and Gilles (1963) that the nephrotic syndrome might be due to glomerular damage caused by the immune-complex deposition. There is now much evidence to suggest this point of view (Ward and Conran, 1966; 1969, Berger et al, 1967; Allison et al, 1969; Edington and Gilles, 1969; Ward and Kibukamusoke, 1969; Houba et al, 1971; Voller et al, 1971a; Boonpucknavig, 1972; 1973; Bhamarapravati et al, 1973; Voller et al, 1973; Houba et al, 1973; Houba and Lambert, 1974; Lambert and Houba, 1974; Voller, 1974; Houba, 1975; Allison and Houba, 1976). Houba et al (1971) described the immunological aspects of the syndrome in great detail and they showed that the coarse granular deposit of immunoglobulin (from fluorescence and electronmicroscopy studies) usually consisted of IgG, IgM and complement; these often showed some response to treatment with corticosteroids, but those deposits of a more diffuse character usually contained IgG alone and may even be more progressive with no response to treatment. Another useful contribution to the quartan malaria nephrotic syndrome story was the

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demonstration of P.malariae antigen in renal lesions in 25% of cases (Houba and Lambert, 1974), and also eluates from kidneys of these patients confirmed the presence of specific antibodies to P.malariae in most cases (WHO, 1975).

All the above findings do help to strengthen the association between P.malariae and chronic nephrotic syndrome, but do not really confirm a causal relationship. It rather raises some relevant questions: Why should P.malariae and not P.falciparum be the causative agent of these lesions since P.falciparum is most prevalent in all the areas where malarial nephrosis do occur? How do the lesions start? What predisposing factors are important in the establishment and chronicity of these lesions?

Why P.malariae seems to be implicated in the chronic progressive lesions and not P.falciparum has yet to be answered. It has been suggested that the nephrotic patient may be more susceptible to P.malariae and not vice versa (Allison and Houba, 1976). The possibility that renal lesions are due to localised reactions between parasite products, antibodies,and complement, in renal capillaries has been suggested from simian and rodent experiments (Ward and Conran, 1966; Boonpucknavig et al, 1972; 1973). An inherent ability to produce aberrant reactions to P.malariae may be implicated. The production of low affinity antibody which is more likely to lead to immune complex formation has also been suggested (Soothill and Steward, 1971;Steward and Voller, 1973).

An autoimmune mechanism for the perpetuation of the lesions has been suggested with a possible switch from an exogenous antigen which initiates the lesion and subsequent perpetuation by endogenous antigen (Houba, 1977). More studies are required to confirm the factors implicated in such a mechanism.

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TROPICAL SPLENOMEGALY SYNDROME Tropical splenomegaly syndrome (TSS) has been considered a sequal to malaria on clinical and epidemiological grounds. That malaria antibody titres are often greatly enhanced and patients respond to prolonged anti-malaria therapy are well documented features (Sagoe, 1970; McGregor, 1972). TSS has been reported in several African countries and is often referred to as 'big spleen disease' (Mustafa, 1965; Lowenthal et al, 1966; Edington, 1967; Watson-Williams and Allan, 1968; Sagoe, 1970; Stuiver et al, 1971), and also reported in New Guinea (Pryor, 1967; Crane et al, 1971; Crane and Pryor, 1971; Crane et al, 1973; Crane, 1974). The condition is characterised by persistent splenomegaly associated with anaemia, lymphocytosis in the peripheral blood and bone marrow, hepatic sinusoidal lymphocytosis and raised serum levels of IgM (Pitney, 1968). Abnormal immune reactions in malaria has been regarded as an aetiological factor.

Parasitaemia is not usual, although small numbers may be detected. Marsden et al (1965) recorded a higher than normal rate of P.malariae infections in TSS patients in Uganda, but studies in New Guinea (Marsden et al, 1967; Crane and Pryor, 1971) and Vietnam (Butler et al, 1973) have not confirmed this observation, rather these studies reveal that TSS parasite rates for all malaria species are not significantly different from controls of the same age group. Epidemiologically, TSS is more common in New Guinea than Africa, and with P.vivax as the predominant malaria species in New Guinea, a causal relationship might be sought, but this has not been confirmed (Crane, 1974).

Serologically, subjects with TSS show an aberrant, although effective, immune response to malaria. This is reflected in the highly elevated levels of circulating IgG, IgG antibody titres and IgG synthesis rate, and it seems likely that the IgG antibodies are effective at eliminating parasites (British Medical Journal, 1969; Crane et al, 1971; Crane et al, 1974;

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Malfunction of T cells, or T-B cell co-operation is another favoured hypothesis, although peripheral T and B cell function in TSS has not been shown to be impaired (Mustafa, 1965; Sagoe, 1970; Ziegler et al, 1969; Crane, 1974). However, Fakunle et al (1978) reported alterations in proportions and numbers of a lymphocyte subpopulation which reverts back to normal after anti-malarial treatment. The role of immune complexes in the pathological sequelae is much favoured, especially as cryoprecipitins in TSS show closest relationship with spleen size and elevated serum IgM (Houba, 1977). Also, detection of IgM in Kupffer cells of TSS patients, but not in matched controls (Wells, 1970; Ziegler and Stuiver, 1972) correlates with the presence of cryoproteins.

The balance of evidence, however, favours a genetic basis for the development of the syndrome, that TSS results from the production of circulating immune complexes in individuals genetically predisposed to produce an IgM antibody response. However, the role of individual immunogenetic difference in the outcome of malaria infection as reported by Piazza (1972) should not be overlooked.

Malaria as the initiating factor of the syndrome remains undisputed and the substantiated evidence of improvement of TSS after prolonged anti-malarial therapy, and the significant reductions seen in serum IgM and autoantibodies confirms the above. However, further work is required to confirm the role of immune complexes and autoimmune phenomena in TSS.

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AUTOANTIBODIES AND AUTOIMMUNITY IN MALARIA Autoimmune antibodies are used as markers of autoimmune diseases. A wide range of autoantibodies has been found in association with high titres of malaria antibodies and with high serum levels of IgM in African sera from endemic malarious areas. These include heterophile IgM antibodies (Adeniyi-Jones, 1967; Kano et al, 1968; Greenwood,1970; Faulk and Houba, 1973; 1974), and also in sera of animals with experimental malaria (Zuckerman,1945; Barnett et al, 1970; Meuwissen et al, 1972), rheumatoid factor (Houba and Allison, 1966; Greenwood et al, 1971b). Similarly anti-nuclear factor (ANF) (Greenwood et al, 1970a; Voller et al, 1972). Shaper et al (1968) also reported high prevalence of autoantibodies to heart, thyroid and gastric parietal cell in sera from Africans with malaria. The mode of production of these antibodies is not clear; their association with malaria antibody and serum IgM needs to be clarified, and their importance in the immunopathology of malaria assessed.

Wells (1970) suggested that the presence of heterophile antibodies is due to the enhanced immune response to parasitised red cells which are altered due to their parasitisation or because they are affected by antibody. That rheumatoid factors detected in malaria are a result of altered immunoglobulin rendered antigenic in the chronic stimulation of polyclonal immunocompetent 'B' cells has been discussed (Crane, 1977). And the possibility that ANF, common in malarious areas especially in adults, may represent a cross-reacting antibody induced by malarial nuclear material (Voller et al, 1972).

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All the autoimmune markers mentioned above are usually associated with connective tissue damage. Thus, their high prevalence in African sera would lead to the expectation of an equally high incidence of autoimmune diseases. In fact, diseases associated with autoimmunity, i.e. systemic lupus erythematosus, scleroderma, rheumatoid arthritis, primary thyrotoxicosis, ulcerative colitis, and myasthenia gravis, although shown to occur in Africans (Goodall, 1956; Shaper and Shaper, 1958; Lauckner et al, 1961; Shaper, 1961; Patel, 1962; Davy and Ogunlesi, 1963; Michaux and Sonnet, 1965; Billinghurst and Welchman, 1966; Greenwood and Larbi, 1966; Greenwood and Francis, 1967) are uncommon in tropical Africa (Greenwood, 1968; Muller et al, 1972). However, the fact that they do occur in Africans suggests that racial or ethnic differences cannot be implicated in autoimmunity, but the correlation of high titres of malarial antibodies with autoantibodies (Shaper et al, 1968) and the effect of anti-malarial therapy in the subsequent reduction of the autoantibodies in tropical splenomegaly syndrome (Wells, 1970) indicates that malaria is an important factor in the reduced incidence of autoimmunity noted.

Greenwood (1968) suggests that given the complex nature of the processes involved in immunity to malaria, the wide serological responses observed may reflect only one of the many immunological abnormalities induced by the parasite, and that malaria infection could lead to tolerance to alteration in host antigens that would normally lead to autoimmunity. Thus, the suppression of autoimmune diseases in certain strains of mice by infection with malaria observed by Greenwood et al (1970b)would support the above hypothesis. Current immunological knowledge suggests that immunological regulation of host cellular integrity may depend on the balance of suppressive and stimulatory subsets of T cells (Rose, 1978). Experimentation in this field would suggest that T and

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B cells capable of reacting with many self-antigens are present and waiting to be triggered off. Prevention of autoimmune response therefore requires continuous and active suppression. It is therefore possible that the enhancement of this active suppression resulting from malaria infection plays a role in the lowered incidence of autoimmune diseases in malarious areas. There is also evidence that acute malaria causes depletion of circulating T and B cells (Wyler, 1976; Ade-Serrano and Osunkoya, 1977),but Greenwood et al (1977) pointed out that since T cell function is not detectably suppressed, the depletion may be a mark of adaptation rather than of immunosuppression. The mechanisms involved here require further elucidation.

IMMUNOSUPPRESSION IN MALARIA Malaria immunosuppression has been well documented,but the mechanism by which this state is effected remains speculative. Hayasaka (1933) first recorded the immunosuppressive effects of malaria to heterologous bacterial antigen , his syphilitic patients on malaria fever therapy were unduly susceptible to salmonella infections. The next report was by McGregor and Barr (1962) who found a higher incidence of non-reactors to tetanus toxoid among malarious than non-malarious children in the Gambia. Mice infected with P.berghei were also shown to have an increased susceptibility to Salmonella typhimurium (Kaye et al, 1965). Salaman et al (1969) later observed that P.yoelii infections in mice severely depressed responses to sheep erythrocytes at the height of parasitaemia. Since then Greenwood et al (1971c), Barker (1971) and Poels and van Niekerk (1977) have amply confirmed the depressed effect of murine malaria on antibody and plaque forming cell responses to sheep red blood cells. In general, the degree of suppression was related to the species of parasite, the virulence and the time when antigen was given.

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Responses to thymus-dependent antigens, keyhole limpet haemocyanin and bacteriophage were largely unaltered, whereas those to tetanus and human gammaglobulin were reduced (Terry, 1977). Antibody responses to thymus-independent antigen pneumococcal polysaccharide (S111) on the other hand were profoundly depressed at the height of parasitaemia (Wedderburn and Draycott, 1977). Depression found in cell-mediated responses was variable, and this was dependent on parasite species used. P.berghei infections caused more depression than P.yoelii infections (Greenwood et al, 1971c; Sengers et al, 1971; Wedderburn, 1974; Jayawardena et al, 1975). In man, Greenwood et al (1972) found that some humoral but not cell mediated responses were depressed and the depression was greatest in those individuals with high parasitaemia. However the cause and effect relationship in malaria immunosuppression was not clear. Studies by Greenwood et al (1971a) indicated that malaria altered the capacity of the macrophages to process antigen. Terry et al (1973) put forward a hypothesis of deficiency of T cell/B cell co-operation to explain a similar immunosuppression observed in Trypanosomiasis and Voller (1974) suggested that the same mechanism might be implicated in malaria.

The relevance of malaria immunosuppression to human disease was first recorded by Greenwood (1968). He observed that recognisable autoimmune disease was less common in Africans living in malarious endemic areas, and suggested that immunological disturbances produced by malaria were responsible. This observation was experimentally confirmed with the demonstration that P.yoelii infection suppressed arthritis in rats and autoimmune disease in MZB and MZB/MZW F hybrid mice (Greenwood et al, 1970b; Greenwood and Voller, 1970a; b; Greenwood and Greenwood, 1971; Greenwood et al, 1972).

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Of equal relevance was the demonstration of lowered antibody affinity in mice with malarial infection (Steward and Voller, 1973). The above work coincided neatly with the demonstration that malarial infection increased the susceptibility of mice to lymphomagenic viruses (Jerusalem, 1968; Wedderburn, 1970; Bomford and Wedderburn, 1973), and to other infectious agents where resistance is known to be T cell dependent (Strickland et al, 1972; Cox, 1975). This observation added strength to the hypothesis linking Burkitt's lymphoma of African children and Epstein Barr virus to malaria (Burkitt, 1969; Lancet, 1970). The work of de-The et al (1978) in Uganda further confirmed this association.

However, on occasions immunosuppression may also affect the outcome of malarial infection (Salaman, 1970), and it is probable that other immunopathological sequels of malaria combined with malaria have yet to be identified. Thus the importance and consequences of malaria associated immunosuppression in man require further study.

Immunity and immunopathology resulting from malaria is largely a composite of complex immune responses and mechanisms.

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CHAPTER TWO

GENERAL INFORMATION ON GHANA

A. THE GEOGRAPHY

POSITION AND EXTENT Ghana lies along the Gulf of Guinea on the West coast of Africa. To the east of its land mass lies Togoland, beyond which are Benin and the Republic of Nigeria. On the west is the Ivory Coast, and Upper Volta lies along its northern borders (Figure 1).

The southern coast of Ghana extends between 4° 30' N in the west and 6° 30' N in the east. From the coast the country extends inland to about latitude 11° N, covering a distance of 420 miles from north to south. From the east to the west the country lies between 1° 30' E and 3° 30' W, and measures 334 miles. It has a total area of 92,100 square miles and for administrative purposes, the country is divided into nine regions (Figure 2).

TOPOGRAPHY Figure 3 shows the patterns of relief in the country. More than half the country consists of a series of plateau surfaces at different elevations. These were often ancient fold mountains flattened by the forces of denudation. Thus very few mountains remain, but there are several hills which rise to a maximum of 900 feet. For the most part, the land is less than 500 feet above sea level.

Structurally, the land mass is composed of voltaic rocks which consist mainly of sandstone and covers nearly 45% of the country. The rest is made up of a variety of rock forms, among which granite, quartzite and phyllites predominate.

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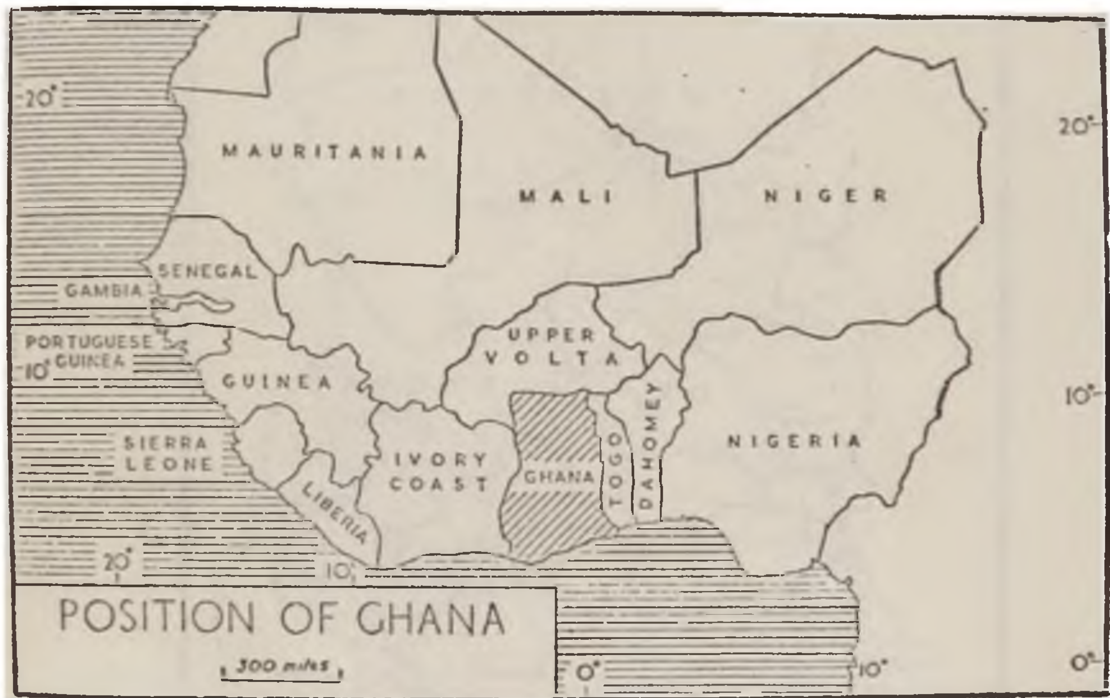

FIGURE 1 Position of Ghana in West Africa

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FIGURE 2 The nine administrative regions of Ghana

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FIGURE 3 Relief map of Ghana

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CLIMATE Lying as it does between 4° N and 11° 50' N, Ghana is situated in the belt of tropical and equatorial climates. Annual mean temperatures for the entire country range from 26°C to 29°C, with the lowest near the coast and highest in the north. Average maximum temperatures are highest in March, with 42.8°C being the highest ever recorded in the north. Lowest temperatures occur in August in the southern areas, and in December in the northern region. 10.6°C is the lowest recorded temperature in December.

There are distinct wet and dry seasons with great variability in monthly and yearly totals. Rainfall is heaviest in the south-west with an average of 215 cm, and least in the far north and in the eastern coastal belt with an average of 82.5 cm. The south has two wet seasons. The first is from March to July, with the major rains in May and June. The second, less wet season, is in September and October. In the north, the intensity of the rains is reversed and there is only one wet season from April or May to October (Figure 4).

Ghana is generally humid with relative humidities of 90 to 100 per cent at night and 45 to 75 per cent during the day. However, during the day during harmattan, a period of cool dry north-easterly winds occurring in December to February, the relative humidity can drop to as low as 12 per cent.

DRAINAGE There is a network of water courses all over the country (Figure 5). However, relatively few rivers or streams maintain an even flow all the year round. Some rivers are supplied by both rainfall and natural springs, and therefore they are rarely completely dry. Others are intermittent - that is, they flood in the rainy season and may dry up in

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FIGURE 4 Rainfall distribution map of Ghana

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FIGURE 5 Drainage map showing network of water courses in Ghana

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the long dry season, especially those in the north. Flooding often creates stagnant pools which are ideal breeding grounds for vectors of malaria, trypanosomiasis and schistosomiasis.

VEGETATION Four broad vegetation types are distinguished in Ghana. These are the high forest, savannah woodland, coastal scrub and grassland, and strand mangrove (Figure 6). The high forest occupies the entire south-western part of Ghana and covers an area of approximately 25,000 square miles. The combination in this region of high rainfall, high humidity and high temperature creates an environment most suitable for insect vectors of many communicable diseases, with malaria on top of the list.

Savannah woodland covers the region north of the high forest, a 65,000 square mile stretch, composed mainly of short trees widely spaced with continuous carpet of grass. The strand mangrove is confined to the immediate coastal area, both along the sea front and beds of lagoons. The lagoons also present ideal all year breeding grounds for mosquitoes.

B. POPULATION

Ghana had a population of 8,559,000 in 1970. This compared with the population in 1960 (first population census in Ghana) of 6,726,800, shows a growth rate of 27 per cent in the ten years. Assuming the same growth rate prevails, the present population should number approximately 10,870,300. Table 1 shows the distribution of the population in the nine regions of Ghana. In 1980, 18 per cent of the population was under 5 years, 64 per cent were under 25 years, and 41 per cent of the females were in the

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FIGURE 6 Map of different types of vegetation in Ghana

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TABLE 1 Population distribution in the nine administrative regions of Ghana

Region	Population [a]
Western	770,087
Central	890,135
Greater Accra	851,614
Eastern	1,261,661
Volta	947,268
Ashanti	1,481,698
Brong-Ahafo	766,509
Northern	727,618
Upper	862,723
All regions	8,559,313

a 1970 Population Census

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reproductive age group. The age structure of the population is important with respect to malaria because mortality and morbidity due to malaria are greatest in the young age group of under 5 years.

In 1970 about 30 per cent of the population lived in the 49 urban areas (an urban area is defined as a town with a population of over 10,000), and the majority lived in rural and semi-rural surroundings. However, a yearly migration of young adults from the villages has created over-population in some industrial and commercial towns.

C. HEALTH SERVICES IN GHANA

The provision of integrated health services for the whole country falls on the august body of the Ministry of Health. A Regional Medical Officer of Health is responsible for the supervision and co-ordination of all health activities in each region. The country has one main referral hospital situated at Korle-Bu which provides specialised in-patient and out-patient care. There is a government hospital in each region and altogether 43 government hospitals, 12 government affiliated hospitals, and 34 mission hospitals. Mining companies set up their own hospitals for the care of employees , these number 12, and there are 17 private hospitals. There are 52 rural health centres and 40 health posts. It must be appreciated that all the hospitals are in urban centres of the country and very few in the rural areas.

The programme for communicable disease control is enforced by the Epidemiology Unit of the Ministry of Health.

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D. PARASITIC DISEASES OF MAJOR PUBLIC HEALTH IMPORTANCE

Ghana's population is subjected to the usual core pattern of infectious diseases due to viral and bacterial agents. These diseases, measles, poliomyelitis, meningitis and yellow fever have high fatality rates, especially in children and their prevalence is often associated with poverty and under-development. In addition to this, an endemic overlay of micro- and macro-parasitic diseases, namely malaria,trypanosomiasis, onchocerciasis, schistosomiasis and many species of intestinal helminths affect large numbers of the population, often with serious sequelae.

MALARIA Malaria remains by far the major public health problem in Ghana. It is the main objective of this study and a fuller account of the seriousness of this disease will occupy subsequent pages.

TRYPANOSOMIASIS Trypanosomiasis is now considerably reduced as a result of control measures, which entail fly surveys, clearing of riverine vegetation of population centres at risk, and barrier clearing of river crossings. Reduced cases indicate the success of the control measures. In 1967, 196 cases were confirmed; in 1969, 170 cases; in 1970, 99 cases; 1976, 57 cases; and in 1977 only 42 cases were discovered.

ONCHOCERCIASIS The disease is widespread in the northern part of Ghana, particularly along the tributaries of the Volta River, where the incidence rate ranges from 5 per cent to 8 per cent; and 0.1 per cent of these suffer from blindness due to the disease. In an effort to control and eradicate the disease, a WHO inter-country project has been set up in which Ghana is participating in undertaking feasibility studies in the Volta River basin in conjunction with neighbouring states to find a solution to the problem.

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SCHISTOSOMIASIS Schistosoma haematobium is the only schistosome species that is commonly seen in Ghana. Occasionally S.mansoni is seen. The disease has a focal distribution, although the prevalence is estimated at 1 per cent of the population.

INTESTINAL HELMINTHS In many rural areas of Ghana multiple infections with two or more parasites is the rule, especially in children. Quite often hookworms, roundworms and other filarial infections occur together with malaria.

The following articles and books provided excellent sources of reference (Dickson and Benneh, 1970; Ghana Government, 1960, 1969, 1970, 1977; Annual Report of the Medical Services 1967; Ghana Monthly Epidemiological Bulletin Jan 1971-Dec 1977; Ghana Epidemiological Yearly Field Report Ministry of Health 1974; Quao, 1976).

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CHAPTER THREE

MALARIA IN GHANA

A. CURRENT MALARIA SITUATION

Malaria is endemic with year round transmission in Ghana although towards the end of the dry season in early March, transmission is reduced. High transmission has been recorded in various parts of the country (Colbourne and Wright, 1955; Ministry of Health Annual Project Reports Afro Year Book, 1958-1960). The predominant malaria species is P.falciparum. It accounts for more than 90% of all malaria infections. Plasmodium malariae is the second species of importance and found mostly in young children within the age group 0-5 years. It appears as single or mixed infections primarily with P.falciparum in 3-6% of cases. Plasmodium ovale infections are rare and also occur as single or mixed infection primarily with P.falciparum in 0.5-1.0% of cases (Ghana Ministry of Health Current Information on Malaria Risk for International Travellers). The Ministry emphasises that P.vivax does not occur in Ghana. The only cases of P.vivax ever recorded in Ghana were by Colbourne and Edington (1950) in Kwansakrom. This, however, has not been confirmed.

B. PREVALENCE OF MALARIA

The most commonly used methods for the classification of malaria prevalence are spleen rate and parasite rate.

The spleen rate is the percentage of children aged 2-10 years in a community who have enlarged spleen. Covell, Russel and Swellengrebel (1953) recommended a method for the measurement of spleen size. Spleen

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rates thus obtained can be used to measure the degree of malaria transmission in a given area. WHO (1951) proposed the following classification of malaria prevalence using spleen rates to reflect the degree of endemicity.

I Hypoendemic malaria

Spleen rate in children 2-10 years = 0-10%

II Mesoendemic malaria

Spleen rate in children 2-10 years = 11-50%

III Hyperendemic malaria

Spleen rate in children 2-10 years = constantly over 75%, with high adult spleen rates.

IV Holoendemic malaria

Spleen rate in children 2-10 years = constantly over 75%, with low adult spleen rates and high tolerance.

In Ghana spleen rates between 50% and 75% have been recorded in children, but low rates were recorded in adults with corresponding high levels of tolerance (Ghana Ministry of Health Afro Year Book, 1958-1960). Colbourne and Wright (1955) provide data on the spleen rates in the three different epidemiologic conditions found in Ghana. These are shown in Table 2. The Ghana Ministry of Health/WHO malaria eradication pilot project in the Volta Region (Afro Year Book, 1958-1960) provides similar results to those found by Colbourne and Wright in the coastal plains. These workers assert from their results that the recognised definition of

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endemicity (WHO, 1951) seemed inapplicable to the Ghanaian situation, because from their evaluations, the endemicity in the coastal plains varied between mesoendemic to hyperendemic, and yet tolerance in adults was very high. Similarly, by the WHO standards neither the spleen rates in the forest belt and northern savannah could be classified as hyper-endemic or holoendemic, and yet the malaria rates in both regions indicate heavy infection with little variation in seasonal transmission and a degree of development of immunity before adult life. Bruce-Chwatt (1952b), faced with similar problems, classified the endemicity observed at Ilaro in Nigeria as holoendemic. The spleen rate is really only an indirect method of malaria investigation, and may be invalidated by the presence of other diseases.

Parasite rates reported for Ghana are high, and quite often constantly over 50% for most regions, with little seasonal variation. Colbourne and Wright (1955) recorded parasite rates for the different epidemiological conditions of Ghana (Table 3). From this data, the coastal plains and the forest belt regions of Ghana could be classified as hyperendemic for malaria, but holoendemic for the northern savannah region. The hyper-endemicity of malaria in the coastal plains is confirmed by the Ghana/WHO feasibility studies for a malaria eradication pilot project at Ho and also data from malaria investigation carried out at 17 health posts and health centres in the Volta Region from January to December 1972 (Saakwa-Mante, unpublished data) with an average of 64% recorded for the age group 2-10 years.

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TABLE 2 **Spleen rates recorded by Colbourne and Wright (1955) in the three epidemiological conditions in Ghana during minimum and maximum parasite transmission periods**

Epidemiological condition	Spleen rates			
	Dry season Period of minimum malaria transmission		Wet season Period of maximum malaria transmission	
	Urban	Rural	Urban	Rural
Coastal plains	18%	43%	31%	57%
Forest belt	–	58%		72%
Northern savannah	–	51%	–	65%

TABLE 3 **Parasite rates of different epidemiological conditions in Ghana (Colbourne and Wright, 1955)**

Epidemiological condition	Parasite rates			
	Dry season Period of minimum malaria transmission		Wet season Period of maximum malaria transmission	
	Urban	Rural	Urban	Rural
Coastal plains	26%	61%	45%	70%
Forest belt	–	58%	–	72%
Northern savannah	–	96%	–	98%

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C. <u>ANTIMALARIAL ACTIVITIES</u>

Attempts at malaria control were carried out in Ghana in the early 1940's. These consisted of antilarval operations and house spraying with insecticides, mainly pyrethrum, which results in significant reductions in vector populations and malaria incidence (Eddey, 1944; Buxton, 1945, Muirhead-Thomson, 1947).

The parasite rate gives a more direct measure of the presence of malaria in the blood, and alone enables a differentiation into species. Thus, the percentage of subjects of a well defined group whose blood contains malaria parasites is a better index of endemicity. Metseler and van Thiel (1959) taking all the above considerations into account proposed that parasite rates be used instead of spleen rates to classify malaria endemicity. The following classification of malaria by parasite rate (WHO, 1951) has been widely accepted.

I <u>Hypoendemic malaria</u>

If the absolute parasite rate in the age group 2-10 years is as a rule under 10%. It may be higher for part of the year.

II <u>Mesoendemic malaria</u>

If the rate in the age group 2-10 years is as a rule between 11 and 50%. It may be higher for part of the year.

III <u>Hyperendemic malaria</u>

If the rate in the age group 2-10 is constantly over 50%.

IV <u>Holoendemic malaria</u>

If the rate in the age group 2-10 is constantly over 75% in the one year age group.

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A national malaria unit in Ghana was formed in 1959. It was to be a collaborative pact between Ghana/WHO/UNICEF for the planning and implementation of total eradication of malaria in Ghana, and to this end a pilot eradication programme was established. The pilot project was suspended in 1967. Until a national control and eradication programme can be met, there are at present no organised national antimalarial measures in the country apart from routine larviciding by municipal sanitary authorities and a medicated (chlorinated) salt experiment in the northern part of Ghana.

D. MALARIA CHEMOTHERAPY AND CHEMOPROPHYLAXIS

In Ghana most antimalarial drugs are easily available to the public in general, and consequently are widely and indiscriminately used, especially in the urban areas. All the three parasite species which occur in Ghana are sensitive to chloroquine and amodiaquine, and there is no evidence of resistance to the 4-aminoquinolines in malaria parasites in the country (Beausoleil, 1965), but there is resistance to pyrimethamine and cross-resistance to proquanil and chlorproquanil in *P.falciparum* in some parts of the country.

Immunity to malaria is an integral part of a host-parasite relationship and the fact that Ghanaian adults are less susceptible to malaria and often do harbour parasites in the absence of clinical symptoms confirms that a certain amount of immunity is developed if not complete. Also, where malaria is prevalent, re-infection and super-infection are common, and immunity can only be assessed adequately at the population level by carrying out well organised parasitological and serological surveys. Such surveys

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which would give a measure of a population's recent past experience with malaria, and yield information on endemicity and elucidate the level of immunity enjoyed by the different age groups in the population, has never been carried out in Ghana.

In this study, current serological methods used in malaria have been applied in Ghana. A comparison of ELISA and IFA has been made, and the result of serological surveys carried out in selected Ghanaian populations will be presented. A rapid IFA test for detecting antibodies to several parasitic antigens, at the same time, on a single microscope slide, has been assessed in Ghana. The usefulness of this method for polyvalent studies especially in Ghanaian populations exposed to poly-parasitism will be explored. Local reference standards for serum IgG, IgA, and IgM have been defined for an urban Ghanaian population. Data will be presented on the contribution of malaria on raised serum IgG and IgM in the Ghanaian population. Several other immunological tests were utilized in order to observe the in vivo and in vitro interactions of malaria antigen, malarial antibody, antigen-antibody complexes and complement (C3). The prevalences of immune complexes and autoimmune antibodies have also been determined. Data will be presented for all these immunological parameters observed, which may be important in the immunopathological sequelae of malaria.

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SECTION II : EXPERIMENTAL TECHNIQUES

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CHAPTER FOUR

MATERIALS AND METHODS

A. AREA OF STUDY

Serological surveys were carried out on urban and rural populations in four of the nine main administrative regions in Ghana (Figure 7). This area was chosen for ease of accessibility, similarity in topography, climate and housing conditions; and also similarity in the malariometric (entomological and parasitological) indices (Colbourne and Wright, 1955; Ghana Ministry of Health Afro Year Book, 1958-1960). This survey was organised and carried out in order to ascertain something of the sero-epidemiological characteristics of malaria in Ghana. Such a survey has never been carried out in Ghana.

B. SURVEY METHOD

The surveys were carried out in conjunction with immunisation programmes, medical field unit health campaigns, health education clinics, ante-natal clinics and child welfare clinics organised in the four regions. Specific clinical sera with proved diagnoses and their relevant controls were obtained from regional hospitals from in-patient and out-patient clinics, and also from patients at the main referral hospital in Ghana, Korle-Bu Teaching Hospital, Accra. However collection of clinical sera was not limited to the four regions designated. Materials were collected over a period of two years from January 1975 to January 1977. A total of 150 villages and 15 towns were visited during this period, and samples were obtained from as many people as would co-operate. Surveys were carried out during the dry season when roads to villages were accessible.

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FIGURE 7 The survey area comprising four of the nine main administrative regions in Ghana

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The survey entailed the collection of blood for plasma and/or serum, and the preparation of thick and thin blood films. General information was obtained from each donor on age, sex, length of residence in area, any previous immunisations received, and history of past or present parasitic infections of major public health importance. Blood samples were taken from all age groups. Voller (1974) reported that antibody response in malaria was to some extent age dependent. Thus the sampling procedure was designed to allow valid comparison of results to be made between different age groups of the urban and rural populations. Blood samples collected in the field were obtained from a finger-stab since this method was more acceptable to the populations sampled. All clinical sera were obtained by venepuncture.

The equipment and field procedure used was that recommended by Voller and O'Neill (1971). Requirements for finger-stab blood collection were disposable lancets (Dade, Merz and Dade AG, Bern), heparinised capillary tubes (Volac), 75 mm x 1 mm (John Poulten Ltd., Barking, Essex, UK), portable blow-lamp, vacuum flask, modelling clay (Seal-Ease by Clay Adams Parsppary, N.J., USA), adhesive labels, haematocrit centrifuge (MSE, UK) and washed, cleaned,ready to use microscope slide 76 mm x 25 mm (Chancer Propper Ltd., Warley, UK).

Thin and thick films were prepared from finger-stab and slides were marked with a diamond pencil. Heparinised capillary tubes were filled with blood from the finger-stab and the tubes sealed at one end, and appropriately labelled with adhesive labels. Slides were placed in slide boxes and the labelled tubes were placed in cooled vacuum flasks. At the end of a day's collection all samples were transported to the nearest

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health post, health centre or regional hospital laboratory. These often had basic electricity and refrigeration facilities. On arrival at these centres the thin blood films were fixed in methanol, dried and returned into the slide boxes. The capillary tubes with blood were centrifuged in a microhaematocrit centrifuge. The tubes were cut at the interface between the plasma and the packed red blood cells. The tubes were sealed at both ends with the seal-ease, labelled and stored in small plastic containers. Plasma samples were kept in domestic refrigerators at +4°C until they were transported to Korle-Bu Accra, where serum or plasma samples were stored in the most reliable freezer in the Department of Microbiology, University of Ghana Medical School, at -20°C. There was daily transportation from regional health centres to Accra, thus enabling all samples to be stored at -20°C within 2-3 days of collection. Blood films were stained with Giemsa revector stain at Accra and stored in air-tight slide boxes until examined.

Venepuncture samples were allowed to stand at room temperature for two hours for clot retraction, then centrifuged. If this was not convenient, samples were kept at +4°C overnight, centrifuged, and serum pipetted into sterile containers and stored within 2-3 days of collection at -20°C.

At the end of the survey, all collected sera were transported by air in dry ice to the Nuffield Laboratories of Comparative Medicine (NLCM).

C. SUBJECTS AND SAMPLES COLLECTED

The total number of Ghanaian subjects sampled including those from whom clinical sera were obtained was 4,443 and comprised seven main groups as listed below. Their ages ranged from 0 (cord) - 75 years. The location of major cities and towns visited in association with the serological surveys, including areas from which clinical sera were obtained is shown in Figure 8.

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FIGURE 8 The location of major towns and cities blood sample storage and collection areas

1 - BOLGATANGA
2 - WORAWORA
3 - HO
4 - AKOSOMBO
5 - KOFORIDUA
6 - BEKWAI
7 - OBUASI
8 - DUNKWA
9 - SEKONDI - TAKORADI
10 - ELMINA
11 - CAPE COAST
12 - SALTPOND
13 - WINNEBAH
14 - ODA
15 - ACCRA
16 - TEMA

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The information obtained from subjects on their immunisation status and history of post-parasitic infections was not considerered reliable. However, it was felt that information on age and length of residence in an area could be reasonably relied upon. Subjects in the rural areas had been resident in the area since birth. The urban population was a more mobile population and residency in an urban area for more than five years was the main criterion for inclusion in the group.

GROUP A These were samples obtained from ambulant and apparently healthy rural and urban people in order to provide information on baseline malaria epidemiology. Sixty percent of the urban samples were obtained from Central Accra, and the other 40% were made up from samples from the regional capitals in the survey area. All rural samples were from villages and small towns in the survey area. Table 4 shows the age and sex distribution in each population.

GROUP B Samples collected from a healthy urban adult population. These constituted 225 samples from blood donors taken at the Korle-Bu Transfusion Centre, Korle-Bu Teaching Hospital, Accra. These comprised 190 males and 35 females, aged 18 to 51 years.

GROUP C Samples obtained from women attending ante-natal clinics (at 30 weeks' gestation). Some of these women received regular weekly anti-malarial prophylaxis during their pregnancy, and others were unprotected (Table 5). Their ages ranged from 16 to 40 years.

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TABLE 4 The age and sex distribution in the urban and rural populations

Age group in years	Number in population					
	Rural	Sex distribution M	Sex distribution F	Urban	Sex distribution M	Sex distribution F
Cord sera				122	64	58
0 - 5	650	210	450	450	200	250
6 - 15	150	80	70	114	64	50
16 - 29	242	110	132	260	110	150
30 and above	340	140	200	320	130	190
Total	1,392	540	852	1,266	568	598

TABLE 5 Rural and urban protected and unprotected pregnant women and their non-pregnant control groups

Pregnant women	Rural	Urban
Unprotected	100	250
Protected (chloroquine 300 mg base weekly)	100	220
Total	200	470
Non-pregnant controls	50	107

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GROUP D To observe the general effects of malaria prophylaxis on specific humoral malaria antibody titres, a small study was carried out on 200 Army officers attending the Military Academy at Teshie-Nungua, Accra. One hundred officers received weekly anti-malarial prophylaxis (chloroquine 300 mg base), for eight months, but the remaining 100 were unprotected. Samples were taken at the beginning and end of the project.

GROUP E Seven hundred and thirteen sera were obtained from three groups of rural infants before and after vaccination against tuberculosis, measles, smallpox, diphtheria, pertussis and tetanus. These comprised 250 sera from age group 3-8 months, 250 from age group 9-14 months and 213 from age group 15-24 months. These children formed part of the population covered in the central region during the expanded immunisation programme of 1974 and 1976 (this was a pilot project organised by the collaborative efforts of the Swedish International Development Agency (SIDA), WHO and the Ghana Government). A half of the children in age groups 3-8 and 9-14 months received a weekly dose of chloroquine syrup 75 mg base, while the other half received 5 ml of vitamin syrup weekly, as shown in Table 6. Prophylaxis could only be carried out for 2 months. Samples were obtained before and after the short period of protection.

GROUP F These comprised clinical sera of special interest. All diagnoses were based on clinical laboratory and epidemiological data. They were divided into two main groups as shown in Table 7.

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TABLE 6 Schedule used for malarial chemoprophylaxis in rural children aged 3-14 months

Group examined (rural children)	Study group 3-8 months		Control group 9-14 months	
	Male	Female	Male	Female
Protected	54	71	57	68
Unprotected	63	62	60	65
Total	117	133	117	133

TABLE 7 Components of clinical sera of special interest

Clinical group	Patients			Controls		
	Number	M	F	Number	M	F
1) Malaria associated diseases						
Acute malaria	43	20	23	30	10	20
Nephrotic syndrome	50	27	23	50	25	25
Tropical splenomegaly	21	13	8	21	10	11
Burkitt's lymphoma	28	18	10	20	14	6
2) Other diseases of interest						
Schistosomiasis	20	14	6	36	26	10
Onchocerciasis	30	20	10	30	20	10
Chronic tuberculosis	50	28	22	50	25	25
Total	236			241		

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The sources from which clinical sera were obtained

Patients with acute malaria Sera from patients with clinical malaria and slide-proven parasitaemia (20 males and 23 females aged between 15 and 42 years) were obtained from the Korle-Bu Poly-clinic, Korle-Bu Teaching Hospital, Accra, Ghana, with the kind permission of the medical officer-in-charge, Dr. E. Bruce-Tagoe.

Patients with tropical splenomegaly syndrome Sera from 20 patients (13 males and 8 females aged 14 to 48 years) were provided by Dr. A. Bruce-Tagoe, Head, Department of Haematology,Korle-Bu Teaching Hospital, Accra, Ghana. The syndrome was characterised by massive splenomegaly in patients with anaemia, jaundice punctuated with episodes of haemoglobinuria (often suggestive of hyperhaemolysis Gravis Syndrome) with reticulocytes, and erythrophagocytosis.Hypergammaglobulinaemia especially marked serum IgM increases were a constant feature. Some patients were febrile, but often overt parasitaemia was absent and no cause other than malaria appeared responsible for splenomegaly.

Nephrotic syndrome patients Sera from 50 patients with nephrotic syndrome were provided by Dr. D. Adu of the Department of Medicine, Korle-Bu Teaching Hospital, Accra, Ghana. They comprised 14 children (8 males and 6 females aged 3 to 9 years), and 36 adults (21 males and 15 females, aged 16 to 50 years). The diagnosis of nephrotic syndrome was made from the presence of heavy proteinuria, hypoalbuminaemia, oedema and in many cases, of hypocholesterolaemia.

Histological examination by electron and light microscopy of renal biopsies obtained from these patients showed a wide range of glomerular changes. In children the variety of lesions observed included minimal changes , membranous glomerulonephritis with spikes on methenamine

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silver stained sections, and also proliferative and mesangiocapillary glomerulonephritis were seen. However, the lesion of greatest incidence was minimal change glomerulonephritis which occurred in 65% of the children.

Adults showed a wider variety of renal lesions. Proliferative glomerulonephritis was the single most common lesion and occurred in 25% (9/36) patients. Six patients each had a minimal change lesion and membranous glomerulonephritis. Five patients had a focal segmental sclerosing glomerulonephritis, and in the three the underlying lesion was a chronic interstitial nephritis with many obsolete glomeruli.

Multiple thin and thick blood films examined for blood parasites revealed only one patient with patent malaria parasitaemia, with P.falciparum the infecting species.

None of the patients clinically or parasitologically showed evidence of schistosomiasis.

Burkitt's lymphoma patients Twenty-eight patients with confirmed Burkitt's lymphoma were obtained from Dr. F.K. Nkrumah's Burkitt's Lymphoma Clinic. Patients comprised 18 males and 10 females, aged 4 to 11 years. Histological and cytological diagnosis of Burkitt's lymphoma was made on each patient according to the criteria established by WHO (Berrard et al, 1969).

Patients with chronic tuberculosis (TB) Sera from confirmed chronic TB patients (28 males and 22 females, aged 15 to 68 years) were provided by Dr. Chowdry of the TB Chest Clinic, Korle-Bu Teaching Hospital, Accra, Ghana. Diagnosis was made by means of the radiological features of tuberculous broncho-pneumonia on chest X-ray, and the detection of acid fast bacilli in patient's sputum.

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Patients with schistosomiasis Sera were obtained from patients attending the schistosomiasis clinic at Akosombo (an endemic schisto-somiasis foci where a WHO control programme is currently underway), Ghana. All patients had Schistosomiasis haematobium infection. Diagnosis was based on clinical symptoms, the presence of eggs in urine and in stools, and haematuria. There were 14 males and 6 females, aged 16 to 39 years.

Patients with onchocerciasis Sera from 30 patients with onchocerciasis (20 males and 10 females, aged 16 to 40 years) were obtained from Dr. Sowa, Onchocerciasis Clinic, Blogatanta, Northern Ghana. All patients had palpable nodules and high densities of microfilariae in their skin.

Chemoprophylactic administration of antimalarial drugs Chloroquine was the drug used for all prophylactic measures in this study. The drug was supplied in a dosage of 300 mg base per week for adults. Children below age 24 months were given chloroquine syrup 75 mg base per week.

Army officers were given their dosage as part of a weekly military exercise. Pregnant women were given theirs when attending the ante-natal clinic. For the infant groups, syrup was given to mothers and trained health attendants supervised their weekly administration.

GROUP G

i) Sera from 500 Caucasian blood donors were obtained from Milton Keynes Blood Transfusion Centre, UK. None had visited a malarious endemic area. This group comprised 250 males aged 19 to 59, and 25 females aged 19 to 51 years.

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ii) Sera from 63 Caucasians (40 males and 23 females) with malaria infections admitted to the Hospital for Tropical Diseases (HTD), London, were also included. Thirty-one had P.falciparum, 24 with P.vivax, and 8 had P.ovale. All samples of serum and EDTA plasma were taken at the time of admission. Before treatment, patients had parasitaemia ranging from 0.05% to 39%. There were three children, aged 6, 7 and 9 years, and the rest were adults of 19-65 years. Samples were obtained with the permission of Dr. A.P. Hall (HTD).

iii) Four hundred sera from Nigerian adults and children taken before and after one year's WHO control activity. Two hundred sera were obtained from babies under 24 months old, 100 before control measures (pre-control), and 100 after control (post-control). Similar groups were obtained from adults, 100 before and 100 after the control operation. These were supplied by Dr. A. Voller (NLCM).

iv) Sera from Caucasians with proven non-tropical and tropical diseases were obtained:

a) A panel of sera from people with a wide range of connective tissue diseases, including positives for rheumatoid factor and antinuclear antibodies were provided by Dr. G.D. Johnson of the Bone and Joint Research Unit, The London Hospital Medical College, Whitechapel, London.

b) Sera from the following patient groups: 25 rheumatoid arthritis, 30 systemic lupus erythematosus, 20 pallendromics and 20 laboratory staff were obtained from the serum bank of the Bone and Joint Research Unit with the kind permission of Professor E.J. Holborow.

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c) Sera from patients with the following diseases: 40 chronic tuberculosis, 15 cholera, 30 brucellosis, 50 syphilis, 40 toxoplasmosis, 15 amoebiasis, 20 trypanosomiasis, 15 schistosomiasis and 30 toxocariasis were provided by Dr. G.J. Kane of the Wellcome Research Laboratories.

v) Fourteen sera were obtained from two Macaca mulatta monkeys vaccinated with Plasmodium knowlesi merozoite antigen at twice monthly intervals, and challenged three times at monthly intervals with P.knowlesi parasitised red cells. Sera were taken before study and after each experimental point. Seven samples per monkey were obtained. Sera were supplied by Dr. A. Voller (NLCM).

D. PRACTICAL DETAILS OF LABORATORY METHODS

1. EXAMINATION OF BLOOD FILMS

Thick and thin blood films were prepared from finger-stab. Venous blood was also used when available. The blood films were air dried, thin films were fixed with methanol for 5 minutes, and both the thick and thin films were stained in Giemsa revector stain (BDH) diluted 1:50 in buffered water (pH 7.2) for 15 minutes, then differentiated in tap water for 5 minutes and dried. The films were examined with an oil immersion x100 objective. The parasite rate was estimated as the percentage of smears showing parasites of any species of plasmodium. The thick films were particularly useful when low parasite densities were encountered. When thick films were examined 100 oil immersion fields were examined before a slide was declared negative. Parasites were

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enumerated against 10,000 red blood cells (using the thin films) and this enabled approximate densities to be calculated. Species classification was also carried out on the thin films. The positive parasite density index (PPDI), a parameter of mean parasite density per age group (the arithmetic mean of density indices of positive blood smear) was determined for the age groups in the urban and rural populations.

2. METHODS USED FOR THE DETECTION OF MALARIAL ANTIBODIES

Malaria antigen

Three human malarial parasites, Plasmodium falciparum, Plasmodium vivax and Plasmodium malariae were used in this study. Strains of these species were preserved at the NLCM as stabilates in 30% glycerol in phosphate buffered saline (PBS) pH 7.2, and stored in 0.5 ml aliquots at $-70^{o}C$. These strains had been adapted to Aotus monkeys by repeated blood passages.

i) The Indirect Fluorescent Antibody (IFA) Test for malarial antibodies

Reagents Antigen for IFA was prepared from infected Aotus monkeys as described by Voller and O'Neill (1971). Fluorescein-isothiocyanate (FITC) labelled sheep anti-human immunoglobulin IgG, IgA and IgM obtained from Wellcome Reagents Ltd. (Beckenham, Kent. UK) were used throughout this study. The freeze-dried conjugate preparation was reconstituted in distilled water according to the manufacturer's specifications. Further conjugate dilution was made in PBS and a working dilution of the conjugates was obtained by titration. For the malarial system, the best conjugate working dilution was 1:20 for anti-human Ig; 1:40 for anti-human IgG and 1:5 to 1:10 for anti-human IgM and IgA.

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<u>Reference sera</u> Positive reference serum was pooled sera from a Nigerian population living in a highly endemic malarious area with an IFA titre of 1:1280. This was stored in 1 ml and 20 ml aliquots at -20°C. The negative reference serum was a pool from caucasian blood donors living in Britain, negative for malaria (<20 titre).

<u>Microscopical system</u> A fluorescent microscope equipped with a suitable light source and filter systems was used for malarial IFA. The microscope was a standard WL with a large fluorescence illuminator obtained from Carl Zeiss (Oberkochen, West Germany) and fitted with a high pressure mercury lamp, HBO 200W. Primary (excitor) filters BG 38/2.5 mm, BG 12/4 mm, UG 1/3 mm and interference filter - Balzer's FITC No.3 were used in combination with a secondary (barrier) filter 44/50. The microscope had a fitting for both bright field and dark field condensers. The nose cone carried neofleur objectives with magnifications of x6.3, x16, x40, and x100 oil immersion with numerical apertures of 0.20, 0.40, 0.70 and 1.25 respectively. The ocular lenses used were x10 magnification.

<u>Test procedure</u> The principle of the IFA is outlined in Figure 9. The apparatus (Figure 10) and method used was that recommended for large scale malaria serology by Voller and O'Neill (1971). The IFA titre obtained for each serum tested was the last serum dilution yielding detectable fluorescence. In this study, sera that showed fluorescence below 1:20 dilution were classified as negative. Figure 11 shows a positive fluorescence of malaria schizonts.

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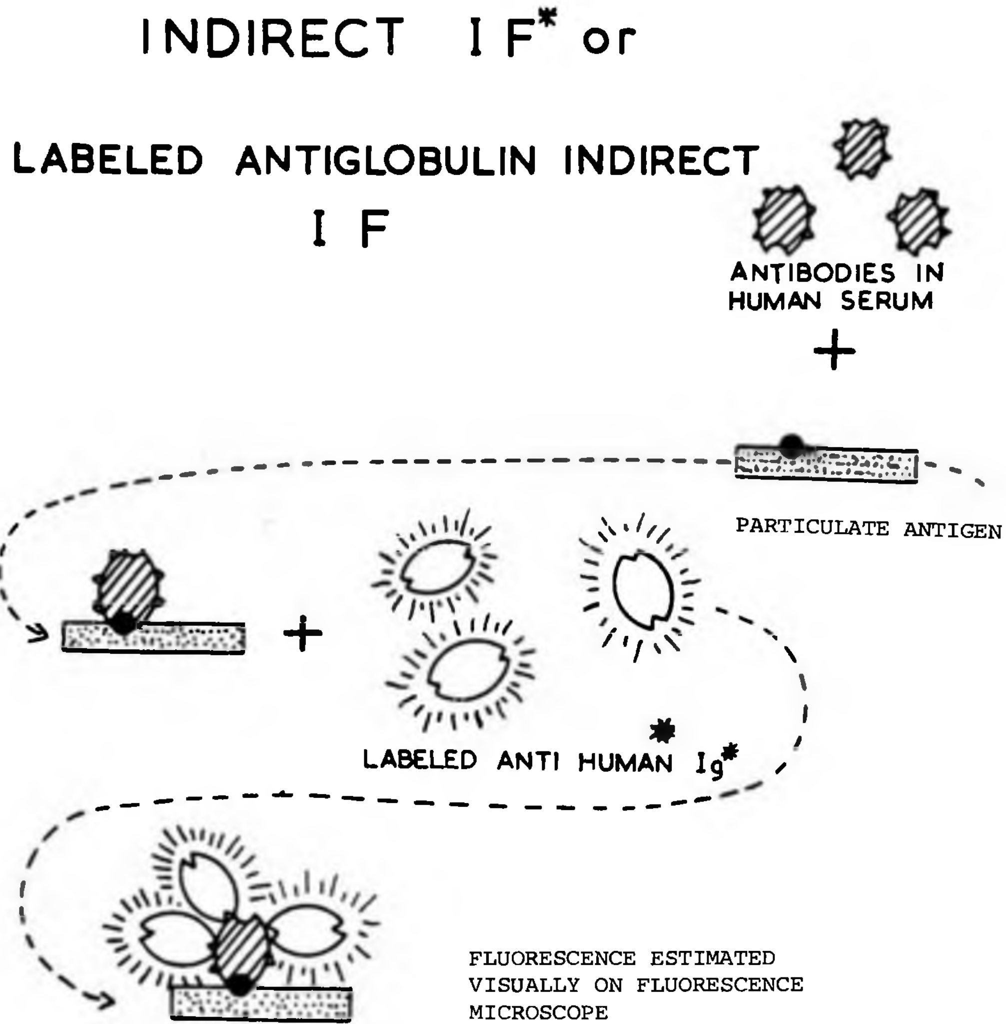
FIGURE 9 Diagrammatic representation of the indirect immunofluorescence antibody (IFA) test

Photograph reproduced with the kind permission of Dr. G.D. Johnson, Bone and Joint Unit, The London Hospital Medical College.



* I F = IMMUNOFLUORESCENCE

I g = IMMUNOGLOBULIN

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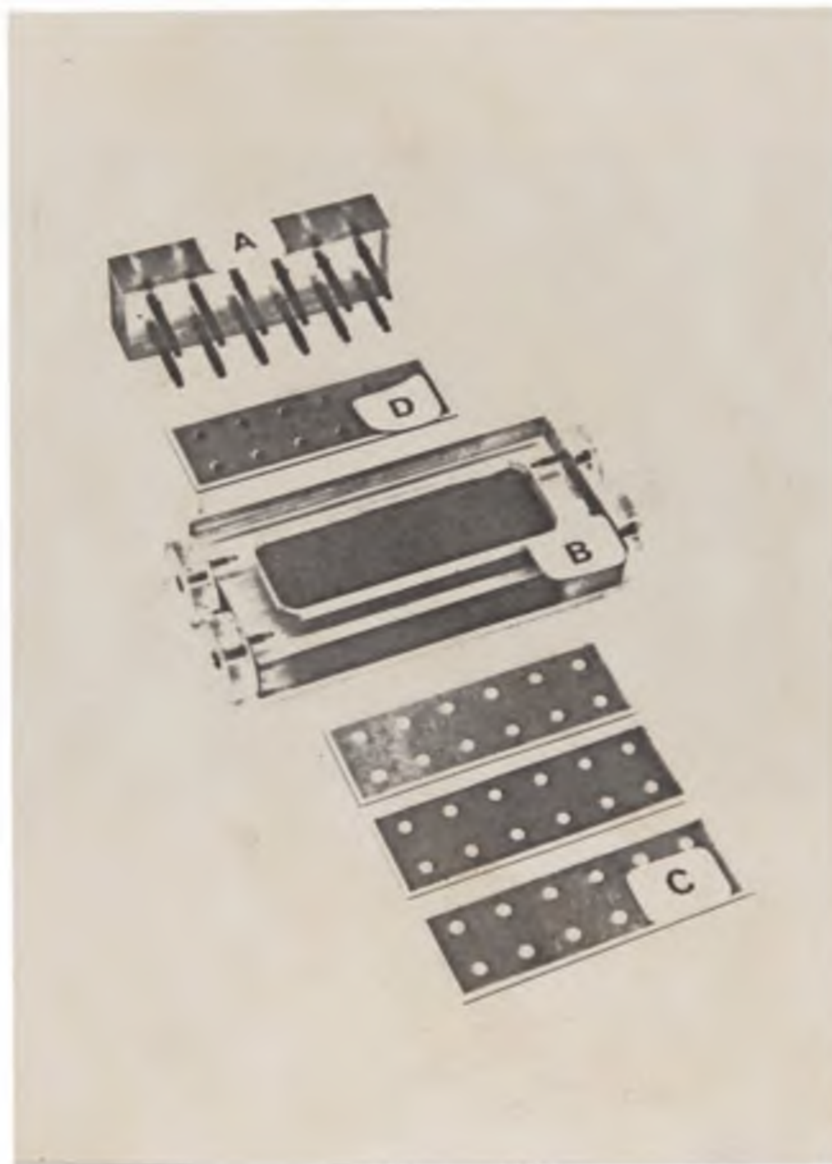
FIGURE 10 The apparatus used in the preparation of malaria antigen slides: A, iron applicator, B, 'haemobile', C, microscope slides sprayed with hydrophobic material, D, completed slide with antigen in wells.



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<u>FIGURE 11</u> Positive fluorescence of <u>Plasmodium falciparum</u> schizonts using positive control serum at 1.320. FITC-labelled sheep anti-human globulin serum was used as the indicator.

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<u>Photography</u> The Zeiss photographic unit 35 mm camera fitted to the above microscope was used. Colour film Kodachrome ASA 200, a magnification of x40 and exposure times between 1-2 minutes produced the best photographs. The exposure time depended on the intensity of the fluorescence. Fluorescence quite often faded after 2 minutes. The film was developed at a rate of 800 ASA.

ii) <u>Immunofluorescent method for detecting complement fixing malarial antibodies</u>

A slight modification of the IFA procedure enabled study of the complement-fixing ability of antibodies that gives rise to identifiable staining patterns with antiglobulin conjugates.

Malaria antigen slides were prepared as described by Voller and O'Neill (1971). Test sera were heated at 56°C for 30 minutes in order to inactivate complement. One drop of serum was added to 6 drops of PBS and 3 drops of fresh normal serum (unheated and heated at 56°C for 30 minutes, and known to be negative for malaria antibody). IFA was carried out as described by Voller and O'Neill (1971) but anti-human complement conjugate (anti C3; $\beta_{1A}/_{1C}$) was substituted for anti-immunoglobulin. Positive and negative sera were included in the assay, and the antibody activity of sera with heated and unheated normal human serum was evaluated.

iii) <u>The Enzyme-Linked Immunosorbent Assay (ELISA)</u>

The indirect microplate ELISA for the detection and measurement of antibody (Voller et al, 1976) was employed in this study. The microplate method with <u>P.falciparum</u> antigen described by Voller et al (1974) was modified as follows:

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Antigen Two antigen preparations were used, one from an infected human placenta and the other from infected Aotus monkey.

a) A placenta heavily parasitised with *P.falciparum* was collected under aseptic conditions and stored on ice at +4°C (freezing placenta at this stage affected the antigenicity of the plasmodial antigen preparation). Infected red cells were washed from placenta with isotonic phosphate buffered saline (PBS) pH 7.2, 0.15 M by centrifugation in a mistral high speed centrifuge at +4°C. The washing of the placenta was carried out 3 times. The supernatant from all the washings was pooled and centrifuged at 3000 g for 30 minutes at +4°C. The buffy coat containing the *P.falciparum* parasitised red cells was collected and made uo to 15 ml with PBS. Thin and thick blood films were made and stained with Giemsa to estimate the parasite density. A count of 10-20 schizonts or mature trophozoites per one oil immersion field of a thin film preparation was considered suitable for soluble antigen preparation. The parasitised cell preparation was disrupted in a Hughes press and freeze dried in 5 ml aliquots and stored at -20°C until used.

To prepare soluble antigen for ELISA, 4 vials of freeze dried parasitised cells were reconstituted in PBS, washed three times in PBS, 15 minutes each wash, and centrifuged at 1,500 g at +4°C. Supernatant fluids containing haemoglobin and red cell membrane were discarded, and the pellet containing parasites was used to prepare soluble antigen. The pellet was suspended in minimum

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quantities of PBS and sonicated at maximum setting of an MSE sonicator for 20 seconds at 4°C, then centrifuged at 10,000 rpm for 30 minutes at +4°C. The residue was discarded and the supernatant (the soluble antigen) was stored at -70°C in 50 µl aliquots.

b) Soluble antigen from Aotus monkey infected with P.falciparum was prepared as in Voller et al (1974).

Reference sera The positive and negative reference sera used in the IFA procedure were used for ELISA.

Conjugates Two enzymes, alkaline phosphatase (ALP) and horseradish peroxidase (HRP) were used for the preparation of enzyme-labelled anti-human globulin conjugates. Antisera used in this study were rabbit antiserum to human IgG (Dako Mercia, Denmark), sheep antiserum to human immunoglobulin (Wellcome Reagents, Beckenham, Kent, UK) and goat antiserum to human IgM (Microbiological Associates, Maryland, USA). These antisera were all supplied as IgG fractions.

Alkaline phosphatase-labelling The one-step glutaraldehyde method as described by Avrameas (1969) was used. The conjugate was stored in the dark at 4°C.

Peroxidase-labelling For the preparation of this conjugate either the two-step glutaraldehyde method by Avrameas and Ternyck (1971) or the periodate method of Wilson and Nakane (1978) was used. Conjugates were preserved in an equal volume of glycerol and stored at 4°C.

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Test procedure A diagrammatic representation of the indirect ELISA method is shown in Figure 12. The materials as described by Voller et al (1979) for alkaline phosphatase-labelled conjugates were used. The substrate used for peroxidase conjugate was ortho-phenylenediamine. This was made up immediately before use; 40 mg orthophenylenediamine (powder from Sigma, USA) was made up in 100 ml of phosphate-citrate buffer pH 5.0 to which 40 µl H_2O_2 is added. This substrate is very light-sensitive and must be used immediately after it is prepared. Reaction stopping solution was 2.5 M H_2SO_4.

Colour reaction produced by HRP conjugate was read in a spectrophotometer at 492 nm.

Carrier surface Microtitration plates polystyrene M29,AR Dynawell Cooke Microtitre cuvettes, and polyvinyl Cooke Microtitre plates 220-29 were used.

Spectrophotometer An ELISAreader obtained from Dynatech (Sussex, UK) was used.

Optimal test conditions for malarial ELISA Suitable dilutions for the two antigen preparations were determined by a chequerboard titration. Serial dilutions of the antigens were made up in coating buffer and 200 µl of each dilution (1:50, 1:10, 1:200, 1:400, 1:800 to 1:6400) were added to a horizontal row of the wells in the microtitre plate. Incubation of varying times and temperatures was carried out (allowing the antigen to absorb to the plastic surface). The plates were washed by emptying and filling with PBS Tween from a wash bottle. The washing process was repeated 3 times, 3 minutes for each wash. The plates were

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FIGURE 12 A diagrammatic representation of the indirect enzyme linked immunosorbent assay (ELISA) of antibody

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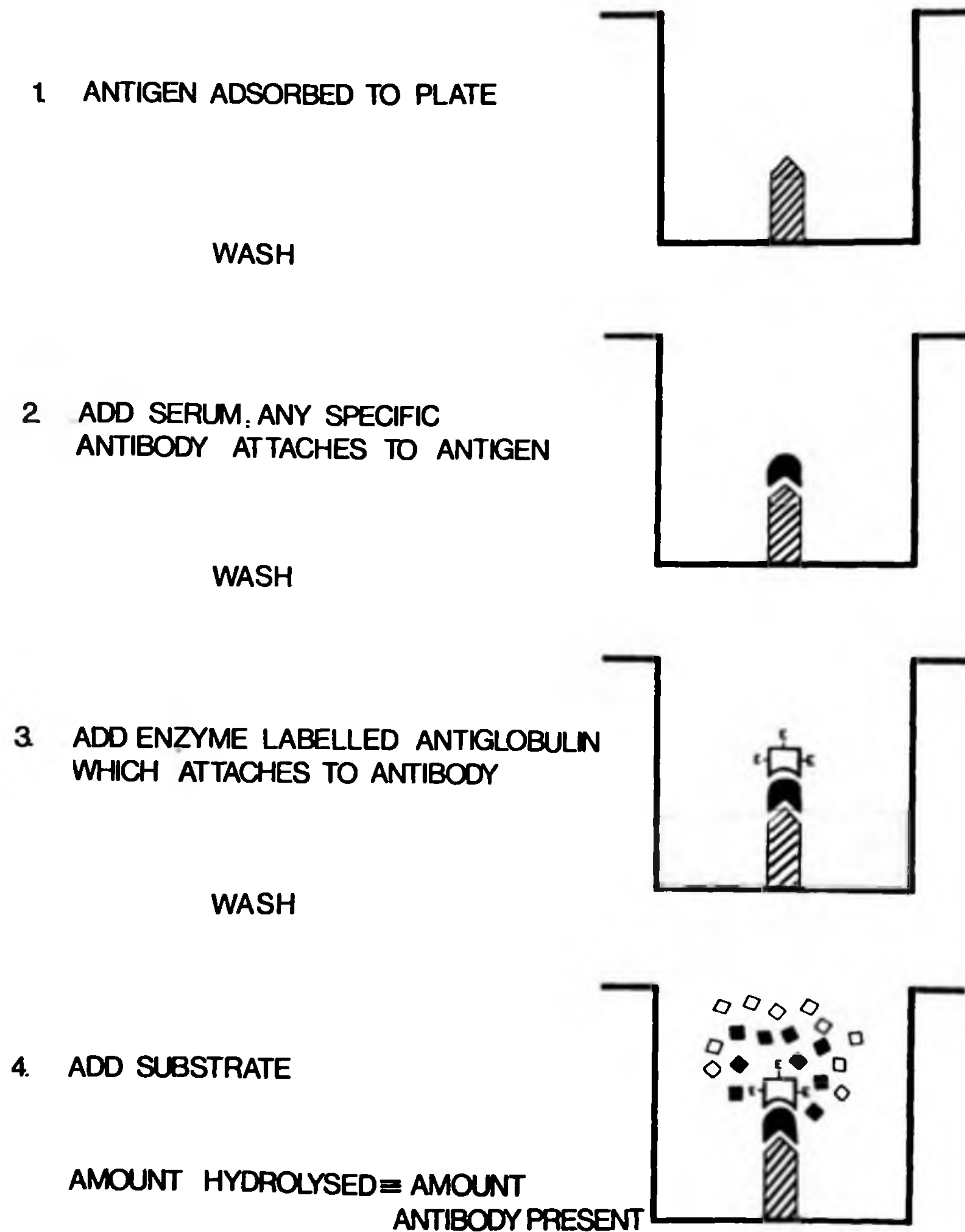

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then shaken dry. Two-fold serial dilution of 2 reference sera (positive and negative for malaria) were made in PBS-Tween and 200 µl amounts were added to vertical rows of wells so that each serum dilution was reacted with each antigen dilution. A row containing only PBS-Tween was included. Plates were incubated for two hours at room temperature in a humid box, then washed. The conjugate of alkaline phosphatase or peroxidase-labelled sheep or rabbit anti-human immunoglobulin was used at the working dilution and 200 µl amounts were added to each well and incubated at +4°C for 18 hours (overnight). Plates were washed as before. Substrate solution was made on day required and 200 µl amounts were added to each well and incubated at room temperature (20°C). The reaction was stopped with 50 µl amounts of 3 M NaOH for ALP conjugate or 2.5 M H_2SO_4 for HRP conjugate after 30 minutes. The reaction in each well was read in a spectrophotometer, and that combination of the antigen/serum dilution which gave maximal separation between positive and negative test samples but still gave negative values below 0.2 was chosen for subsequent tests.

<u>Antigen coating</u> There was no detectable difference in the malaria system between antigen coated at 20°C after 4 hours and overnight coating (18 hours) at +4°C. Plate sensitisation was therefore carried out at 20°C for 4 hours and plates were washed immediately.

<u>Standardisation of substrate reaction</u> A positive reference serum was included in several wells of each plate. The reaction of the enzyme with its substrate (p-nitrophenyl phosphate) in these wells was monitored at 5 minute intervals and the reaction stopped with 3 M NaOH, when the reference positive had reached a value of 1.70 at E405.

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Determination of specificity of malarial ELISA test The specificity of the assay for soluble P.falciparum antigen was assessed by inhibition of malaria antibody using soluble malaria antigen in a range of dilutions added to sera with known ELISA values. These were incubated overnight at 4°C. The anti-malarial activity was assessed by ELISA and IFA tests.

Immunoglobulin class specificity of malarial antibodies Monospecific and polyvalent conjugates, alkaline phosphatase-labelled anti-human IgG and IgM and anti-whole immunoglobulins were used in the malaria ELISA to test for immunoglobulin class specific antibody bound to antigen in ELISA. Similar tests were carried out by IFA test using FITC-labelled anti-human IgG, IgM and anti-whole globulin conjugates.

Gel filtration procedure Separation of sera was carried out using sephadex G-200 or sephacryl 200 (Pharmacia, Uppsala, Sweden), as described by Fahey and Terry (1978).

Method of 2-mercaptoethanol reduction 2-mercaptoethanol (2-ME) 0.2 M was prepared in PBS pH 7.2. This was added to serum in 1:1 ratio. The mixture was incubated at 37°C for one hour and then dialysed overnight in PBS instead of 2-ME and similarly dialysed overnight in PBS, the method adapted from Haurowitz (1968).

3. DETERMINATION OF ANTI-PARASITIC ANTIBODIES OTHER THAN MALARIA

i) The rapid IFA test for anti-parasitic antibodies to several antigens on a single microscope slide

The method described here is based on a multiple antigen slide test developed by Dr. G.J. Kane (Wellcome Research Laboratories) in 1973. It is a rapid IFA test which lends itself to simultaneous testing of antibodies to several antigens at single or serial serum dilutions on a

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single microscope slide. In this study specific antibodies to *Mycobacterium tuberculosis*, *Vibrio cholerae*, *Treponema pallidum*, *Entamoeba histolytica*, *Toxoplasma gondii*, *T.gambiense* and *T.rhodesiense*, *Schistosoma mansoni* and *Toxocara canis* were measured.

Antigens The following freeze-dried antigens were provided by Dr. G.J. Kane (Wellcome Research Laboratories): *Mycobacterium tuberculosis*, *Vibrio cholerae*, *Treponema pallidum*, *Entamoeba histolytica*, *Toxoplasma gondii*, *Trypanosoma gambiense*, and *T.rhodesiense*, *Schistosoma mansoni* (cercariae) and *Toxocara canis* (larvae).

Reference sera Positive and negative sera for all the clinical conditions listed above were provided by Dr. G.J. Kane.

Other materials Fluorescein isothiocyanate (FITC)-labelled anti-human globulins were obtained from Wellcome Reagents. Polarfluor B semi-permanent mounting fluid was obtained from Polaron Equipment Ltd., Watford and stored at 4°C. Evans blue dye was prepared as a 1% aqueous solution. Multispot teflon-coated slides were also prepared, as described below.

The area of a standard 76 x 25 mm microscope slide was outlined on a piece of graph paper. Within this area another area 50 x 22 mm was outlined using a Chance No.1 coverslip of this size. An array of dots were marked out within the coverslip area. Figure 13 shows a typical arrangement for preparation of the slides.

A microscope slide was placed on the above design and small drops of glycerol were added exactly above each dot on the design using a pasteur pipette. Drops were spread no more than a 2-3 mm diameter. Each prepared slide is given a reference number, marked towards one

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A TYPICAL ARRANGEMENT OF PARASITE ANTIGENS ON MICROSCOPE SLIDE FOR RAPID IFA TEST

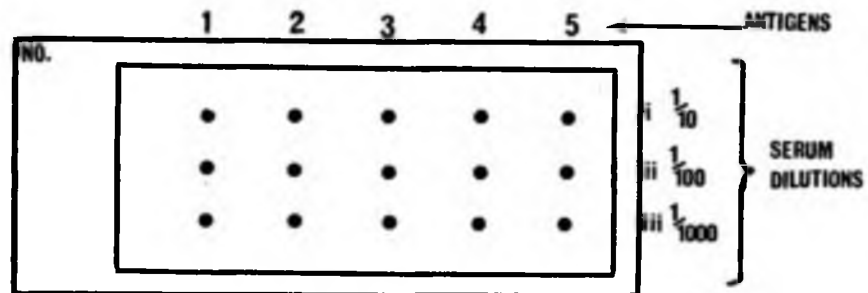

FIGURE 13 Typical arrangement for the preparation of multispot teflon-coated slides for the rapid IFA test for antiparasitic antibodies to several antigens on a single microscope slide. The placement of antigens and serum dilutions have been indicated.

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end of the slide using a water soluble felt-tip pen. The slide was sprayed lightly with a teflon aerosol containing an adhesive (Fisons Scientific Apparatus). The slide was then washed under tap water followed by ethanol, then dried and stored.

Method Freeze-dried antigens were re-suspended in sterile water containing 0.1% sodium azide. A drop of reconstituted antigen (10 μl) was applied to wells of the teflon-coated slides. Five antigens were applied per slide and each antigen was applied to the three wells of the vertical row of wells as shown in Figure 13. The antigens were air-dried and then fixed with methanol for 10 minutes. Sera were reacted with all eight antigens at three dilutions: 1:10, 1:100 and 1:1,000, as indicated in Figure 13. One drop of diluted serum was placed on appropriate antigen wells. Slides were incubated at room temperature for 30 minutes, washed in PBS pH 7.5 and left to stand in PBS with continuous washing for a further 30 minutes. The slides were drained dry and to each test area was added a drop of 1:20 dilution of fluorescein-labelled anti-human immunoglobulin prepared with 0.1% Evans blue stain in PBS. Slides were incubated for 40 minutes and dried and mounted under Chance No.1 coverslips (50 x 22 mm) using Polarfluor B. Slides were examined under a uv illumination at 400x magnification using a dark ground condenser. A positive result was recorded when the antigen particles appeared discrete and showed a distinct yellow-green fluorescence on their outer surface. Negative reactions were dull red. The last titre showing visible fluorescence was the end-point titre of serum.

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A set of standard slides were also prepared with known positive and negative sera for each parasite antigen tested, and these were used for comparison and were found most useful in borderline cases of weak positives.

ii) ELISA tests for onchocercal antibodies

Soluble antigen from *Onchocerca gutturosa* antigen was used and this was provided by Dr.A. Bartlett (NLCM). The method was essentially as described by Voller et al (1976). The *O.gutturosa* antigen was used at a dilution of 1:1000. In each assay a positive serum (from a patient with palpable nodules and microfilariae in skin) and a negative serum were included. Alkaline phosphatase-labelled anti-human conjugate was used at a dilution of 1:3000. Results were obtained as absorbance values at E405.

iii) Detection of viral antigen and antibody in serum

This assay was kindly carried out by Dr. K.C. Ng who has standardised this test at the Bone and Joint Research Unit, The London Hospital Medical College.

Patients' sera were tested for antibodies to Epstein-Barr capsid antigen (VCA) and hepatitis B surface antigen (HBsAg).

The antibodies to VCA were detected essentially as described by Henley and Henley (1966) using P_3HR cells as VCA-positive control and Raji cells as VCA-negative. All sera were tested at a screening dilution of 1:8.

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The HBsAg was measured by a direct passive haemagglutination technique using a test kit from Wellcome Reagents. Sera were tested at four-fold serial dilutions from 1:2 to 1:512 in order to obtain an end-point titre. Positive sera were further tested using the hepatitis B antigen haemagglutination inhibition confirmatory kit (Wellcome Reagents). Neutralisation of HBsAg using specific antiserum inhibits genuine positive agglutination enabling rapid confirmation of positive serum. (Mr. Ian Cayzer of Wellcome Reagents kindly tested all sera for the presence or absence of HBsAg).

4. QUANTITATION OF SERUM IMMUNOGLOBULINS AND ALBUMIN

Four immunochemical methods were used for the measurement of serum immunoglobulins IgG, IgA, IgM, IgE and albumin in healthy and clinical serum samples.

i) Automated Immunoprecipitin Technique (AIP)

This method was based on the principle of fluoronephelometer described by Ritchie et al (1969) and the equipment used in this study was constructed from standard Technicon modules (Technicon Instruments Incorporation, UK).

In the AIP system, specific antibody reacted with specific protein to be analysed, in dilute solution, in antibody excess. The antibody concentration was kept constant while the antigen concentration was varied by using standards of known concentrations and unknown test samples. The amount of complex formation was therefore a function of the antigen concentration. In dilute solution, these complexes remained in solution

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and their concentration was determined by measuring the light scattered at right angles to the incident beam. The amount of light scattered was a function of the number and molecular size of the complexes formed as well as the wavelength of light. Under test conditions the size of the molecules varied only slightly and so the amount of light scattered was proportional to the antigen concentration. The complexes formed were measured on a calibrated chart recorder as reaction peaks. Standard reference sera with known concentrations provided peak heights from which concentration of unknown sera could be calibrated.

Materials The diluent used in this system consisted of normal saline (0.15 mol/litre) containing 0.5 ml/litre of the surfactants 'Tween 20' (Koch Light Ltd., Colnbrook, UK). The diluent was filtered through a Millipore filter (0.22 mm pore size).

Antisera All antisera used were monospecific. Antiserum to human albumin was raised in sheep (Seward Laboratories, London, UK). Antisera to human IgG and IgM raised in goat were obtained from Kallestat Laboratories (Mn 55418, USA), and rabbit anti-human IgE from Behring Diagnostics (Hounslow, Middlesex, UK). All antisera were diluted in nephelometric-grade saline (particle-free normal saline + Tween 20).

Reference sera The International Federation of Clinical Chemistry Immunoglobulin Standard IFCC 74/1 was used. IFCC/1 is a liquid preparation standardised against the WHO international reference preparation code no. 67/99 (Whicker et al, 1978). This preparation was found more suitable for nephelometric estimations of immunoglobulin than the WHO reference standard, which is a freeze-dried product which can become turbid

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when reconstituted and gives very high blank readings in nephelometric assays*. Top standards used for IgG and albumin in this assay were well above the normal range 17.2 g/l and 50 g/l for IgG and albumin respectively, and dilutions were made in nephelometric saline.

Quality control sera To maintain quality control, a normal serum which had been previously assayed on the AIP system and on Mancini radial immunodiffusion with assigned concentration of IgG and albumin was included in every run.

Procedure Albumin and IgG were assayed by AIP, anti-serum to human albumin and IgG were diluted to 1:70 in nephelometric saline. The nephelometer was switched on for an hour before a run was started. At the beginning of each run the top standards were aspirated twice, followed by the remaining standards. After the standards, the samples and quality control sera were aspirated. It was found useful to have a quality control sample first then after every ten samples. The flow rate was 120 samples per hour with a sample to wash ratio of 1:1. The standard curve was repeated for every ten samples. The baseline was adjusted to 90% transmission on the recorder.

ii) Modified Laurell immunoelectrophoresis

Polyclonal IgG and IgM exhibit both anodic and cathodic migration in agarose at pH 8.6. The double peaks thus formed made simple height measurement alone inaccurate for quantitation. Weeke (1968) used potassium cyanate to treat sera prior to Laurell immunoelectrophoresis (Laurell, 1966). This carbamylation process increased the surface negative charge of the proteins, and hence their mobility. After such treatment IgG and IgM moved towards the positive pole (IgA however would travel

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* An Ig standard suitable for use in nephelometry has since become available from WHO (Dr. I. Batty, personal communication).

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anodically without a need for this treatment), forming single peaks allowing a more accurate quantitation. Carbamylation was found to take about 6 hours, so formylation using formaldehyde solution was substituted (Slater, 1975). This produced the same results as carbamylation, but after only a 20 minute incubation at room temperature. IgA was also quantitated with formylated sera for convenience and this produced taller, narrower peaks which could be more accurately measured.

Materials Tank buffer - 0.02 M pH 8.6 barbitone buffer. Plate buffer. - 0.04 M pH 8.6 barbitone buffer Agarose Litex type HSA (Glostrup, Denmark) 20 g/l in distilled water. Antisera- Dakopatts rabbit anti-human IgG, IgA and IgM. Formaldehyde - BDH, Poole, Dorset, 36% solution.

Procedure To prepare plates, agarose was melted. Equal volumes of agarose and 0.04 M plate buffer were pipetted into separate tubes in a water bath kept at 56°C.

	Plate size (cm)	Vol buffer (ml)	Vol agarose (ml)
Small plate	8 x 8	5.5	5.5
Large plate	10 x 20	13.5	13.5

To prepare small plates the required volume of anti-serum was added to the buffer tube which was then mixed quickly with an equal volume of agarose at 56°C. Contents were gently poured on to alcohol cleaned plates, resting on a levelling table. Any bubbles formed were lightly flamed. The plate was left to set for 10-15 minutes.

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To prepare large plates, a perspex mould was sandwiched between two large clean plates. The required volume of antisera was pipetted into buffer, buffer and agarose were mixed and the contents were transferred into the mould. When partially set, the plates were transferred into the refrigerator at +4°C for 20 minutes.

Standards were diluted 2-, 4-, 6-, 8- and 10-fold in formaldehyde solution (20 mmol/litre) prepared by diluting 360 g/litre formaldehyde in barbital buffer (20 mmol/litre pH 8.6). Test sera were diluted 1:10. All samples were incubated at room temperature for 30 minutes.

In order to apply standards and sera to agar gel, origin wells were cut about 5 mm apart along one side of prepared agar plates using a stainless steel gel cutter. A cutter with diameter of 1.7 mm was used for IgA and for IgM a diameter of 2.5 mm was used. Agar plugs were sucked out of the wells, and the plates were placed on the electrophoresis apparatus (Shandon, Cheshire, UK), with the origin wells towards the negative pole. Samples and standards were applied with a micropipetter 6 µl and 9 µl for IgA and IgM respectively. Wicks were placed in contact with the plate ends. The plates were run as described by Weeke (1973) at 2 V/cm for 18 hours. The plates were removed from the electrophoresis apparatus after the run, covered with a piece of filter paper and a thick layer of absorbent paper pressed for 10 minutes dried in a stream of warm air, stained for 10 minutes in Coomasie Brilliant Blue (R.A.Lamb, London, UK), and finally destained in a methanol/acetic acid/water mixture (4/1/4 by volume (Weeke, 1973). Peak heights were then measured from the upper edge of the origin well to the tip of the peak. Peak heights were adjusted to fall in the range of 0.5 to 5 cm. A standard curve was constructed on semi-log paper and unknowns were read from it.

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iii) Double antibody radioimmunoassay

This method proved useful for measuring serum IgE since other immunochemical techniques were not sensitive enough to detect the low levels of IgE present in normal sera. A competitive assay using labelled IgE was carried out.

Reagents ^{125}I-labelled IgE was obtained from Pharmacia in a kit. It was reconstituted in 5.5 ml of distilled water as described in the kit. It was further diluted 1 in 10 in the kit buffer before use. Rabbit anti-human IgE was obtained from Behring Diagnostics (Hounslow,Middx. UK). The working reagent was a 1 in 10,000 dilution of the commercially available antiserum. Normal rabbit serum obtained from Wellcome Reagents (Beckenham, Kent, UK) was diluted 1 in 200 with normal saline and sodium azide added. Donkey anti-rabbit serum from Wellcome Reagents was diluted 1 in 48 in normal saline with sodium azide added. Working standard was diluted initially to give a solution of 400 IU/ml. Serial doubling dilutions were made from this to obtain 8 standards and 0.5 ml was required for each standard. Test sera and quality control sera were also diluted 1 in 10 in normal saline.

Procedure 100 µl of suitably diluted samples, standards and quality control sera were incubated with ^{125}I-labelled human IgE and 100 µl of rabbit anti-human IgE at 1:10,000 dilution at room temperature for 24 hours. 100µl of donkey anti-rabbit serum diluted 1:48 and 100 µl of non-immune rabbit serum diluted 1:200 to act as carrier protein, was then added. Following a further overnight incubation at 4°C the precipitate was collected by centrifugation at 1800 g for 20 minutes. The precipitate

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was washed and finally counted on a gamma counter. A punch tape was generated at the time of counting and this was used with a suitable programme on the Hewlett Packard 9815 desk top calculator for processing the results.

iv) Radial immunodiffusion technique

Materials Oxoid No.1 agar (Oxoid Ltd., Basingstoke, UK); 8 x 8 cm glass plates; diethyl barbital buffer (40 mmol/litre); standard antigen solution for IgG, IgA, IgM; laboratory standards and commercial standards from Hyland (USA), Behring (Hounslow, Middx. UK) and Wellcome Reagents (Beckenham, Kent, UK); flat level surface (spirit level used); stainless steel gel punch; humid chamber (plastic box with wet filter paper).

Procedure Equal volumes of diethylbarbital buffer (40 mmol/litre pH 8.6) containing anti-serum and agar 20 g/litre all at 56°C were mixed together and carefully poured on to cleaned 8 x 8 cm glass plates resting on a flat level surface. The optimal dilution of any monospecific anti-serum used depended on the strength of the antiserum and antigen. 12.8 ml of the diluted agar was used in order to produce a 2 mm gel thickness. When the gel had set the plate was placed over graph paper ruled in centimetres to produce 36 intersections 1 cm apart. With a 1.5 mm stainless steel punch, 36 holes were made in the gel.

Samples were diluted as follows in 0.15 mol/litre sodium chloride. For IgA and IgM assay, samples were used neat and diluted two-, three-, five- and tenfold. For IgG assay samples were used neat and diluted by five-,ten- 15,20 and 40-fold. All dilutions were made with the Oxford sampler (Boehringer, Sussex, UK). A sample volume of 3 µl was applied to the wells and the plates were incubated for 16 hours at 37°C.

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The plates were pressed and stained as described for Laurell immunoelectrophoresis. The rings were read using a perspex calibrated ruler (Boehring, Hounslow, UK) with the aid of a light box.

Commercial immunoplates Commercial immunoplates from Hyland and Behring tri-partigen plates were often used for convenience and when available with their respective standards. European sera were diluted twofold for the assay and Ghanaian sera were diluted three- to tenfold so that precipitin rings produced showed better definition and they were also within the range of standards applied.

Standardisation and quality control All working standards used in above tests for the determination of IgG, IgA, IgM and albumin were calibrated against IFCC 74/1, a recently established International Reference Standard for Immunoglobulins (Whicker et al, 1978; Riches et al, 1979).

5. TESTS EMPLOYED IN STUDIES OF THE INTERACTIONS OF ANTIGEN,ANTIBODY AND COMPLEMENT IN MALARIA

i) Indirect immunofluorescent test for the study of in vitro effects of malarial antibody and malaria antigen on complement

Materials

Antigen Malaria slide particulate antigen (*P.falciparum*) was prepared by the method of Voller and O'Neill (1971) and stored at -70°C.

Sera Positive malaria sera and EDTA plasma were obtained from Caucasians with acute malaria (having slide-proved parasitaemia), healthy adult Ghanaians with no parasitaemia but malaria IFA titres of 1:1280, subjects from malarious endemic areas but resident in Britain for more than two years and having no patent parasitaemia and low IFA titres 1:80.

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Negative malaria sera and plasma were obtained from healthy Caucasians, and Caucasians with systemic lupus erythematosus with high levels of rheumatoid factors and immune complexes, and sera from patients with connective tissue diseases containing high levels of antinuclear antibodies.

Normal human serum (NHS) with normal C3 levels (110 mg %) was a pool of sera from healthy Caucasian laboratory workers. All sera were aliquoted in 50 µl amounts and stored at -70°C.

Other materials Fluorescein-labelled anti-human C3 (BIA and BIC) conjugate was kindly provided by Dr. Shireen Chantler of the Wellcome Research Laboratories, and fluorescein-labelled anti-human IgG and IgM were obtained from Wellcome Reagents Ltd.,

Procedure

The tissue complement fixation immunofluorescent test described by Johnson, Holborow and Dorling (1978) was used to observe complement fixing ability of malarial antibody, and activation by the antigen and complement. Malaria particulate antigen was reacted with all serum and plasma samples. All serum and plasma samples were previously heated at 56°C for 30 minutes in order to inactivate intrinsic complement, before they were further incubated with fresh NHS or heat deactivated NHS (one drop of serum was added to six drops of phosphate buffered saline (PBS) pH 7.2 and three drops heated or unheated sera). The immunofluorescent test was then carried out using anti-complement conjugate at a dilution of 1:20, or anti-immunoglobulin conjugate IgG at a dilution of 1:40 and anti-human IgM at 1:10. Several controls were set up. Also, the effects of pretreating slide antigen and sera with 0.1 M MgEGTA and 0.1 M EDTA were

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observed. Table 8 shows the overall test design used. Each test serum was put through the battery of 18 tests, and the effect of EDTA and MgEGTA pre-treatment with antigen was carried out in all 18 tests designed as well as pre-treatment with serum and plasma. After conjugation slides were washed in PBS and mounted in 10% buffered glycerol pH 8.6. The presence or absence of bright positive fluorescent staining of malaria schizonts were read on a Reichert fluorescent microscope with transmitted light. A Zeiss camera was used for photography, and colour photography with an Agfacolour CT18 35 mm film.

ii) Two dimensional immunoelectrophoresis for the study of complement activation by malaria antigen and in vivo complement activation in acute malaria

Materials

Antigen   Soluble P.falciparum antigen was prepared as described in 00.93-98 and was used at a concentration of 428 μg/ml in barbitone buffered saline (PBS) 0.15 M pH 7.6.

Sera   Positive malaria sera: Sera and EDTA plasma samples were obtained from Caucasians with P.falciparum slide-proven infections and having malarial IFA titres of $>$1:20, also sera from adult Ghanaians (resident in Ghana) with negative parasitaemia but malarial IFA titres of 1:1280 and positive for immune complexes (Clq-BA 20% - baseline levels of normal serum 10%). Sera and plasma from subjects from malarious endemic areas but living in Britain for more than two years with low malaria IFA titres 1:80.

Negative malaria sera: Sera negative for malarial antibodies were obtained from health adult Caucasians, Caucasians with systemic lupus erythematosus (SLE) with high levels of rheumatoid factors and immune complexes (Clq-BA 40%) and sera from patients with connective tissue diseases containing high levels of antinuclear antibodies.

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TABLE 8 Test design used to study the *in vitro* effects of malarial antibody and malarial antigen on complement

Test no.	Slide antigen	Antibody	Source of complement (C3)	Conjugate
1	*P.falciparum*	+ Serum or plasma	NCA	Anti-IgG and IgM
2	*P.falciparum*	+ Heated serum or plasma	NCA	Anti-IgG and IgM
3	*P.falciparum*	+ PBS	NCA	Anti-IgG and IgM
4	*P.falciparum*	+ Serum or plasma	NCA	Anti-human C3
5	*P.falciparum*	+ Heated serum or plasma	NCA	Anti-human C3
6	*P.falciparum*	+ PBS	NCA	Anti-human C3
7	*P.falciparum*	+ Serum or plasma	NHS +	Anti-IgG and IgM
8	*P.falciparum*	+ Serum or plasma	Deactivated NHS +	Anti-IgG and IgM
9	*P.falciparum*	+ Heated serum or plasma	NHS +	Anti-IgG and IgM
10	*P.falciparum*	+ Heated serum or plasma	Deactivated NHS +	Anti-IgG and IgM
11	*P.falciparum*	+ PBS	NHS +	Anti-IgG and IgM
12	*P.falciparum*	+ PBS	Deactivated NHS +	Anti-IgG and IgM
13	*P.falciparum*	+ Serum or plasma	NHS +	Anti-human C3
14	*P.falciparum*	+ Serum or plasma	Deactivated NHS +	Anti-human C3
15	*P.falciparum*	+ Heated serum or plasma	NHS +	Anti-human C3
16	*P.falciparum*	+ Heated serum or plasma	Deactivated NHS +	Anti-human C3
17	*P.falciparum*	+ PBS	NHS +	Anti-human C3
18	*P.falciparum*	+ PBS	Deactivated NHS +	Anti-human C3

NCA = No complement added

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Normal human serum (NHS) with normal C3 levels (110 mg %) was a pool of sera from healthy Caucasian laboratory staff. All sera were stored at -70°C in 50 µl amounts.

Antisera Antiserum to human C3 was obtained from Behring Diagnostics (Behring, Middlesex).

Other reagents Inulin was prepared as a 10 mg/ml solution. Also, 0.1 M EDTA and 0.1 M MgEGTA solutions were made in BBS pH 7.6

Procedure

The two dimensional immunoelectrophoresis by Laurell (1973) and modified by Milford-Ward (1977) was used to detect the presence of C3 conversion products in malarious sera, and in NHS incubated with soluble malaria antigen. For this, a series of tests was set up:

a) 50 ml amounts each of NHS, malarious sera and plasma samples, and malaria negative sera and plasma from healthy and disease associated Caucasian groups was added to 50 µl amounts of BBS in the presence or absence of 5 µl 0.1 M EDTA.

b) Incubations of NHS (50 ml) with equal volumes of soluble malaria antigen, malarious sera, malaria negative sera, or inulin were set up in duplicate. To one set was added 5 µl of 0.1 M EDTA and 5 µl of 0.1 M MgEGTA to the other. Tests were incubated at 37°C for 30 minutes. Gel plates were prepared, 2% agarose in buffer (barbitone buffer 0.08 M in 0.025 M EDTA) pH 8.6. Antibody layers for the second dimension contained 1.2% anti-human C3.

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Electrophoresis was carried out in 0.05 M barbitone EDTA buffer pH 8.6 at 10 V/m for 60 minutes for the first dimension, and 2 V/cm for 18 hours for the second dimension. A Shandon cooling plate was used for both dimensions. Activation of complement by the classical and alternative pathways was demonstrated with immune complexes and inulin respectively, and using fresh NHS and deactivated NHS as controls. A peak height obtained for conversion products more than 30% greater than that obtained with normal serum was considered positive.

iii) Clq-binding test

This test was based on the ability of pure Clq to bind immune complexes and on the precipitability of the bound Clq by 2.5-3% polyethylene glycol (PEG).

Isolation and purification of human Clq A modification of the method of Volanakis and Stroud (1972) was used. 200 ml of freshly drawn blood was allowed to stand at room temperature for one hour. The serum was separated after centrifugation at 30,000 g for 30 minutes, leaving a lipid layer at the top which is removed completely. The lipid-free serum was used for isolating Clq. Four volumes of the serum were mixed with one volume of a solution of 0.1 M EDTA pH 7.5 and incubated at 37°C for 10 minutes in order to dissociate the Clq-rs complex. Subsequent steps were carried out at 4°C and involved the addition of EDTA and NaCl with relative salt concentrations (RSC) of 0.04 M and 0.078 M respectively. A precipitate was formed which was recovered after centrifugation at 12,000 g for 30 minutes at 4°C and washed twice in EDTA/NaCl of RSC 0.04. The pellet was redissolved in 10 ml of 0.75 M NaCl and 0.01 M EDTA, pH 5.0, and allowed to stand overnight at 4°C. This solution was centrifuged at 30,000 g the following day, and the supernatant dialysed against EDTA,

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pH 5.0, RSC 0.078 for two hours, using a magnetic stirrer. This dialysis was repeated after changing the EDTA solution. The precipitate was recovered after centrifugation at 1,200 g for 30 minutes and washed twice in a solution containing EDTA. It was then dissolved in 3 ml of 0.30 M NaCl and 0.01 M EDTA, pH 7.5 and again centrifuged at 30,000 g for 30 minutes. The supernatant was regarded as pure Clq and was stored in 500 µl amounts at -70°C.

Quality control The yield was determined on a spectrophotometer at OD 280, and was 2.0 mg of protein. The purity of Clq was checked by performing immunoelectrophoresis on 1.5% agarose in Michaelis buffer containing 10 mm EDTA using anti-whole serum, as well as monospecific anti-Clq antiserum. There was a single distinct line against the anti-Clq antiserum and a faint line against anti-whole serum.

Radioiodination of Clq This was carried out using lactoperoxidase as described (Morrison et al, 1971; Heusser et al, 1973). The materials required for iodination were:

An aliquot of purified Clq, removed from -70°C just before labelling.

Sodium iodide (mw 149,89 Merck)

Radioactive iodine (Radiochemicals, Amersham) $-I^{125}$

Lyophilised lactoperoxidase (grade B, Calbiochem)

30% Hydrogen peroxide (mw 34.0 Merck)

Sodium azide (mw 65.01 Merck)

Trichloracetic acid (TCA) 20%

Veronal Buffered Saline (VBS)

Gamma counter (Beckman Biogamma)

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Labelling was carried out at 4°C in a 5 ml plastic tube (Bio-vial, Beckman). 200 microcuries of ^{125}I were added to 200 microgrammes of Clq. Five microlitres of a solution of sodium iodide (NaI), 0.6 mg/100 ml in VBS were added, followed by 5 µl of hydrogen peroxidase (30%, diluted 1:10,000). The mixture was shaken carefully and allowed to stand at 4°C for 15 minutes. 10 µl of a solution of NaI 6 mg/ml + 0.03 mg/ml sodium azide (NaN_3) were added to stop the reaction. Veronal buffered saline (VBS) was then added to reach a final volume of 2 ml. The uptake of the Clq-^{125}I fixation was counted and when the count was more than 30% the labelled Clq was dialysed overnight against VBS pH 7.2. The labelled Clq was stored in 100 µl amounts and stored at -70°C. This preparation could be kept stored for up to two months.

The Clq binding test

Reagents An aliquot of ^{125}I Clq (100 µl) stored at -70°C; EDTA buffer made up to Na_2H_2; EDTA 0.2 M adjusted to pH 7.2 with 1 M NaOh plus 0.3% Tween 20. Polyethylene glycol solution (PEG) made up of 30 g PEG (mw 6000) in 1000 ml borate buffer (0.1 M boric acid, 0.025 M disodium tetraborate, 0.075 M NaCl) to make a 3% PEG solution. This was prepared fresh for every assay. Test tubes used were Bio-vial polypropylene (Beckman, Palo Alto, California, USA). Beckman Biogamma gamma-counter was used.

Procedure Figure 14 shows a schematic diagram for the test. An aliquot of the labelled Clq was thawed at room temperature on the day of testing and diluted in 5 ml of VBS containing 1% w/v bovine serum albumin (BSA). The solution was centrifuged at 18,000 g for 40 minutes at 4°C, and the supernatant was used for the test. The test was set up in duplicate using

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FIGURE 14 Schematic diagram of the Clq binding test
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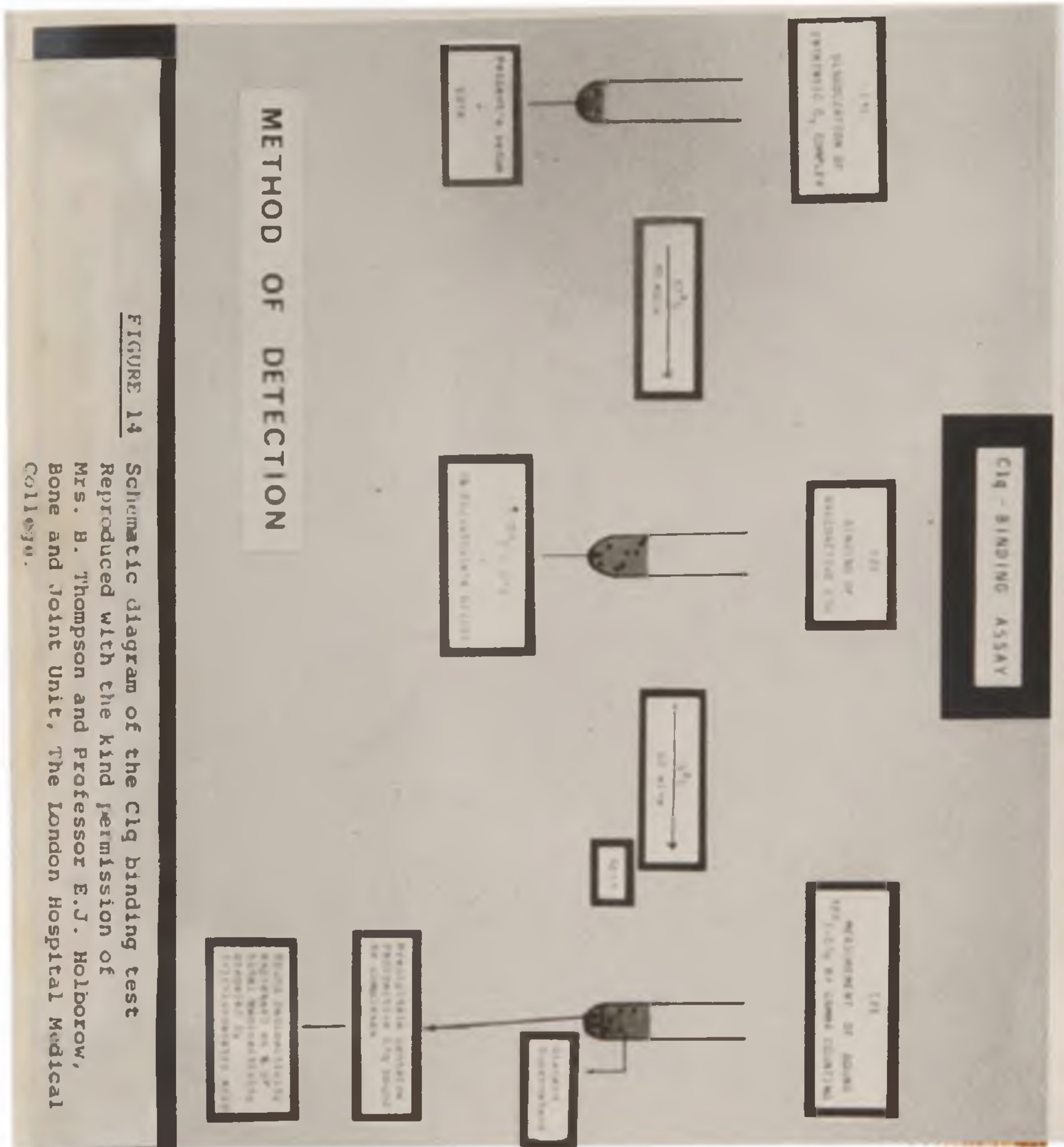
Bone and Joint Unit, The London Hospital Medical
College.



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Bio-vial polypropylene tubes with a set of positive controls consisting of 20% trichloracetic acid (TCA) (which precipitates all proteins and therefore had 100% Clq binding capacity), and decreasing concentrations of heat aggregated human gamma globulin which binds Clq to a decreasing extent. NHS was the negative control. 50 µl of neat test sera were added to each tube, followed by 100 µl of 0.2 M EDTA. The mixtures were incubated at 37°C for half an hour in order to minimise the intrinsic Clq activity in the test sera. The tubes were then transferred to an ice bath in which all subsequent steps were performed. 50 µl of the labelled Clq was added to each tube, followed by 1 ml of 3% polyethylene glycol (PEG). 1 ml of 20% TCA was added to each of the first set of tubes instead of PEG. The tubes were then allowed to stand for one hour at 4°C, and subsequently centrifuged at 2,500 rpm for 20 minutes. The supernatants were discarded and the radioactivity in the tubes counted. The mean of two counts was taken and expressed as a percentage of the average count obtained in the TCA tubes which was always higher than all the others. To ensure that the test had worked, a graph was always drawn for the results obtained with 20% TCA, and various concentrations of aggregated IgG. At a concentration of 3 mg/ml, aggregated human IgG had 90% Clq binding activitity (Clq-BA) whereas NHS had 3-7% Clq-BA. The upper limit of normal was established as 10%.

iv) Polyethylene glycol (PEG) precipitation test

Polyethylene glycol has the ability to precipitate proteins of various sizes depending on the concentration used. At high concentrations it can and does precipitate even small molecular weight proteins like BSA. However, it has been used at low concentrations to precipitate high molecular weight substances of immune complex size (Polson et al, 1964; Creighton

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et al, 1973; Zubler et al, 1975). Creighton et al (1973) used a final concentration of 7.5% PEG in their study, but this concentration produced precipitates in almost all normal controls (Mohammed and Thomson, personal communication). Mohammed and Thompson designed experiments in which decreasing concentrations of PEG were used, until at 4% final concentration no precipitates were obtained in normal sera. The method used in this study is a modification of that of Creighton et al (1973) and it followed close the procedure adopted by Mohammed and Thompson,Bone and Joint Unit, The London Hospital Medical College.

Polyethylene glycol, mw 6000, was dissolved in phosphate buffered saline (PBS) pH 8.4 to a concentration of 8%. The sera were diluted 1:5 in PBS, and equal volumes of these and the PEG solution were mixed to reach a final PEG concentration of 4%. The mixtures were incubated at 37°C for two hours, and then at 4°C for a further 18 hours. They were then centrifuged at 2.800 rpm at 4°C for 30 minutes. The resulting precipitates were washed three times in 4% PEG at 2,800 rpm for five minutes each time. The supernatant was drained off and the precipitates taken up in 0.2 ml PBS, the original volume of diluted serum. The IgG, IgA and IgM contents were determined by Mancini's method (Mancini et al, 1965). The total protein precipitated in each sample was determined by Lorwy's method (Lowry et al, 1951). The amounts of IgG, IgA and IgM in the PEG precipitates were expressed as a percentage of their respective serum levels.

v) Radiobioassay

This method was developed by Onyewotu et al (1974) after observations they made, that guinea-pig peritoneal macrophages possess FC receptors for immunoglobulin G and the third component of complement (C3), but significantly, none for immunoglobulin A. Onyewotu et al (1974) thought it feasible to devise a method in which this property might

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be used to detect circulating immune complexes. Since these macrophages had the ability to take up aggregated human gamma golbulin (AHGG) as well as immune complexes, Onyewotu et al developed a competitive radiobioassay technique in which ^{125}I-labelled AHGG and immune complexes would compete for the receptor sites on the macrophages. This method was used to detect immune complexes in rheumatoid arthritis (Onyewotu et al, 1975) and systemic lupus erythematosus (SLE). Mohammed, Thompson and Holborow (1977) modified the method to make it easily reproducible and more reliable.

Macrophages were obtained from out-bred white guinea-pigs of the Dunkin Hartley strain, three to five days after intraperitoneally injecting them with sterile pre-warmed liquid paraffin. The amount injected was usually 1 ml/20 g. The macrophages were harvested by gentle lavage of the peritoneal cavity with medium 199 using a 10 cc syringe within a 20 cc syringe barrel with holes at the bottom which facilitates the breaking up of macrophage clumps.

The cells were collected in plastic sterilin universal bottles (Sterilin Ltd.) and washed three times in medium 199 by centrifuging at 500 g at 4°C for five minutes in order to remove free liquid paraffin. Cellular debris was removed by treating the macrophages with inactivated (decomplemented) foetal calf serum as described by Shortman et al (1972). Coarse debris sank to the bottom of the tube. The top layer containing 'clear' macrophages was pipetted off and centrifuged once at 500 g at 4°C in a plastic tube approximately 6 xm x 1 cm (Sterilin Ltd.) containing 1 ml foetal calf serum (FCS). Cells were washed twice in medium 199 as previously described to remove FCS.The pellet was resuspended in 2 ml medium 199 and thoroughly, but carefully, mixed by gently tapping the tube until

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no clumps were visible. The cells were counted in a coulter counter, set to count cells with diameter greater than 20 μm. In order to ensure that the cells were actually macrophages, stained smears were examined under the light microscope. The macrophage suspension was adjusted to 1.5 million cells per ml, and kept at 4°C until ready for use.

Preparation of ^{125}I-labelled AHGG Unheated human IgG is first iodinated with ^{125}I by the Chloramine T (Hunter and Greenwood, 1962) and stored as a solution in guinea-pig serum (GPS) at 4°C. Aggregation at 63°C for 15 minutes was carried out immediately after a radiobioassay experiment. The aggregate was spun at 100,000 g for 90 minutes and the pellet resuspended in medium 199, the concentration being adjusted to 1.0 mg/ml.

Treatment of the test tube The falcon tubes used in the experiment were coated by filling with 0.1% GPS and standing at room temperature for 24 hours before use. This reduced the non-specific uptake of radioactivity by the tubes by 75-90%. Coating with higher concentrations of GPS showed no improvement on this.

The test was set up in triplicate sets of precoated plastic tubes. The first set contained PBS, the second normal human serum (NHS) as negative control, and subsequent sets contained sera under test. Sera were diluted 1:5. A set of tubes containing aggregated IgG was used as the positive control. 10 μg of labelled AHGG solution was placed as a drop on the side of each tube and 10 μl of normal or test serum dilution on the opposite side. 500 μl of medium 199 containing approximately 750,000 macrophages were added to each tube so that the suspension of cells came into contact with the labelled AHGG and serum simultaneously. The tubes were shaken gently and incubated at 37°C for 75 minutes and

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shaken every 15 minutes. At the end of incubation period the cells were carefully washed three times with medium 199 and each tube counted for radioactivity for one minute. The mean count of each set of three tubes was taken and the percentage uptake of radio-labelled aggregate calculated as follows:

$$\frac{100 \times \text{cpm test sample}}{\text{cpm PBS sample}} = \begin{array}{l}\%\text{ uptake of }{}^{125}\text{I} \\ \text{labelled uptake}\end{array}$$

When the result was over 100, 100 was subtracted and the result was expressed as a percentage enhancement. When below 100, the value obtained was subtracted from 100 and the result expressed as percentage inhibition.

vi) Detection of immune complexes by measurement of free C3d

Soluble immune complexes in serum can be studied by investigating their reactivity with complement components, especially C3 and its break-down products C3b, C3c and C3d, and the appearance of the degradation products of complement provide indirect evidence for immune complexes. The breakdown products can be seen in trace amounts sometimes in normal persons but elevated levels are associated with complement consuming illnesses such as immune complex states.

In this method the C3d was quantitated in a two step procedure (Perrin et al, 1975). In the first step, C3 and the high molecular weight fragment, C3c, were precipitated with PEG. In the second step, the C3d was measured in the PEG supernatant by single radial immunodiffusion (Mancini et al, 1965) using anti-C3d anti-serum.

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Reagents Anti-human serum monospecific α2D (C3d) was obtained commercially (Central Laboratory of the Netherlands Red Cross Blood Transfusion Centre). Polyethylene glycol (PEG) 22% (mw 6000). Michaelis buffer, pH 8.2 consisted of 80.2 g of Na-S 5-diethylbarbiturate, 52.89 g Na-acetate.$3H_2O$, 9.71 g EDTA, 750 ml 1 M Hcl, 0.25 g NaH_3 in 4.5 litres of distilled water (stock solution) at a working dilution of 1:2. Borate buffer (0.2 M) was made up of 0.1 M boric acid, 0.25 M Na tetraborate and 0.075 M NaCl.

Procedure Agarose 1.5% prepared in Michaelis buffer was melted and temperature maintained at 56°C, then 3.5 ml of anti-C3d was added to give a concentration of 2.8%. The mixture was poured on to alcohol cleaned plates (8 x 8 cm) on a levelling tray and allowed to set. With a 3 mm stainless steel punch 49 holes were punched in gel (1 cm apart). Test and control sera were prepared by adding 100 µl of 22% PEG solution to 100 µl EDTA plasma (11% final PEG concentration), mixed, incubated on ice for two hours, and centrifuged at 300 g for 30 minutes at 4°C. Supernatants (containing C3d) were carefully pipetted and used for the test. C3d standards were prepared from inulin activated normal human serum (NHS). 200 µl activated NHS was added to 200 µl 22% PEG and incubated as before. The supernatant from this preparation was estimated to have 100% C3d. Dilutions of 1:2, 1:4 and 1:8 were made to obtain 50%, 25% and 12.5% standard preparations. 10 µl amounts of standards and supernatants from test and control sera were added to wells in duplicate. Fresh NHS was included as a negative control together with a serum sample with high concentration of C3d as a positive control. Plates were incubated in a humid chamber at room temperature for 48 hours. The plates were pressed and stained as described previously for Laurell rocket immunoelectrophoresis.

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Ring diameters were read using the Behring calibrated ruler for serum immunoglobulins. The per cent concentrations of C3d in test and control sera were read off a standard curve obtained from the standard values plotted (where squared ring diameter was on the ordinate and per cent C3d on the abscissa of a linear/linear graph paper). Positive and negative limits were established and values of 12.5% of inulin-activated NHS were considered positive.

vii) An antibody amplification system investigating malarial antigens in immune complexes

This method was used to look for the presence of malarial antigens in immune complexes detected in acute and chronic malaria sera by the Clq-binding assay as described above. Peritoneal macrophages were obtained from Dunkin-Hartley guinea-pigs, as described above. The final macrophage suspension in medium 199 (M199) was adjusted to 6 x 10^6 cells/ml. For each separate serum studied, 100 µl of the macrophage suspension was taken and made up to 2 ml with M199. Cytochalasin B (Aldrich Chemical Co., UK), dissolved initially at 5 mg/ml in dimethyl sulphoxide, was added to the macrophage suspension to a final concentration of 10 µg/ml. The cells were incubated at 37°C for 20 minutes, washed twice with M199, resuspended in 1.5 ml of M199 together with 1 ml of decomplemented test or control human serum and then incubated at 37°C for one hour. After incubation, all subsequent steps were carried out at 4°C. The cells were washed twice in isotonic phosphate-buffered saline (PBS) pH 7.4, and resuspended in 1.0 ml of M199. This material was then injected immediately into a Dunkin-Hartley guinea-pig. A pre-immune bleed from each guinea-pig used for immunisation had been taken by cardiac puncture. Each guinea-pig was immunised with

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macrophages treated by the above procedure using different aliquots of the same human serum on days 1, 10, 15 and exsanguinated on day 30. All guinea-pig sera were stored in aliquots with NaN_3 at $-20^{o}C$.

The presence of malaria antibodies in the pre- and post-immune sera were determined by the standard and amplified ELISA described above. Soluble P.falciparum antigen was passively adsorbed on to wells in a polystyrene microtitre plate. Test sera were reacted with the sensitised solid phase, incubated and washed. Rabbit anti-guinea-pig serum was used as the antibody bridge and alkaline phosphatase labelled goat anti-rabbit conjugate 1:2000 dilution (pre-absorbed with guinea-pig serum) was added, incubated and washed. The substrate (p-nitrophenyl phosphate 1 mg/ml) was added. The chromogenic reaction of reaction wells was observed and the absorbance at E405 was recorded.

viii) The amplified enzyme-linked immunosorbent assay

This technique is a modification of the standard ELISA as described above, and employs the use of an antibody (bridge). The method described by Sternberger et al (1970) was used.

ix) Localisation of immune complexes in germinal centres of mouse spleen

The method of Brown et al (1970; 1973) was used to investigate the antigenic nature and biological properties of soluble immune complexes detected in malarious sera.

Procedure 200 µl volumes each of a) malarious sera shown to have immune complexes, b) heat aggregated IgG 100 µl/ml (Kabi AB, Sweden) carried out by heating a 1% (w/v) solution at $63^{o}C$ for 15 minutes, and c) a soluble malarial (P.falciparum) antigen prepared as previously described (p.93-98).

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were injected peritoneally into inbred CBA mice of either sex. Mice were killed 24 hours after injection and their spleens were removed immediately, snap-frozen as described before and cryostat sections were prepared as for direct immunofluorescence studies (Johnson et al, 1968). Fluorescein-labelled anti-human IgG, IgM and IgA conjugates were absorbed (1:2) with normal mouse serum and used at a dilution of 1:100. Sections were stained for human IgG and IgM to detect localisation of immune complexes. To test for the localisation of malarial antigen in spleen germinal centres, spleen sections from normal mice injected with normal saline and mice injected with soluble malaria antigen were first reacted with malarial positive serum at a dilution of 1:20, for 30 minutes, washed and then tested for bound malarial antibody using anti-human IgG, IgM and IgA FITC-labelled conjugates. Positive localisation of immune complexes showed a specific dendritic pattern which occupied only the germinal centres, with all other areas of the spleen showing no staining. A Reicherts quartz-halogen lamp fluorescent microscope was used.

6. METHODS USED FOR THE DETECTION OF AUTOANTIBODIES

Several methods were used to detect the presence of a wide range of autoantibodies in Ghanaian sera. These included immunofluorescence, red cell and latex agglutination, enzyme-linked immunosorbent assay and radioimmunoassay.

i) Immunofluorescence (IF) tests for the detection of tissue-reactive autoantibodies

The procedure described by Johnson et al (1978) was used in this study.

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Preparation of tissue section A composite block convenient for screening a wide range of autoantibodies was made from rat liver, stomach and kidney. Small pieces of rat liver, stomach (fundal region) and kidney (transverse section) were obtained from a freshly killed rat (Black Hoodyoung rat). The tissues were trimmed with a sharp scalpel knife and blade with minimal pressure and orientated on a small square of saline-wetted filter paper placed on a microtome chuck. This was carefully snap frozen by means of a CO_2 freezing chuck assembly (SLEE, UK) which discharged CO_2 jet on to composite block tissue without disturbing the relative positions of tissue on chuck. The whole procedure was completed within 20 minutes of the animal being killed. Chuck with attached tissues was immediately stored in polythene bags at -70°C. Once frozen, care was taken to ensure that tissues were not allowed to thaw before use.

Biopsy samples were snap-frozen in tissue-Tek OCT compound in iso-pentane chilled by liquid nitrogen as described by Johnson et al (1978).

Sections from unfixed tissues snap-frozen in one of the above two methods, were obtained by cutting in a cryostat (SLEE, UK). Sections were cut at 6 μm thickness and placed on a 'multi-spot' teflon coated slides prepared as described by O'Neill and Johnson (1970) enabling four sera to be tested at two dilutions on a single slide. The sections were immediately dried at room temperature by placing the slides under an electric fan for 30 minutes. This ensured that they remained attached during the staining procedure. Sections were stained on the day they were cut.

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Evaluation of conjugates Fluorescein isothiocyanate (FITC) - labelled sheep anti-human immunoglobulin was obtained from Wellcome Reagents (Beckanham, Kent, UK). To determine appropriate conjugate dilutions chequerboard titration of conjugates was carried out. Cryostat sections of rat liver were prepared. Fourfold dilutions of a serum containing antinuclear antibody were made, starting from 1:10. Serum dilutions were applied to the liver sections. Slides were incubated in a humid chamber at room temperature for 30 minutes. Slides were washed with a jet of PBS pH 7.2, from a wash bottle. Slides were washed for a further 20 minutes in a bath of PBS on a magnetic stirrer. Surplus fluid was removed from the slide with tissue paper, and the sections were left moist. Twofold conjugate dilutions, starting from 1:10 to 1:1280 were applied to moist tissue sections. Each conjugate dilution covered a set of sections treated with the range of serum dilutions (1:10 - 1:10240). Slides were incubated in a humid chamber for 30 minutes at room temperature. They were rinsed with a jet of PBS and further washed in a bath of PBS on a magnetic stirrer for one hour. Surplus fluid was removed with tissue paper and slides were mounted in 10% buffered glycerol pH 8.6. Results with a satisfactory conjugate are shown in Figure 15. A maximum serum titre of 1:64) **was obtained** with a conjugate end-point titre of 1:80 -the'plateau end-point'.The working dilution of 1:80 was chosen so that no non-specific background staining occurred.

Staining procedure (for indirect method) This was used to detect antibodies, immunoglobulins, and complement components in tissue specimens. Sera were tested at two dilutions, 1:20 and 1:40. A further titration was carried out when positive levels of autoantibodies in sera exceeded 1:40. A large proportion of Ghanaian and malarious Caucasian sera often

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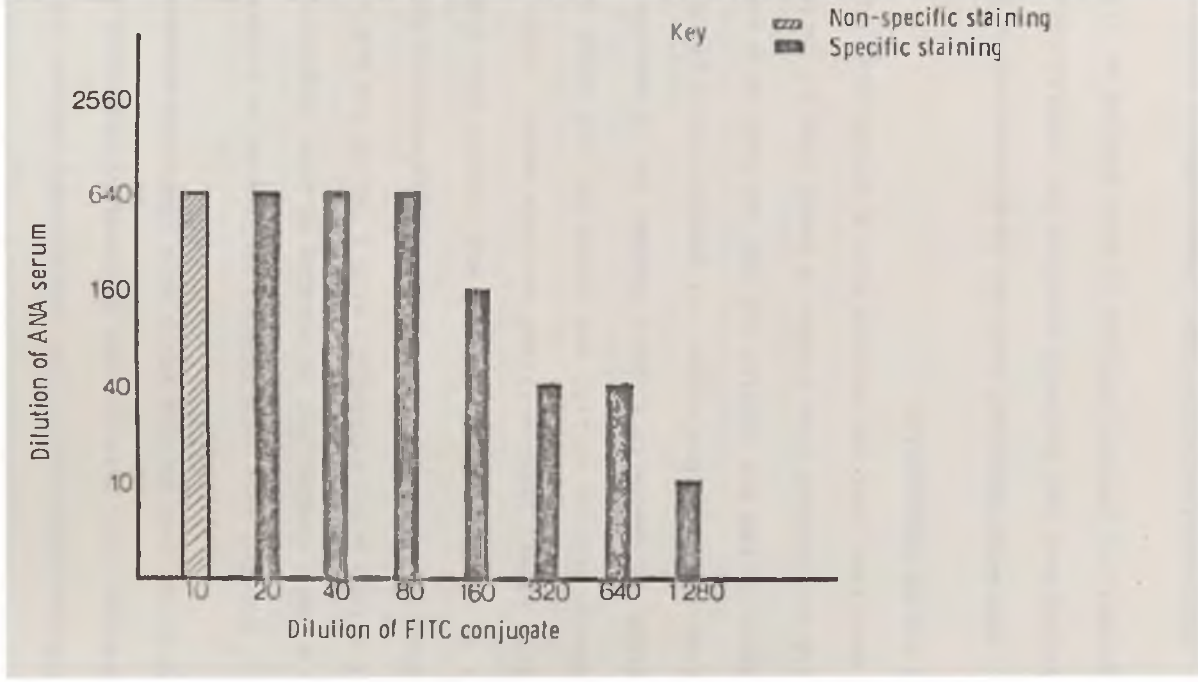
FIGURE 15 Block titration of antiglobulin conjugate and serum containing antinuclear antibody (ANA): the maximum titre for the ANA serum is obtained with dilutions of conjugate extending to 1:80 - the 'plateau end point'



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showed high non-specific background staining at 1:10 dilution, and this made the discrimination of other patterns difficult. However, using a screening dilution of 1:20 considerably reduced this non-specific background staining. All sera, except for healthy Caucasian sera (tested at 1:10 and 1:40) used in this study were therefore screened at 1:20 and 1:40.

Diluted sera were applied to tissues on slides. Slides were left in a humid chamber for 30 minutes at room temperature. Sera were rinsed off slides after incubation with a jet of PBS and slides were further washed in a bath of PBS with continuous staining on a magnetic stirrer for 30 minutes. Surplus fluid was removed from slides carefully with tissue paper, leaving tissue sections moist. FITC-labelled anti-human globulin diluted at 1:80 was placed on test areas on slides. Slides were further incubated in a humid chamber for 30 minutes at room temperature. Excess conjugate was rinsed off gently with a jet of PBS, and continuous washing in PBS was carried out for one hour on a magnetic stirrer. Surplus fluid was removed from slides. A small drop of buffered glycerol was added to each test area and mounted with a coverslip. The slides were read on the day of preparation.

For every batch of test set up a titration of a known positive and negative sera was included ensuring the sensitivity, specificity and comparability between batches of sera stained on different days.

FITC-labelled anti-whole immunoglobulin conjugate was used when screening for the presence of tissue-reactive autoantibodies. However, IgG, IgA and IgM class-specific conjugates were used to determine the class specificity of a wide range of anti-nuclear antibodies observed in this study.

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ii) Method demonstrating complement fixing ability of autoantibodies)as applied to tissue reactive autoantibodies

A modification of the indirect staining procedure allowed complement fixing ability of some autoantibodies to be studied. Taking antinuclear antibody as an example, the test was carried out as follows: Sera staining positive for antinuclear antibody was heat-deactivated (56°C for 30 minutes). An equal volume of fresh serum shown to be negative for ANA at a dilution of 1:2 was added. The indirect method was carried out as detailed above. The only difference was the use of anti-human complement labelled with FITC in place of FITC-labelled anti-human immunoglobulin. Control tests were also carried out using heat-deactivated fresh sera. The test was positive if positive staining occurred with unheated fresh serum, but not with heat-deactivated fresh serum. Sections were read on a Reichert fluorescent microscope with transmitted light. A Zeiss camera unit was used for photography, and colour photography with an Agfacolour CT18 35 mm film. Exposure times of 15-30 seconds were used at x 40 magnification.

iii) Detection of antibodies to native DNA

The non-pathogenic haemoflagellate *Crithidia luciliae* was used for the detection by immunofluorescence of antibodies specific for native DNA. The method used was that described by Aarden et al (1975).

Preparation of antigen The organism was cultured in Bacto tryptose medium pH 7.4 at 24°C. The organisms were recovered from culture when in their logarithmic growth-phase by centrifugation and washed three times with PBS at 300 g for 10 minutes. A suspension of 20 x 10^6 organisms

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per ml was made in distilled water containing 0.1% bovine serum albumin (BSA). The distilled water caused distension of the organisms which facilitated recognition of the kinetoplast, addition of BSA improved the morphological appearance of the organisms. The morphology was checked by staining with ethidium bromide (1 mg/ml) followed by a quick wash (1 minute). Ethidium bromide intercalated in ds-DNA, and the resulting fluorescence of the kinetoplast and the nucleus of the organism was visualised by fluorescence microscopy with the FITC setting as detailed above. Small drops of suspension (10 µl were dried on multi-spot microscope slides under a fan and fixed in 96% ethanol for 10 minutes at room temperature. Slides not used immediately were stored at -20°C in air-tight plastic bags.

Test procedure The indirect staining method as described above was carried out. The sera under investigation were diluted 10 times in PBS.

iv) Detection of autoantibodies to cytoskeletal cells

Cultured fibroblasts are ideal substrates for detection of autoantibodies to cytoskeletal cellular structure (microfilaments, microtubules and intermediate filaments), and by incubating fibroblasts with colchicine, vinblastin and cytochalasin B, the morphological characteristics of smooth muscle antibodies observed in Ghanaian sera could be studied.

Monolayers of cultured foetal lung fibroblast (Toh and Hard, 1977) were examined after 1-3 subcultures. Cells were then subcultured on glass coverslips in 35 mm culture dishes containing Dulbecco's modified Eagle's medium and 5% foetal calf serum. The cells were examined after incubation for 24-72 hours at 37°C in a humidified atmosphere containing

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5% CO_2 and 95% air, and when satisfactory, were used for immunofluorescence. Cells were also tested after incubation with Colchicin (0.5 mg/ml) for 12 hours, vinblastin (10 mg/ml) for four hours, and cytochalasin B (10 mg/ml) for 30 minutes. Before testing, monolayers were rinsed with warm phosphate buffered saline (Coon's PBS), fixed in absolute acetone for 15 minutes at -20°C and air dried. All sera tested on cultured fibroblast were screened at a dilution of 1:8 in PBS. FITC-labelled anti-human immunoglobulin was used to detect bound antibody.

v) Determination of the solubility of nuclear staining antigens

A number of Ghanaian sera gave positive ANA staining with a wide range of nuclear patterns. The characteristics of the nuclear antigens in the varied patterns observed was studied as follows: Freshly cut and dried rat liver cryostat tissue sections were pre-treated in PBS at room temperature at varying times (20, 30, 40 and 60 minutes) before the indirect immunofluorescent staining test was carried out. Control slides of untreated cryostat sections were used.

vi) Haemagglutination (HA) tests for the detection of thyroid microsomal and thyroglobulin antibodies

Procedure The 'Thymune T and M' haemagglutination test kits (Wellcome Reagents Ltd.) were used for the detection of thyroid microsomal and thyroglobulin antibodies in serum. (These kits were kindly donated by Mr. Ian Cayzer of the Wellcome Research Laboratories, Beckenham).

Both haemagglutination tests were carried out using microtitration apparatus with U-welled plates. For the thyroglobulin HA test, unheated serum samples were titrated in doubling dilutions using standard 0.025 ml volumes starting at a dilution of 1:10. To each 0.025 ml of diluted serum

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was added 0.025 ml of sensitised cells. A control for each serum was included at a dilution of 1:10 by adding 0.025 ml of unsensitised cells. The microsomal HA test was performed by making fourfold dilutions of serum inactivated at 56°C for 30 minutes, starting at a dilution of 1:100. To each 0.075 ml of diluted serum was added 0.025 ml of sensitised cells. Similarly a control for each serum using unsensitised cells was included at the 1:100 level. The contents of the wells were gently mixed using a microplate shaker. Trays were covered with plastic lids and the cells were allowed to settle at room temperature. Cell sedimentation patterns were read after an interval of 30-60 minutes.

vii) The latex agglutination test for detection of IgM rheumatoid factor

Rheumatoid factors (RF) - serum macroglobulins with anti-IgG specificities were detected by the agglutination of IgG coated particles. Human immunoglobulin was coupled to latex particles in a suitable buffer solution. This preparation when reacted with dilution of patients' sera positive for RF, showed flocculation of latex particles which settled out to the bottom of the tube. Sensitised latex in negative serum remained in homogeneous suspension.

Procedure Sensitised latex was prepared by adding a 1:4 dilution of latex suspension (polystyrene latex 15% solids - Yarsley Research Laboratories) to 110 ml of latex buffer (25 g NaCl, 18.75 g glycine, 6.25 g NaN_3, made up to 1 litre with distilled water and pH adjusted to 8.2), and 50 mg of human IgG prepared by salt precipitation by the method of Heide and Schwick (1978). The mixture was stirred at room temperature for 20 minutes.

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Serial twofold serum dilutions, starting from 1:20 to 1:320 were made. Negative and positive control sera were also suitably diluted.

1 ml of sensitised latex was added to each tube containing 1 ml of diluted serum. The mixture was placed in a 56°C water bath for two hours and further incubation was carried out overnight (18 hours) at 4°C. End point titre of sera was taken as the last dilution which showed flocculation of latex particles.

viii) Enzyme linked immunosorbent assay (ELISA) for the detection of rheumatoid factors

The method described by Voller et al (1976) and modified for the detection of rheumatoid factors by Vejtorp et al (1979) was used. The method was essentially as described before. Plates were coated overnight with 0.1mg/ml of human gamma globulin. Test sera were diluted to 1:200 and incubated for twohours at 37°C with the coated plates. Alkaline phosphatase was used to label human gamma globulin which was then added to each well and incubated for three hours at 37°C. Serum positive for RF (by latex agglutination) titre 1:640, and sera negative for RF were used as the positive and negative controls. In each assay, a positive and negative sera were included. A wide range of sera were tested in order to establish the positive and negative limits of the assay as shown in the results.

ix) Enzyme linked immunosorbent assay (ELISA) for the determination and semi-quantification of antibodies to denatured single stranded DNA (ss DNA) in sera

A micro-ELISA for the detection of ss-DNA was developed and evaluated in this study. This method was essentially that of the indirect ELISA by Voller et al (1976) and adapted for detection of ss DNA in serum by Gripenberg et al (1978) with certain modifications. Freeze-dried highly polymerised calf thymus DNA was diluted to 100 µg/ml and dissolved by

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gentle stirring in PBS at $4^{o}C$. The DNA solution was denatured by quickly immersing the volumetric flask into boiling water for 15 minutes. The solution was quickly chilled in an ice bath for 10 minutes and stored in 200 µl aliquots at $-20^{o}C$.

<u>Sera</u> Serum positive for ANA (as tested by indirect immunofluorescence, and positive for both ss-DNA and ds-DNA by the ammonium sulphate precipitation (Farr, 1968)) was used as the positive control serum. Serum from a healthy adult shown to be negative for ANA, ss-DNA and ds-DNA was used as the negative control. Alkaline phosphatase-labelled rabbit anti-human IgG conjugate with a working dilution of 1:3000 was used. The enzyme substrate was p-nitrophenyl phosphate (1 mg/ml).

<u>Procedure</u> The basic procedure for indirect ELISA as outlined above (p.93-98 was carried out. Optimal dilutions of antigen and sera were established by a chequerboard titration, dilutions of 1:1000 and 1:200 were obtained for antigen and sera respectively. Polystyrene microtitre plates (Dynatech) were used as the carrier surface. Results of the test were read spectrophotometrically at 405 mm using the Dynatech ELISAreader.

Anti-ss-DNA activity was inhibited by adding increasing amounts of ss-DNA and ds-DNA (0.1 - 1 mg/ml) to equal volumes of positive sera diluted 1:10. The solutions were incubated overnight at $4^{o}C$. The anti-ss-DNA antibodies present were then tested in the ELISA.

x) <u>Radioimmunoassay for double stranded DNA</u>

In this study, the Farr method (Farr, 1958) as modified by Hughes et al (1971) was used in conjunction with the <u>Crithidia luciliae</u> immunofluorescent test previously described to screen for antibodies to ds-DNA in a wide range of healthy and clinical sera from Ghanaians.

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Reagents for DNA binding

Borate buffer 0.05 M pH 8.0 11.60 g NaCl, 6.184 g boric acid, 3.738 g tetrasodium borate made up to 2 litres with distilled water, and stored at 4°C.

Scintillation fluid Two volumes of toluene and one volume of Triton was made up with PPO 7 g (2, 5, dimethyloxazole); dimethyl Popop 50 mg/l (1, 4-Dl-2 (5-phenyloxazoyl) benzene). This mixture was stored in a dark bottle. Saturated ammonium sulphate solution was also prepared.

Labelled DNA ^{14}C-labelled DNA was obtained from the Radiochemical Centre, Amersham, Bucks, UK. A concentration of 1 µm ^{14}C-DNA per ml was used for the test.

Procedure Sera to be tested were deactivated at 56°C for 30 minutes in a water bath at a dilution of 1:10 (5 µl serum in 50 ml of borate buffer). Duplicate tubes per serum were set up. Deactivated serum dilutions were cooled and 50 µl of labelled DNA solution was added to all tubes and mixed well. Tubes were incubated in a water bath at 37°C for 60 minutes. Further incubation of tubes was carried out for 24 hours at 4°C. Tubes were placed on ice and 100 µl amounts of cold saturated ammonium sulphate solution were added to each tube and mixed well. Tubes were incubated on ice for one hour and centrifuged, 2,000 rpm at 4°C for 45 minutes. 100 µl of the supernatant was pipetted into a scintillation bottle containing 900 µl of borate buffer. Precipitates containing ^{14}C-DNA-anti-DNA complexes were washed in borate buffer by centrifugation at 2,000 rpm for 45 minutes at 4°C. The precipitate was resuspended in 100 µl of borate buffer and was added to 900 µl of borate buffer in scintillation bottles. 10 ml of scintillation fluid was added to each bottle and cooled at 4°C for 15

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minutes. Bottles were counted for 10 minutes on an β emitter liquid scintillation counter. The 'per cent binding activity' was calculated from the formula:

$$\frac{\text{Precipitate CPM} - \text{Supernatant CPM}}{\text{Precipitate CPM} + \text{Supernatant CPM}} \times 100$$

Normal sera usually bound less than 20%.

7. STATISTICAL ANALYSIS

All data were analysed on the computer at the University of London Computer Centre. Programme used was SPSS/Statistical Package for the Social Sciences Version 7.0 for CDC computers. This invaluable service was provided by Mr. Stephen Evans, Lecturer in Computer Sciences, The London Hospital Medical College, Whitechapel, E.l.

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SECTION III : RESULTS AND DISCUSSIONS OF IMMUNO-EPIDEMIOLOGY OF MALARIA IN GHANA

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CHAPTER FIVE

PARASITOLOGY

In order to obtain information on malaria endemicity in Ghana, cross-sectional surveys were carried out in rural and urban populations in Ghana as described above. Malaria parasitological and a wide range of immunological assays were carried out. The parasitological results obtained are discussed here. Serological results and other immunological parameters will be discussed in subsequent chapters.

Parasitological data were obtained on samples from all the Groups A - G of the survey population. The findings are summarised in Tables 9 - 12. The predominant species found throughout was P.falciparum which occurred in 98.5% of all the positive cases. Plasmodium malariae occurred in only 3% often among the age group 1 - 5 years and usually as a mixed infection with P.falciparum. Neither P.vivax nor P.ovale were observed in Ghanaian samples in any of the seven groups of sera. The gametocyte rate of the P.falciparum carriers was very low. In fact throughout the examination only five slides from the adult population showed the presence of gametocytes for P.falciparum.

Table 9 (Group A) shows a higher parasite rate for rural than the urban population. The highest parasite rates occurred in the 3 - 5 years age group in both the rural and urban populations. Interestingly parasitaemia was not detected in children less than three months old. The results show a gradual decline of parasite rate and density with age in both populations. Also placentae from which the 122 cord sera were collected were all parasitologically negative.

The blood donor population (Group B) comprising 225 adults, between 18 - 45 years, were all parasitologically negative.

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TABLE 9 Parasitological data for survey population group A rural and urban populations

Age group in years	No.of slides examined	Slide positivity rate (%)	Parasite density (%) Mean	Range
Rural				
0 - 2	250	43.0	2.0	0.01 - 20
3 - 5	410	55.0	2.4	0.01 - 20
6 - 15	150	18.0	1.2	0.01 - 5
16 - 29	242	8.3	0.9	0.01 - 2
30 and over	340	2.1	0.2	0.01 - 0.9
Total	1,392	27.9	1.1	
Urban				
Cord	122	-	-	-
0 - 2	250	10.0	1.0	0.01 - 10
3 - 5	200	37.0	1.6	0.01 - 12
6 - 15	114	8.7	0.8	0.02 - 2
16 - 29	260	-	-	-
30 and over	320	-	-	-
Total	1,266	8.6	0.6	-

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Table 10 shows the parasitological data for Group C. Only the unprotected rural pregnant women had parasitaemia with a parasite rate of 25% accompanied by a high mean parasite density for the group. The protected rural pregnant group, protected and unprotected urban pregnant women and the rural and urban unprotected non-pregnant controls were all negative.

The Army group of sera (Group D) were all parasitologically negative both before and after chemoprophylaxis was given.

Tables 11a and 11b show parasitological data obtained for Group E before and after vaccination and two months chemoprophylaxis. The high parasite rates for this infant rural population reflected the higher prevalence of malaria in the rural areas. There was some reduction in parasite rates and parasite densities after chemoprophylaxis. This reduction was less marked in the age group 3 - 8 months.

Table 12 shows results for the clinical group of sera (Group F). Acute malaria patients in this group were selected because they showed clinical and parasitological evidence of malaria infection at the time of sampling. The group therefore had 100% parasite rate, and a mean parasite density of 4.50. Multiple thick and thin films (seven slides per patient) were examined for each of the nephrotic syndrome patients and only one of the group was positive for P.falciparum. None showed evidence of P.malariae infection. Of the 28 Burkitt's lymphoma patients examined only three showed parasitaemia, with a parasite rate of 10.7%.

Group G consisted of Caucasian and other miscellaneous Caucasian groups. The 63 Caucasians with malaria forming part of this group were selected because they all showed parasitaemia,ranging from 0.01 to 39% with a mean parasite density of 1.10. Thirty-one of these had P.falciparum infection, 24 had P.vivax and eight had P.ovale. There were three children, aged 6, 7 and 9 years, and the rest were adults of 19 - 65 years.

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TABLE 10 Parasitological data on rural and urban protected and unprotected pregnant women and their age-matched non-pregnant unprotected female control group

Population tested	No.of slides examined	Slide positive rate %	Parasite density (%)	
			Mean	Range
Rural pregnant (unprotected)	100	25	1.20	0.10 - 3.40
Urban pregnant (unprotected)	250	-	-	-
Urban pregnant (protected)	200	-	-	-
Rural pregnant (protected)	50	-	-	-
Rural non-pregnant	50	-	-	-
Urban non-pregnant	107	-	-	-

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TABLE 11a Parasitological data of rural infants at the first survey before malaria prophylaxis and vaccination were initiated (125 slides from individual patients were examined in each group)

Population number and age group in months	Slide positivity rate (%)	Parasite density (%) Mean	Range
Study group			
Group 1 : Age 3 - 8	21.6	1.90	0.01 - 11
Group 2 : Age 9 - 14	42.4	2.50	0.01 - 20
Total 250	32.0	2.05	0.10 - 20
Control group			
Group 1 : Age 3 - 8	30.4	1.4	0.01 - 8
Group 2 : Age 9 - 14	44.0	2.1	0.01 - 20
Total 250	37.0	1.92	0.01 - 20

TABLE 11b Parasitological data of the same group of rural infants at the second survey after vaccination and 2 months of malaria chemoprophylaxis

Population number and age group in months	No.of slides examined	Slide positivity rate (%)	Parasite density (%) Mean	Range
Study group				
Group 1 Age 7 - 10 protected	107	21.1	1.04	0.1 - 3
Group 2 Age 11 - 16 protected	103	22.3	2.11	0.1 - 6
Total	210	21.9	1.90	0.1 - 6
Control group				
Group 1 Age 7 - 10 unprotected	106	25.5	1.60	0.1 - 3
Group 2 Age 11 - 16 unprotected	105	28.6	2.10	0.09 - 9
Total	211	27.0	1.57	0.09 - 9

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TABLE 12 Parasitology data for clinical group of sera

Malaria associated diseases	No.of slides examined	Slide positivity rate (%)	Parasite density (%) Mean	Range
Acute malaria	30	100	4.5	0.01 - 10
Nephrotic syndrome	50	2	0.1	0.10
Tropical splenomegaly syndrome	20	-	-	-
Burkitt's lymphoma	28	10.70	1.1	0.01 - 5
Other disease				
Schistosomiasis	20	-	-	-
Onchocerciasis	30	-	-	-
Chronic tuberculosis	50	-	-	-

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DISCUSSION

Seasonal variations in transmission of malaria are reflected in results obtained from parasitological surveys (Bruce-Chwatt, 1952b; Colbourne and Wright, 1955; Voller and Bruce-Chwatt, 1968; Cornille Brogger et al, 1978). In this study, the surveys were carried out during the dry season, December to May, ending before the start of the rainy season, and during periods when roads to villages are easily accessible. The survey area is also part of the coastal plains with mixed vegetation (coastal grassland, scrub and strand mangrove) shown to be perennial for Anopheline (A.gambiense) breeding (Colbourne and Wright, 1955), although transmission due to lowered vector breeding during the dry season may have influenced the parasitological results obtained in this study.

Results of the present study based on examination of a single blood film, indicate a higher parasite rate and parasite density in rural than urban populations, and would imply that malaria transmission is higher in the rural than in the urban area in Ghana. This confirms observations made by Colbourne and Wright (1955) and the Ghana Ministry of Health Survey (Afro Year Book 1958 - 1960) although the parasite rates reported were higher than those observed here. Parasite rates peaked in children in both populations but occurred earlier in the rural than in the urban children. Levels fell to lower rates by adult life and this fall occurred earlier in the urban than the rural population. These differences between urban and rural populations reflect the lower transmission rates in urban areas and possibly the availability, and a higher intake of malaria drugs among the urban populations. The striking decrease of mean parasite density with age would confirm the development of a more effective acquired immunity with age.

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The negative results obtained with Group B (blood donors) reflect the high adult resistance to the infection suggesting a very high protective immunity, or maybe a combination of acquired immunity and lowered parasite transmission as shown in the urban population, also coupled with an indiscriminate use of malaria drugs.

The interaction between malaria and pregnancy has always been of great interest. Malaria is severe during pregnancy (Colbourne, 1956), and the high placental parasitaemia and lowered resistance of women during gestation has been noted (Clark, 1915; Blacklock and Gordon, 1925; Garnham, 1938). It was therefore surprising that the 122 placentae from urban primigravidae from which cord sera were obtained showed no evidence of parasitaemia. It is possible that the improved socio-economic status of these women and a wider usage of malaria chemoprophylaxis might be responsible. Also, urban pregnant women at 30 week gestation period were parasitologically negative compared to the rural pregnant women who showed a positive rate of 25 per cent. This may reflect the shortcoming of the methods used. Examination of a single slide may not be adequate to show overall prevalence. Colbourne (1956) showed in his study of pregnant women in Accra, Ghana, that when 10 thick smears of blood are examined per each woman tested (100 women) the parasite rate rose from 30 to 41% and the gametocyte rate of P.falciparum rose from 7 to 20%. Thus the probability of detecting low parasitaemia increases with the examination of a large sample of blood. But as Raghavan (1966) observed, even the dedicated among expert microscopists can fail to detect subjects with intermittent parasitaemias.

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Adult non-pregnant females in endemic areas have lowered parasite rates and densities which are rarely accompanied by clinical symptoms (Lawson, 1967; Pingoud, 1969). In this study negative results were obtained from all urban and rural adult non-pregnant females.

Negative results were also obtained for the military officers (Group D) which may reflect the high degree of environmental sanitation practised at the Military Academy which results in elimination of the breeding sites for the Anopheline vectors. Ribbands (1946) showed that environmental sanitation contributed to a reduction in vector breeding during a bush clearing project in Sekondi, Ghana. The exclusion of vectors mainly through the use of mosquito nets and screening of houses (a popular practice at the military academy) reduces transmission of parasites.

Effective malaria chemoprophylaxis has been shown to reduce the transmission of malaria parasites in a highly endemic area (Cornill Brogger et al, 1978). The marginally lowered parasite rate after eight weeks prophylaxis in a group of rural infants 3 to 16 months old in the study (Group E) confirms the above observation. Because in this age group parasitaemia increases with age, and not a decrease as observed after chemoprophylaxis in this group.

These results however should be interpreted with caution since the period of protection with chemoprophylaxis (eight weeks) was not long enough to provide decreases in parasite rates which would be statistically significant. This important point is further stressed by observations made by Cornille Brogger et al (1978) in their study of protected and unprotected Nigerian populations, that in the absence of control the parasite rate can vary by year and season between 30% and 65% for P.falciparum. The marginal

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fall of parasite rate in the protected Ghanaian children may not therefore be due to a decrease associated with suppression by chemoprophylaxis. On the other hand, the absence of parasitaemia in the protected rural pregnant groups (protected by chemoprophylaxis from time of first reporting at ante-natal clinic 8 - 12 weeks of gestation to the time of sampling 30 weeks gestation) in an environment of high parasite transmission rate is a significant finding. It further confirmed that chloroquine, the drug used in this study with known malaria suppressive activity managed to eliminate the asexual forms of the erythrocytic stage of the parasite.

No parasites were observed in the slides of most of the clinical groups of sera and their matched hospital controls (Group F) except the acute malaria patients who had a mean parasite density of 4.50. All the slides for the tropical splenomegaly syndrome patients were negative, but it is their response to malaria chemotherapy, and other clinical features as described by Sagoe (1970) and reported in subsequent chapters of this study that justified their inclusion in the malaria associated disease group. Only one patient was positive among the nephrotic syndrome group and P.falciparum was the parasite species. This observation is contrary to reports from Nigeria and Uganda (Hendrickse and Gilles, 1963; Hendrickse et al, 1972; Kibukamusoke, 1973) where the association of P.malariae with nephrotic syndrome has been confirmed. It is possible that P.malariae levels in the patients in this study were too low to be detected by the parasitological procedures used in this study.

The Burkitt's lymphoma (BL) group showed a parasite rate of 20%. These patients were mainly children within the age group 4 - 11 years of age, and reside in the rural area so that a 20% parasite rate is in keeping with the observed parasitological data from rural populations where children

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show a marked susceptibility to infection. However it has been suggested that BL arises as a result of immunological disorders in children exposed since early infancy to heavy malarial infection (Dolldorf et al, 1964; Burkitt, 1969; O'Conor, 1970), although the actual contribution of malaria to the aetiology of Burkitt's lymphoma is still obscure. There is also considerable circumstantial evidence that BL is caused by Epstein Barr Virus (de The et al, 1978). It is also possible that BL may lead to an enhancement of susceptibility to malaria infection in affected children. However, the mystery of the interaction between virus, malaria parasite and host lymphoid tissue remains to be solved.

Concentration techniques, staining methods and improved microscopy have been developed (Dowling et al, 1966; Sodeman and Jeffery, 1968; Dowling, 1968) in order to improve the microscopical diagnosis of malaria. Despite all these measures positive slides are frequently missed and often loss of parasites occur during thick film staining procedures (Dowling and Shute, 1966). It becomes evident that since parasites cannot always be detected by direct microscopical examination of blood smears other methods must be sought. In this respect the utilisation of serological methods in malaria has provided additional sero-epidemiological tool with which to study the intricate biological events that occur in malaria.

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CHAPTER SIX

SEROLOGY

This study was divided into two main parts: A. (1) An evaluation and validation of the indirect enzyme-linked immunosorbent assay (ELISA) for malaria was made in terms of optimal conditions for the assay, its reproducibility, sensitivity and specificity, together with a comparison of ELISA with the standard immunofluorescent antibody (IFA) test for malaria. (2) The diagnostic potential of malaria ELISA was assessed as was its ability. (3) to reflect changes in transmission following a control programme. B. The above assay was then applied to the study of malaria in the Ghanaian population. Using ELISA as well as the standard IFA test, the following aspects of malaria were investigated:-

1. The prevalence of malaria in Ghanaian rural and urban populations.

2. Changes in immunity to malaria, which are known to occur during pregnancy.

3. The use of malaria antibody levels in differentiating between clinical malaria, malaria associated chronic diseases and other parasitic diseases occurring in patients with chronic exposure to malaria.

4. The effect of malarial chemoprophylaxis on malarial antibody levels.

5. The degree of persistence of malarial antibody in the absence of exposure to malaria, as well as susceptibility to infection and the changes in antibody levels on re-exposure after prolonged absence from endemic area.

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EVALUATION AND VALIDATION OF ELISA FOR MALARIA

The materials and methods used have been described in detail in Chapter Four (pp. 93-98). To establish the optimum conditions for the assay the following investigations were carried out, based on the approaches recommended by Voller et al (1979).

To find the best carrier surface

The first factor of crucial importance to the assay is the type of carrier plate used. The three most widely used microtitre plates in ELISA assays are polystyrene Cooke Microtitre plates M29AR, Dynawell Cooke Microtitre cuvettes, and polyvinyl Cooke Microtitre plates 220-29. Identical tests for malaria antibody were set up on all three and Figure 16 shows results of the tests using positive and negative reference sera at a dilution of 1:200, with a series of antigen dilutions. All three plates showed good discrimination between positive and negative values. The positive/negative ratio (P/N ratio) was highest with polystyrene plates (Table 13) and these were therefore the carrier surface of choice for malaria ELISA.

To find the effect of changing antigen coating time

There was no detectable difference in the malaria system between antigen coated at 20°C after four hours and overnight coating (18 hours) at +4°C. Throughout the study plates were sensitised for four hours at 20°C.

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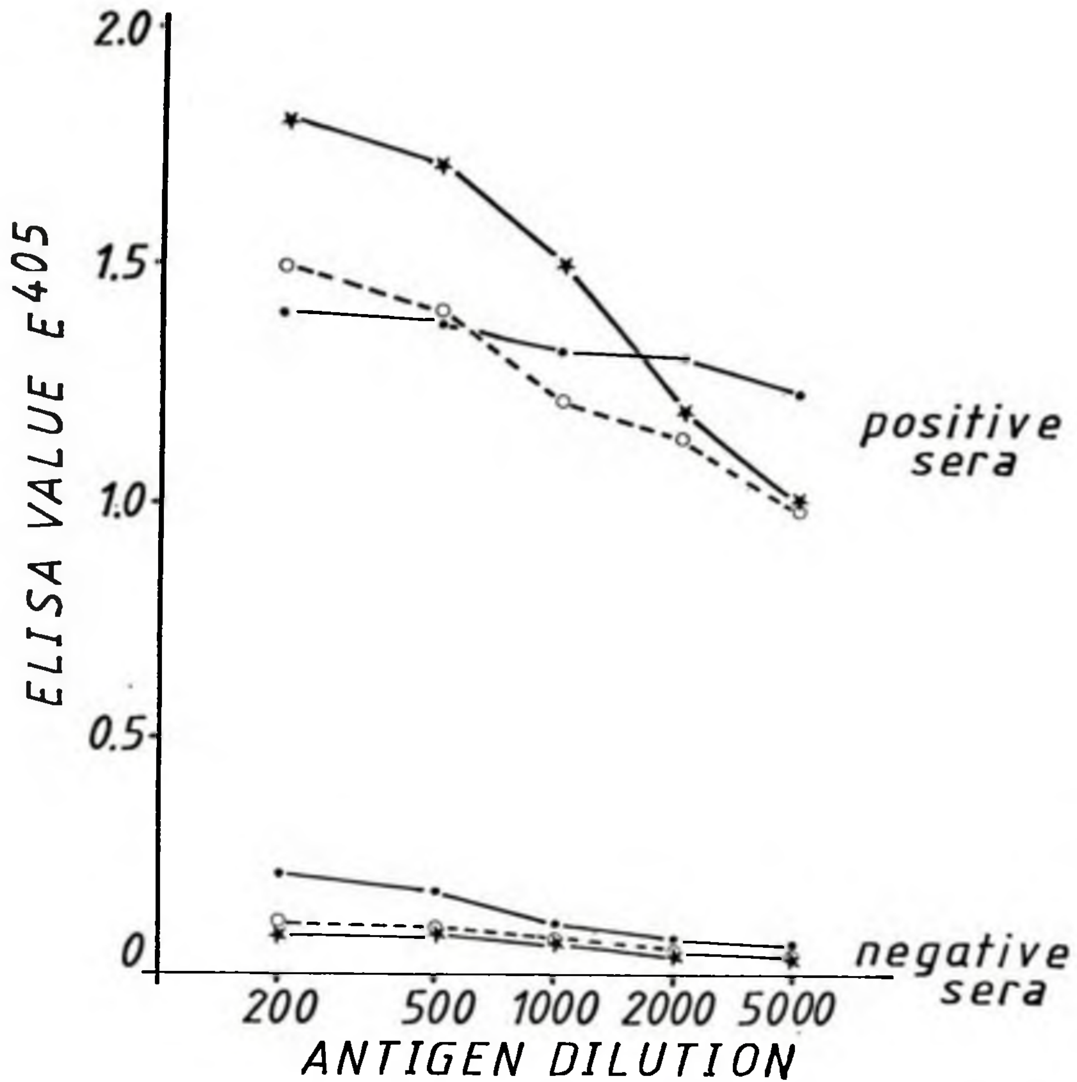

FIG. 16: RESULTS OF THE ENZYME LINKED IMMUNOSORBENT ASSAY FOR MALARIA ON DIFFERENT TYPES OF CARRIER PLATES USING REFERENCE SERA AT A SINGLE DILUTION AGAINST A RANGE OF ANTIGEN(P.FALCIPARUM) DILUTIONS. ABSORBANCE AT E405.

X—————X POLYSTYRENE M29AR

O- - - - - O POLYVINYL 220-29

■————■ DYNAWELL POLYSTYRENE CUVETTES

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TABLE 13 The Positive to Negative (P/N) absorbance ratio at different antigen dilutions at a single serum dilution (1:200) observed with the three carrier surfaces

Type of carrier surface	The ratio of the absorbance value obtained with positive to negative serum (used at 1:200) at a range of antigen dilutions 1:200 - 1:4000				
	1:200	1:500	1:100	1:2000	1:4000
Polystyrene	23	21	30	30	25
Dynawell Polystyrene cuvettes	15	16	24	29	33
Polyvinyl	7	8	14	19	25

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To find the effect of changing serum incubation times

Incubation times of 30 min, 1 hour, 2 hours, 18 hours, and 24 hours were tested. The optimum reactivity of sera was obtained after one to two hours' incubation at $20^{o}C$. Thus all serum incubations for malaria ELISA were subsequently 2 hours at $20^{o}C$.

To find the working dilution of the conjugate

The optimum working dilutions of conjugates were established by titration against human globulin. Plates were coated with 100 μg/ml human γ globulin. Dilutions of conjugate were reacted with the coated plate for 18 hours at $+4^{o}C$. The working dilution was that which gave a value of 1.0 after 30 minutes' substrate incubation.

Determination of antigen and serum working dilutions at optimal incubation times

The optimal working dilutions for antigen and serum were found by a chequerboard titration. The aim was to determine the most economical dilution of antigen, which would give the best separation of positive and negative sera, so that the ratio of the absorbance reading of a highly positive sample to that of a negative sample was at least 10:1. Table 14 shows the results of the chequerboard titration. An antigen dilution of 1:1000 and serum dilution of 1:200 gave the best positive/negative (P/N) ratio.

Standardisation of substrate reaction and endpoint determination

Several methods have been used to standardise the substrate reaction and to determine and interpret the end result in an ELISA test. A set time for the substrate reaction has been used, but this introduces considerable variability from day to day due to technical factors and

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TABLE 14 Results of chequerboard titration of reference sera

Antigen dilutions	Positive and Negative Serum Dilutions																				
	1:100			1:200			1:500			1:1000			1:2000			1:4000			1:8000		
	+ve	-ve	P/N Ratio	+ve	-ve	P/N Ratio	+ve	-ve	P/N Ratio	+ve	-ve	P/N Ratio	+ve	-ve	P/N Ratio	+ve	-ve	P/N Ratio	+ve	-ve	P/N Ratio
1:100	2.96	.12	19	2.34	.08	28	1.81	.07	26	1.46	.06	25	1.26	.05	24	.83	.04	19	.65	.04	15
1:500	2.54	.12	20	1.95	.07	30	1.76	.06	30	1.39	.06	25	1.10	.06	19	.77	.05	16	.54	.05	12
1:1000	2.00	.08	24	1.75	.06	30	1.50	.06	27	1.16	.05	24	.89	.05	17	.62	.04	15	.45	.05	10
1:2000	1.81	.08	22	1.58	.07	24	1.20	.05	23	.92	.05	20	.74	.05	16	.52	.04	14	.36	.04	10
1:4000	1.66	.07	22	1.16	.05	22	1.00	.05	21	.85	.05	18	.62	.04	14	.41	.03	12	.31	.03	9
1:8000	1.20	.06	21	.89	.04	20	.74	.04	19	.62	.04	16	.50	.04	12	.30	.03	10	.25	.03	8

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differences in the ambient temperature. In the present study reference positive and negative serum samples were included in several wells of each plate. In each day's assays the enzyme substrate reaction in these wells was monitored at 5 minute intervals and the reaction stopped in the whole plate, with 3 M NaOH, when the reference positive absorbance reached 1.70 at E405 - a value predetermined by chequerboard titration. The daily substrate incubation time varied between 30 and 40 minutes. This enabled valid comparisons to be made between tests done on different days.

To determine the absolute ELISA value of any serum tested, the differences between the absorbance ratio of test serum and that of the same dilution of the negative control serum examined in the same plate were calculated.

Determination of positive and negative limits of ELISA test

Absorbance values obtained at the end of malaria ELISA are values obtained under defined test conditions and controlled by inclusion of reference samples. These values therefore are a direct measure of the reactivity of the samples. In order to relate ELISA values to antibody reactivity in any test serum, operational definitions of positive and negative threshold limits of assay need to be set. In this study the threshold level for malaria ELISA was determined by testing a large number of samples (500) from non-immune Caucasian blood donors from south-east England. There was no difference between ELISA values for males and females; results were therefore assessed with male and female as a combined group. The relative frequency distribution of ELISA values in this group (Figure 17) was log normal with a sample medium of 0.06 and a range of values from 0.00 to 0.14. It was assumed that this population consisted of individuals totally lacking in any antibody

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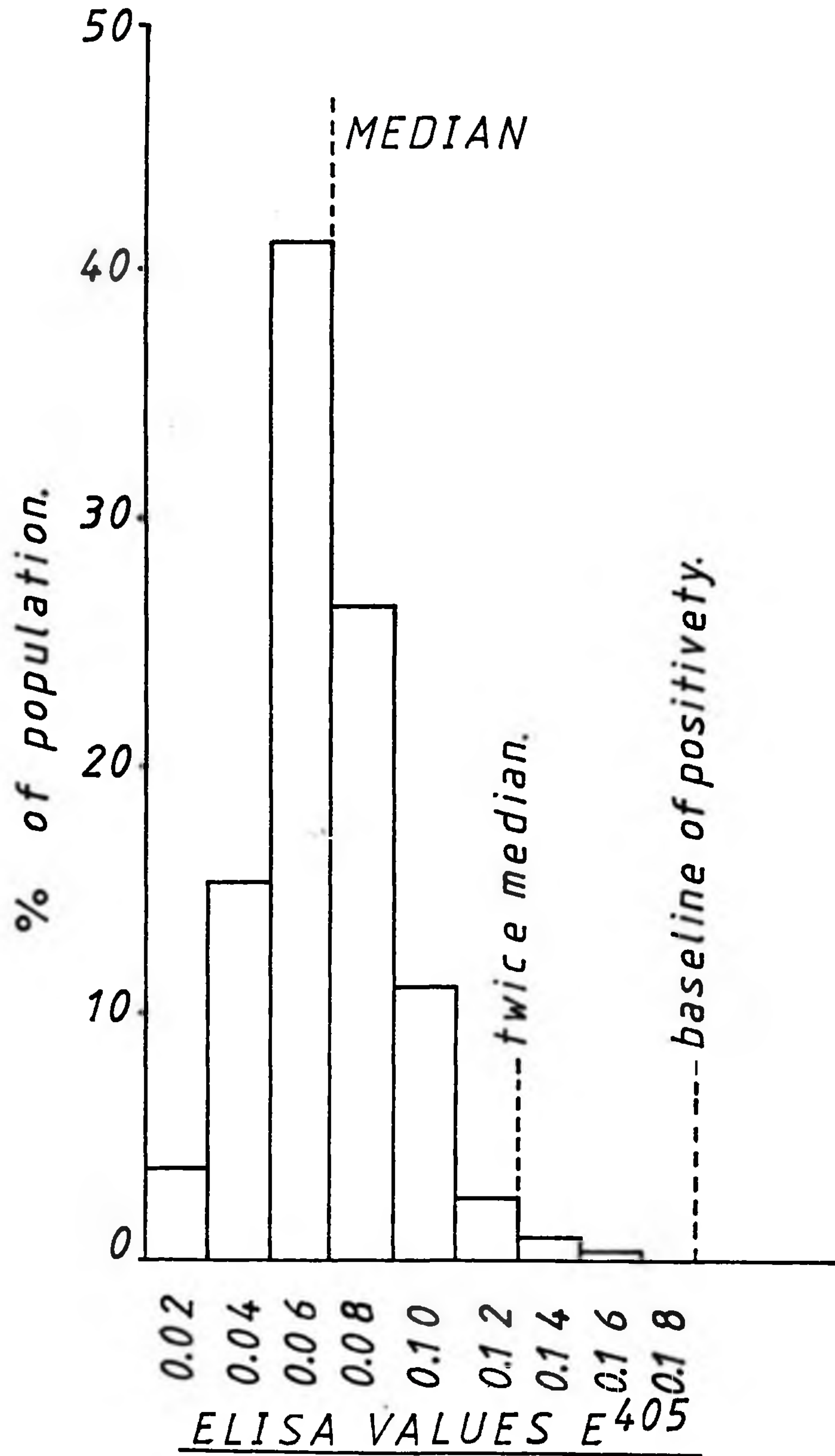
FIGURE 17 Frequency distribution of malaria antibodies by ELISA in 500 Caucasian blood donors never exposed to malaria



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to malaria. The absorbance value of 0.20 was chosen as the baseline level of positivity for malarial antibodies. This value was more than three times the median and mean plus standard deviation of the ELISA values of the unexposed population. Also at this value the non-immune blood donor population were all negative.

Reproducibility of malaria ELISA

This was assessed from the day to day variation in absorbance value of reference sera included with every 20 sera tested. An estimate of the precision of the ELISA test was shown by the coefficient of variation (CV%) of the results of reference sera measured in duplicate in 100 separate assays. This did not exceed 4.5%, a value which indicates a high assay precision.

Detection rate of malaria ELISA

The detection rate of the assay was assessed by results obtained from 63 Caucasians with clinical malaria and slide-proven parasitaemia, 100 Nigerian infants, and 100 Nigerian adults living in malarious areas. Frequency distribution of ELISA values in these three groups was compared with that of the 500 non-exposed Caucasian blood donors (Figure 18). An ELISA value of 0.20 or over indicates positivity; it can be seen from the figure that antibody could be detected in 80% of the Caucasians with malaria. The Nigerian infants show 85% positivity rate and the Nigerian adult population a positive rate of 100%. The mean ELISA value of the Caucasian non-exposed population (0.06) was significantly different from the mean values obtained for the Caucasians with malaria (0.3), the Nigerian children (0.40) and Nigerian adults (1.52) ($P < 0.01$). The differences in means between Caucasians with a single and early malaria infection and Nigerian adults from endemic areas were statistically significant as were differences between Nigerian children and Nigerian adults ($P < 0.01$).

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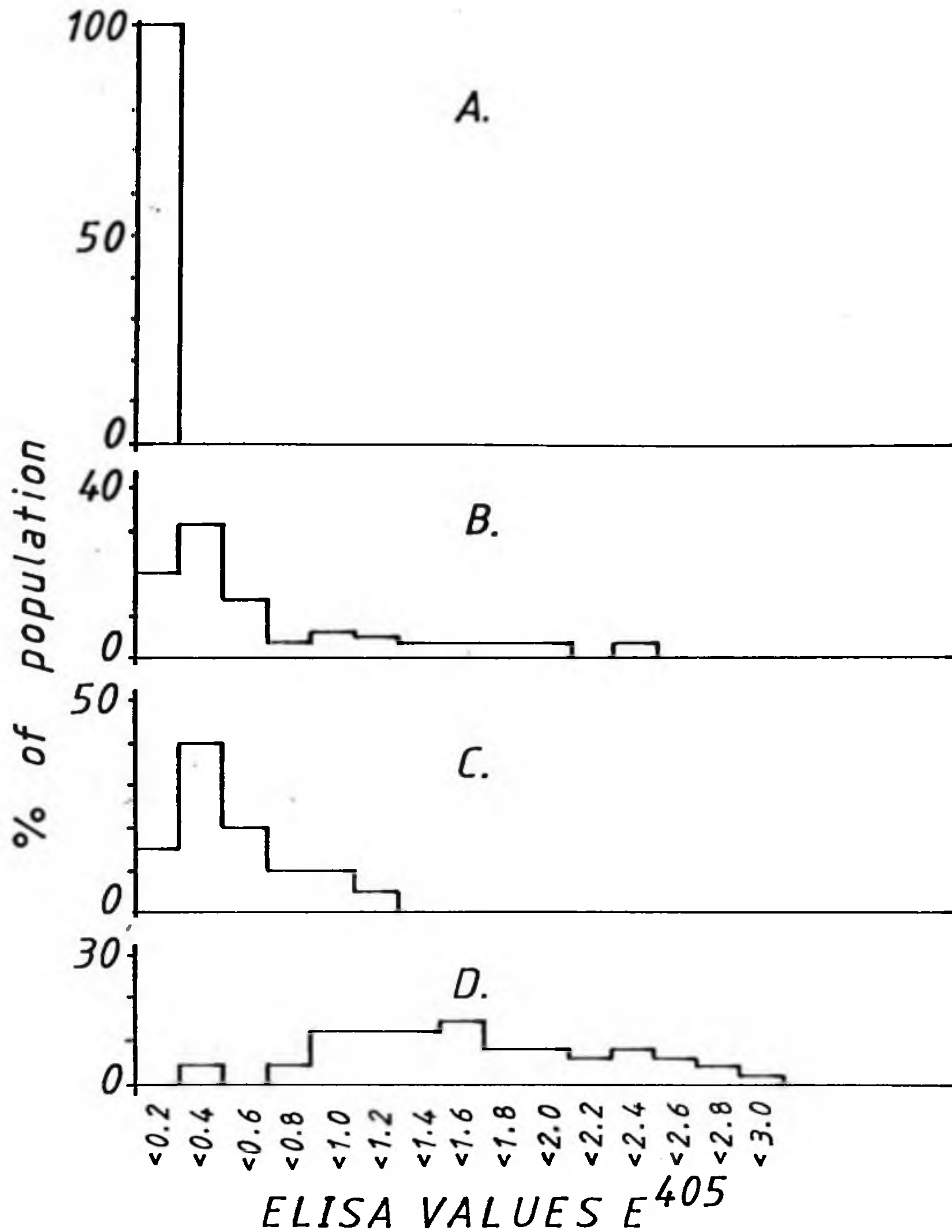

FIG. 18: FREQUENCY DISTRIBUTION OF MALARIA ANTIBODIES IN 3 GROUPS OF SERA FROM SUBJECTS EXPOSED TO MALARIA COMPARED TO DISTRIBUTION IN THE BLOOD DONOR UNEXPOSED POPULATIONS.

A- CAUCASIAN UNEXPOSED POPULATION

B- CAUCASIAN WITH CLINICAL AND SLIDE PROVEN MALARIA

C- BABIES RESIDENT IN ENDEMIC AREA

D- ADULTS RESIDENT IN AN ENDEMIC AREA

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Specificity of malaria ELISA

Serum samples from areas where malaria does not occur were consistently negative in malaria ELISA. The specificity was further assessed using sera from 540 Caucasians with single parasitologically proven and serologically positive bacterial, protozoal (amoeba, toxoplasma, trypanosomes), helminthic infections, and also sera from patients with a variety of connective tissue diseases (Figure 19). Sera from these groups of patients were chosen because they might contain antibodies against substances antigenically related to plasmodia and because other serum factors, such as rheumatoid factors, might affect the test specificity. The distribution pattern for this group was similar to that observed for the unexposed healthy Caucasian blood donors, and the difference in means between these two Caucasian groups was not significant. Since all Caucasian groups, healthy or diseased (and unexposed to malaria) gave ELISA values below 0.20, this indicated that ELISA malaria has a practical specificity of 100%.

A few Caucasian sera with known P.ovale, P.vivax and P.falciparum infections were tested for malaria antibodies by ELISA using P.falciparum soluble antigen. Unfortunately P.ovale and P.vivax soluble antigens were not available for testing. Figure 20 shows the relative distribution of ELISA values in the three groups of sera tested. Results show that malaria ELISA is not species-specific. Although the numbers tested here were few, the results suggest greater sensitivity for the homologous antigen. The higher mean ELISA value, 0.57, obtained for P.falciparum sera as compared to 0.43 for P.vivax and 0.16 for P.ovale suggests a stronger reaction with the homologous antigen - P.falciparum.

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FIGURE 19 Comparison of geometric means of E405 levels of different categories of sera.
Vertical lines indicate range
Horizontal lines indicate means
Values are shown in relation to baseline positive value

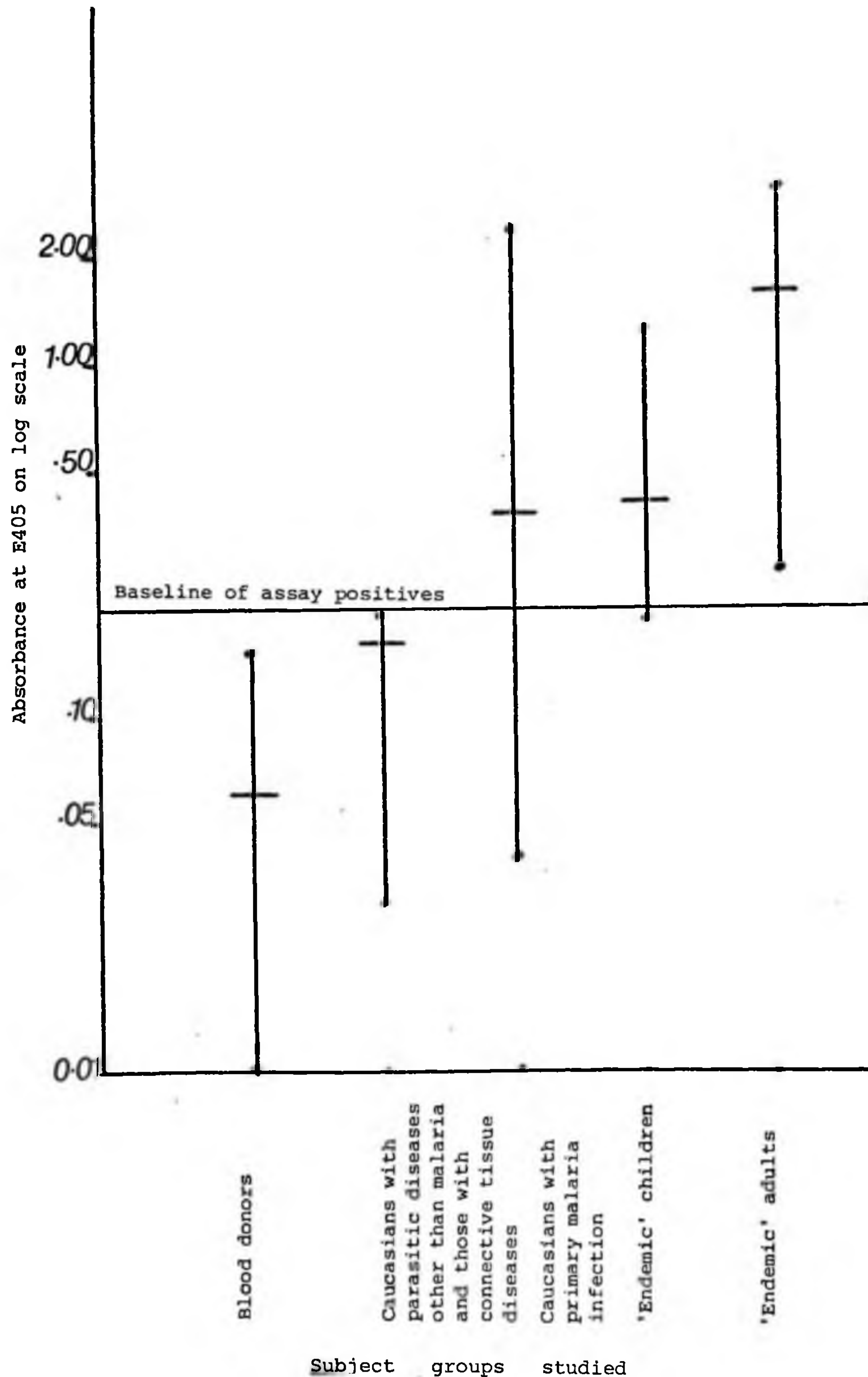

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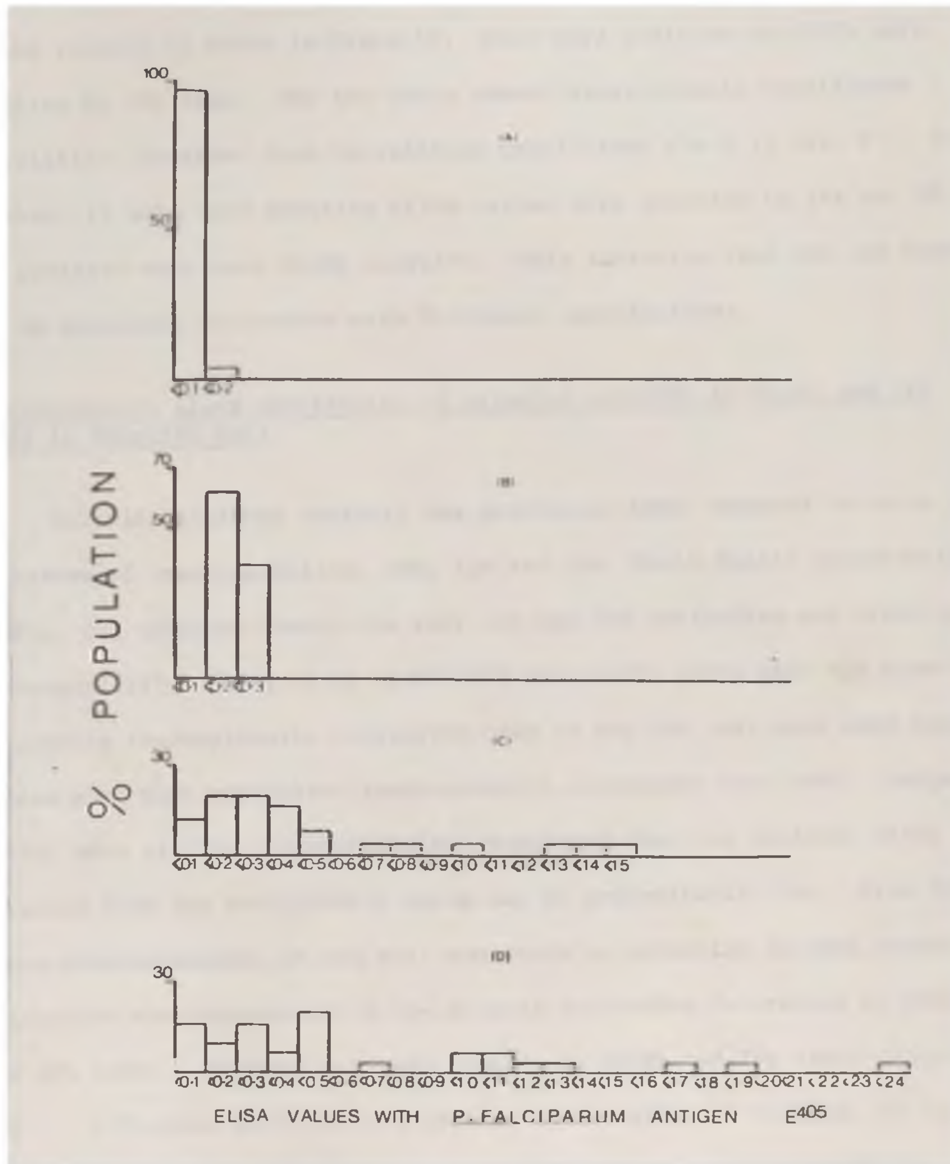
FIGURE 20 Distribution of ELISA values with P.falciparum as antigen in Caucasian population (A = blood donors, B = Caucasians with P.ovale, C = Caucasians with P.vivax, D = Caucasians with P.falciparum infections)



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Do malaria ELISA results correlate with malarial immunofluorescent antibody (IFA) titres?

963 sera with an expected range from negative to strong positive for malarial antibodies were tested by ELISA and IFA tests. A comparison of the results is shown in Table 15. Most sera positive by ELISA were positive by IFA test. The two tests showed statistically significant correlation (Spearman Rank Correlation Coefficient r = 0.71 with P = 0.01). However, 13 sera with positive ELISA values were negative by IFA and 58 IFA positive sera were ELISA negative. This indicates that the two tests may be measuring antibodies with different specificities.

Immunoglobulin class specificity of malarial antibody by ELISA and IFA tests in Ghanaian sera

Malarial antibody activity has previously been reported to occur in 3 classes of immunoglobulins, IgG, IgM and IgA (World Health Organisation, 1968). IgA antibody levels are very low and IgM antibodies are transient (McGregor, 1974). Rey et al (1967) had previously shown that the usual anti-whole immunoglobulin conjugates used in the IFA test gave much lower titres than when monovalent immunoglobulin conjugates were used. Targett (1970) made similar observations and suggested that the antibody being detected with the antiglobulin serum may be predominantly IgG. From the above considerations, it was felt necessary to establish to what extent IgM antibodies are represented in the malaria antibodies determined by ELISA and IFA tests. Malarial antibody results by ELISA and IFA tests obtained for 170 urban Ghanaian sera comprising 50 cords, 50 infants, 50 adolescents and 40 adults, using monospecific conjugates, were assessed for immunoglobulin class specificity.

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TABLE 15 Comparison of malaria antibodies detected by ELISA and IFA tests on 963 sera

ELISA E405	IFA titres						Total
	20	20	80	320	1280	5120	
0 - < 0.2	536	43	13	2	-	-	594
> 0.2	12	27	26	13	4	-	82
> 0.4	1	6	28	7	6	1	49
> 0.6	-	4	26	17	6	-	53
> 0.8	-	-	10	23	6	-	39
> 1.0	-	-	6	13	9	-	28
> 1.2	-	-	6	20	2	-	28
> 1.4	-	-	2	13	6	-	21
> 1.6	-	-	8	13	2	-	23
> 1.8	-	-	-	15	1	-	16
> 2.0	-	-	2	7	1	-	10
> 2.2	-	-	-	2	4	1	7
> 2.4	-	-	-	5	2	2	9
> 2.6	-	-	-	-	2	-	2
> 2.8	-	-	-	-	-	-	-
> 3.0	-	-	-	2	-	-	2
Total	549	80	127	152	51	4	963

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Malarial IgG antibodies There was little difference between ELISA values observed with alkaline-phosphatase labelled anti-human IgG and anti-whole immunoglobulin conjugates. On the other hand, IFA titre showed a 2 to 4 fold drop in titre when FITC-labelled anti-human conjugate was used instead of monospecific FITC-labelled antihuman IgG conjugate.

Malarial IgM antibodies Malaria ELISA was standardised for the determination of IgM antibodies. Reference sera were; unexposed Caucasian blood donor pooled sera which gave an IgM ELISA value of 0.09 and IFA titre of $<$1:20, and a Caucasian with *P.falciparum* infection with an IgG ELISA value of 1.04 with an IFA titre of 1:320; these were used as negative and positive reference sera respectively. The threshold of positive was established as 0.20 (as for IgG antibodies). The 170 serum samples were subsequently tested for IgM malarial antibodies using monospecific IgM conjugates. All cord sera tested were negative for IgM malarial antibodies by both ELISA and IFA tests ($<$0.20 and $<$1:20 titre respectively). The sera from infants showed 13% seropositivity by ELISA and 10% by IFA tests, although ELISA values did not exceed 0.40 and IFA titres obtained did not exceed 1:80. Seven per cent of adults were positive for ELISA malaria IgM antibodies and 5% of adults were positive by IFA. Similarly antibody levels were low and did not exceed 0.45 by ELISA or an IFA titre of 1:80.

The results indicated that IgM malarial antibodies did not form a significant part of the total malarial antibodies in immune sera, although it is possible that IgG malarial antibodies rapidly saturated the binding sites on the malaria antigen and thus inhibited the binding of the specific IgM antibodies.

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To find out if high levels of malarial IgG antibody were competitively inhibiting IgM,'immune' (subjects chronically exposed to malaria and showing high serum malarial antibodies) and 'non-immune'(subjects infected with malaria after a brief stay in a malarious endemic area) malaria sera were fractionated and the fractions tested for antibody. The results corrected for dilution effects are shown in Table 16. Separation of the IgG from IgM antibody did not affect the result, which would imply that IgM antibodies represented only a small part of the total IgM in the immune sera and the presence of IgG did not affect its detection.

Absorption studies

The crude extract soluble P.falciparum antigen was used to absorb malarial antibodies from immune sera giving a range of ELISA values, in order to observe for other characteristics of malarial IgG and IgM antibodies. The results obtained before and after absorption are shown in Figure 21 . All sera absorbed showed more than 50% inhibition of antibody binding to soluble malaria antigen. However, there was a residual activity which was not absorbed with 428 ng/ml antigen. It was thought that the presence of IgM antibodies effecting this residual activity might be implicated. Two sera were fractionated to separate IgG and IgM= one serum had very high ELISA IgG and the other high IgM malarial antibodies. It was shown that absorption of IgG fraction with 428 ng/ml gave complete inhibition of IgG activity (Figure 21). This same concentration of antigen only inhibited IgM activity by 77% and only after absorption with 860 ng/ml antigen was IgM activity completely inhibited.

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TABLE 16 Malarial antibody levels in immune and non-immune sera before and after fractionation

Test used	Source of sera used							
	Immune				Non-immune			
	Before		After		Before		After	
	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG
ELISA	0.35	1.70	0.45	1.95	1.04	2.34	1.17	2.98
IFA	80	1280	80	1280	320	5120	320	5120

Immune - sera from subjects chronically exposed to malaria

Non-immune - sera from Caucasians infected with malaria after visit to malaria endemic area

TABLE 17 The effect of treatment with 2-mercaptoethanol on IgM malarial antibodies determined by ELISA and IFA

Sample no.	Untreated		Treated with 2-ME	
	ELISA values E_{405}	IFA titres	ELISA	IFA
1 (positive reference serum)	1.04	320	0.09	< 20
2 Test serum	0.22	80	0.01	< 20
3 "	0.24	20	0.01	< 20
4 "	0.30	80	0.03	< 20
5 "	0.30	80	0.04	< 20
6 (negative reference serum)	0.09	20	0.01	< 20

TABLE 18 Population ranges, mean and 95 percentile confidence limits (95% CI) before and after malaria control programme

Population tested and no. Nigerians from malarious endemic area	Range	Mean	95% CI
Babies pre-control (100)	0.19 - 1.20	0.40	0.36 - 0.44
Babies post-control (100)	0.14 - 1.00	0.27	0.23 - 0.32
Adults pre-control (100)	0.26 - 3.04	1.52	1.40 - 1.64
Adults post-control (100)	0.20 - 2.60	1.11	1.01 - 1.21

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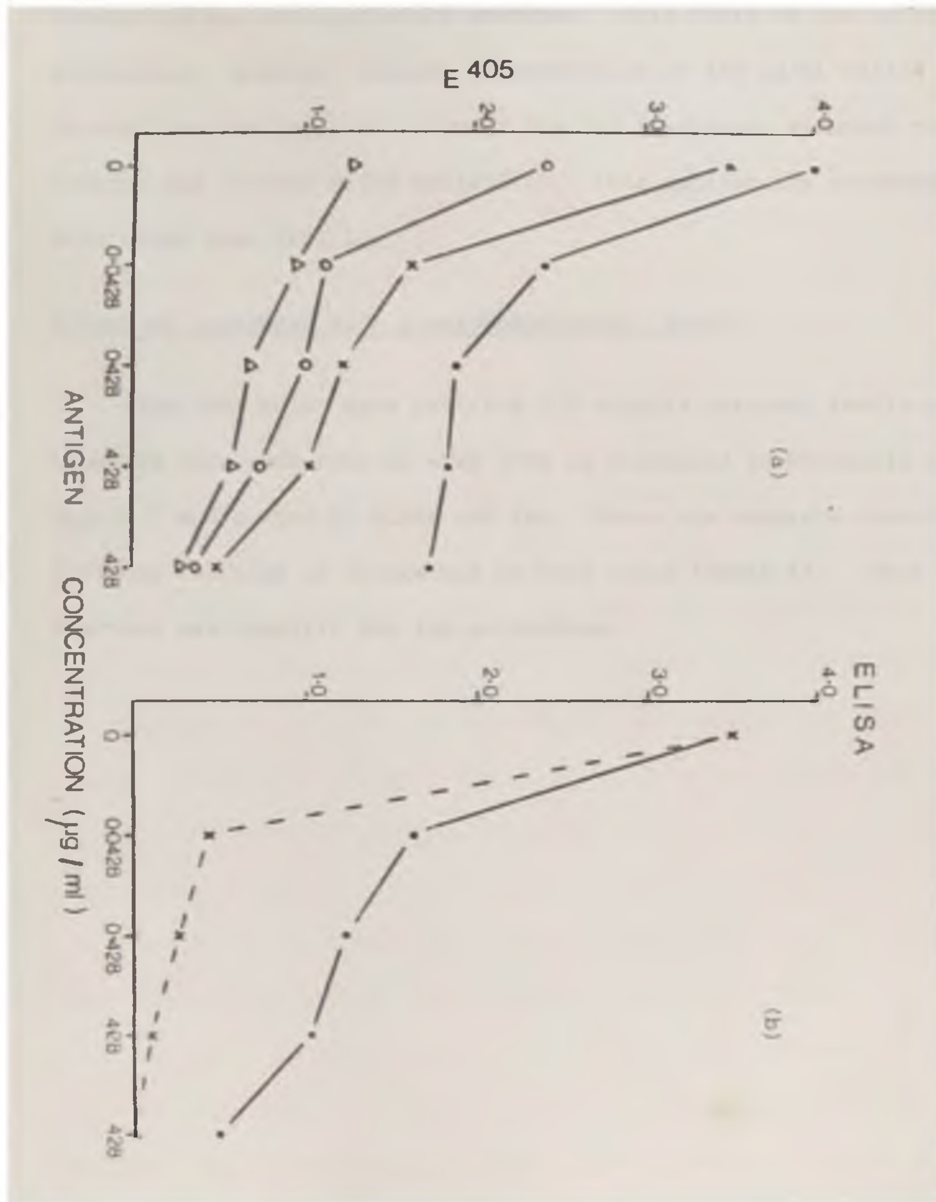
FIGURE 21 Inhibition of malarial antibody activity with *P.falciparum* antigen:

a) Results of 4 sera with varying ELISA values

●——● Caucasian resident in malarial endemic area
✱——✱ Reference positive sera
o——o and Δ——Δ Adult African malarious sera



b) Inhibition of fractionated reference positive serum

●——● Whole serum
x - - -x IgG fraction

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Antigen absorption was also carried out for the IFA tests. IFA titres were inhibited by 80% with soluble malaria antigen, but the antibody reactivity was not completely absorbed. This could be due to IgM malarial antibodies. However, antigen concentration of 428 ng/ml failed to completely inhibit the IFA positive titre of the IgG fractions, although this concentration did inhibit ELISA antibodies. This implied the presence of antibodies with other specificities.

Effect of treatment with 2-mercaptoethanol (2-ME)

Five sera which gave positive IgM malaria antibody levels and one IgM negative sera were treated with 2-ME as described in Materials and Methods (pp.98) and tested by ELISA and IFA. There was complete reduction of IgM antibody reaction as determined by both tests (Table 17). This showed that the test was specific for IgM antibodies.

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IMMUNODIAGNOSTIC POTENTIAL OF MALARIA ELISA

It is well known that estimation of antibody levels by whatever method cannot replace microscopic examination of blood films in patients with clinical malaria for definite diagnosis. All antibody detection methods can give negative results in sera from patients with patent parasitaemia. In the present study the serodiagnostic potential of ELISA was assessed using results from the 63 Caucasians with slide-proven malaria parasitaemia. Results obtained (Figure 22) showed that there was no consistent relationship between parasitaemia and ELISA values. However, an ELISA value of <0.2 and parasite density of $<1\%$ was consistent with a short stay of less than 6 months in an endemic area. On the other hand, ELISA values of >0.2 but with parasite density of $\leq 1\%$ correlated with a longer stay in an endemic area. Positive ELISA values were always obtained with parasite density of 2% and above. Also regardless of parasitaemia, stronger positive reactions ≥ 0.60 were always consistent with residence in an endemic area for more than 2 years without adequate prophylaxis. A patient who had been resident in an endemic area for over 20 years was admitted to hospital a fortnight after his return to Britain with fever of unknown origin; blood films showed parasite density of $<1\%$ and his serum had an ELISA value 2.34. The variable and usually inadequate prophylactic measures taken by these people make it impossible to draw any definite conclusions about the lack of correlation between parasitaemia and antibody. However, the overall seropositivity rate of malaria ELISA of 80% for this group, with 89% seropositivitity as determined by IFA test, in conjunction with above results would suggest that malaria ELISA could be used as an aid to diagnosis.

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FIG.22: RELATIONSHIP BETWEEN PARASITAEMIA AND ELISA VALUES IN CLINICAL MALARIA

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THE ABILITY OF MALARIA ELISA TO DETERMINE CHANGES IN MALARIA TRANSMISSION RATES IN A POPULATION

The efficiency of malaria ELISA in detecting changes in malaria parasite transmission rates was determined by testing sera from a Nigerian population before and 12 months after a (WHO) malaria control programme. The ranges, means and 95% confidence limits of ELISA results are shown in Table 18.

The frequency distribution for pre- and post-control Nigerian babies, and pre- and post-control Nigerian adults is shown in Figures 23 and 24. Fifteen per cent of the pre-control baby group were seronegative compared to 60% of the post-control group. Similarly for the adult group, the pre-control group showed 100% positive rate but post-control sera showed 98% seropositivity. Furthermore sera from the post-control groups showed a greater tendency for lower ELISA values than those observed for pre-control ELISA values. Also the difference in mean ELISA observed between the baby group and the adult group was significant at the 5% level.

DISCUSSION

This work indicates that ELISA tests with P.falciparum show sensitivity and specificity for the detection and measurement of malaria antibodies, and confirms results of preliminary work by Voller et al (1974; 1975).

The reproducibility of ELISA is greatly dependent on the carrier surface used. All three microtitre plates evaluated in this study were found to be useable for malaria ELISA but the polystyrene plates gave the best discrimination between the positive and negative reference sera.

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FIGURE 23 Distribution of ELISA values with *P.falciparum* antigen in Nigerian infant population (A = pre-control, B = post-control)

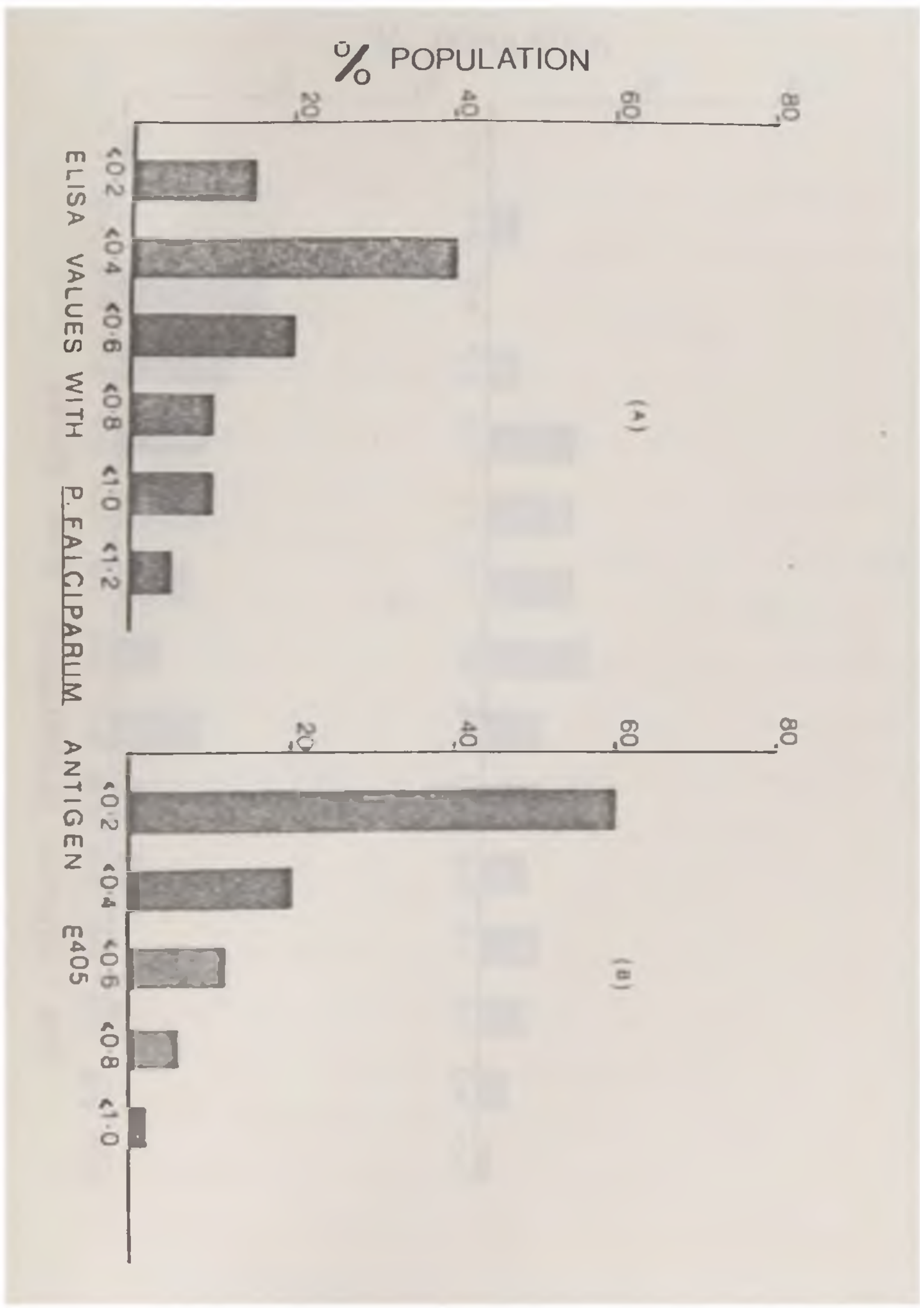

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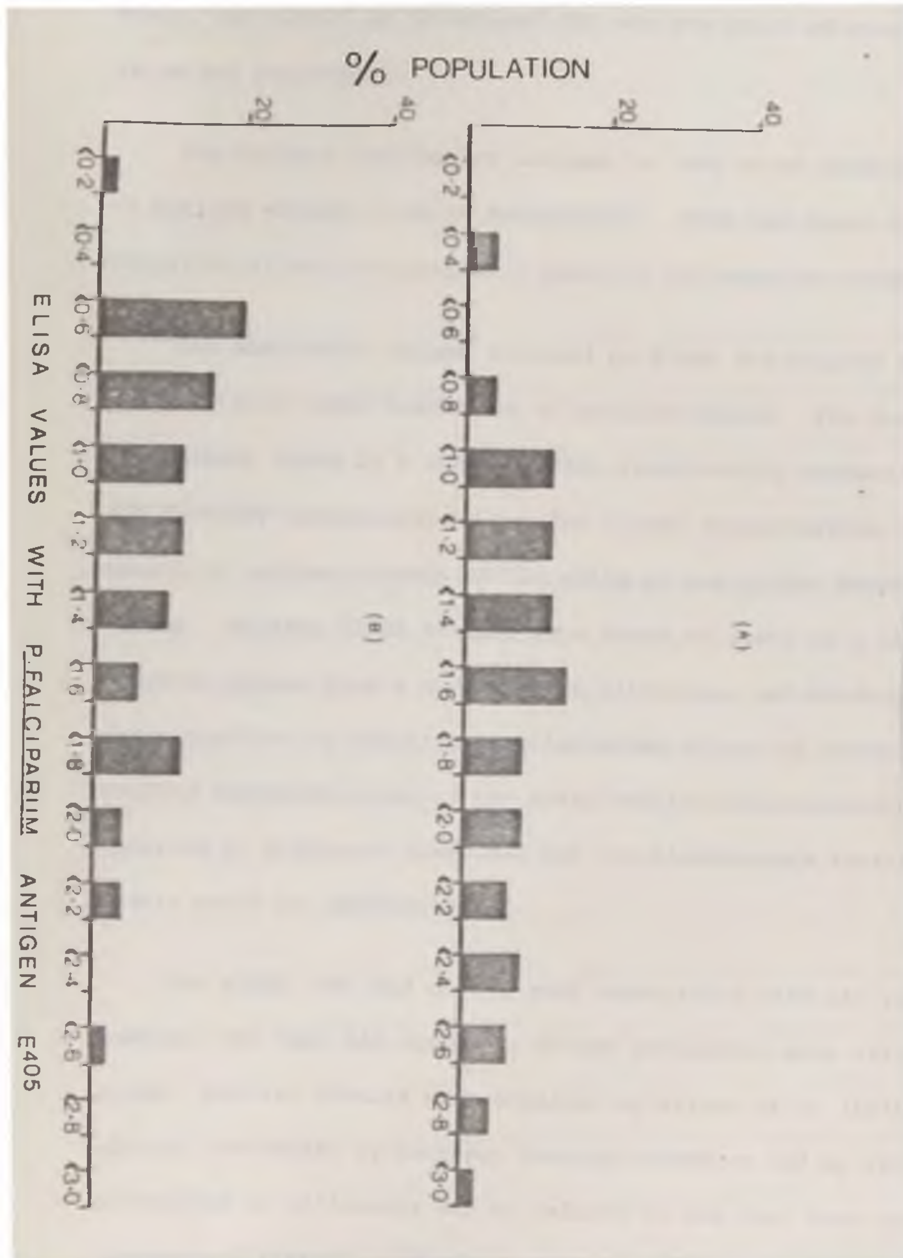
FIGURE 24 Distribution of ELISA values with *P.falciparum* antigen in Nigerian adult population (A = pre-control, B = post-control)



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Incubation times vary for different ELISA systems (Bidwell et al, 1977) and optimum incubation times for this system were obtained by trial and error, and should be determined for any new batch of reagent (antigen, serum and conjugate).

The optimum coating for antigen is that which enables economic use of antigen without loss of sensitivity. This was found by a chequerboard titration of antigen against a positive and negative reference sera.

The absorbance values obtained by ELISA are related to the amount of antibody only under conditions of antigen excess. For low concentration of antibody there is a straight line relationship between enzyme activity and antibody concentration, but for higher concentration of antibody, the amount of antigen present on the wells of the plates becomes a limiting factor. Malaria ELISA results were based on tests at a single serum dilution chosen from a chequerboard titration, and which gave the best serum positive to negative discrimination values of reference sera. This enabled standardisation of the assay and the comparability of results obtained at different times,but had the disadvantage that high antibody levels would be underestimated.

The ELISA test did show a good correlation with IFA reciprocal titres. However, IFA test did appear to detect antibodies more efficiently than ELISA. Similar results were obtained by Wilson et al (1971) who compared malaria antibodies by indirect haemagglutination and by IFA tests. The difference in efficiency may be related to the fact that the two tests determine different antibodies. The use of whole parasite as antigen in the IFA test may provide a wider spectrum of antigen with which antibodies

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in serum may react. The use of soluble parasite extract makes available only selected antigenic specificities. Indeed it is even likely that the two tests may be measuring antibodies that are differently produced, possess different functions and biological properties, but there is little doubt that they both measure antibodies specific for malaria.

Techniques used in malaria serology are not species-specific; however, the test sensitivity is improved when homologous antigen is used (World Health Organisation, 1975). The higher test sensitivity observed for Caucasians with P.falciparum infection as compared to that for P.vivax and P.ovale infections in the present study is consistent with the above observations.

Malarial antibody activity has been reported to occur in IgG and IgM class immunoglobulins and to a lesser extent in IgA (World Health Organisation, 1975). Surprisingly, assay of IgM malarial antibodies has proved quite inadequate even with the use of monospecific conjugated antisera in the IFA test, and levels observed are low compared to IgG antibodies (World Health Organisation, 1975). Targett (1970) observed similar low malarial IgM antibody titres in Gambian immune sera, and there is some evidence that IgG antibodies may interfere with their detection. Results from this study show low malarial IgM antibody levels in 'immune' sera (from malaria endemic population), compared to the significantly raised levels in 'non-immune' (Caucasians infected with malaria after temporary exposure to malaria) sera. The probability that low IgM antibodies in immune sera could be due to the competition between IgG and IgM was not confirmed from the fractionation studies. The possibility that the levels of IgM specific antibodies bear a direct relationship to patent parasitaemia is suggested from the present results, and also that elevated levels may be associated

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with primary infections as noted in the 'non-immune' (with patent parasit-aemia) and not with chronic exposure to malaria as may be the case in the immune sera. Tobie et al (1966) showed that levels of serum IgG, IgA and IgM were markedly raised during primary induced malaria infections in human volunteers, and Abele et al (1965) further confirmed that the increase in IgM during such infections was due in part to malaria antibody. Targett (1970) in a study of Gambian immune population observed significant diff-erences between the mean total IgM concentration of adults who were IgM antibody positive and the mean IgM concentration for those who were IgM antibody negative, and observed that IgM antibodies were significantly higher in the group with positive blood films. McFarlane and Voller (1966) compared IgG, IgM and IgA levels and malaria fluorescent antibody titres of adult Nigerians who were blood film positive with those who were blood film negative. The antibody levels were significantly higher in the group with positive blood films and this antibody increase corresponded with an increase in serum IgA and IgM but not IgG. These results would indicate that an association exists between IgM and malaria, but it is not known what the IgM antibody titres represent in quantitative terms. In the two sera tested here IgM antibodies represent only a small part of the total IgM, at least in immune sera.

Inhibition of the antibody response by preabsorptions with soluble antigen was carried out for both assay methods. Although in ELISA complete inhibition of antibody response was not achieved with serum, when serum was separated into IgG and IgM fractions total inhibition was effected. IgM antibodies required twice the antigen concentration for total inhibition. This difference may be related to their different molecular size and probably to differential specificities. Also the same concentration of

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soluble antigen only inhibited IFA by 80%. This residual activity probably reflects the fact that IFA detects antibodies to both surface and soluble antigens, whereas ELISA only detects soluble antibodies. This may explain the apparent lower sensitivity of ELISA by comparison with IFA.

The quantitative nature of the ELISA allows for the following conclusions to be made on results obtained from clinical malaria patients. Positive ELISA values of >0.2 correlate well with parasitaemia of $>2\%$, and negative ELISA with parasitaemia $<1\%$ in cases of primary exposure to parasites. However, it should be appreciated that there may be many exceptions to the above observed relationship. Furthermore, a correlation existed between higher ELISA values and the age of the infection regardless of parasitaemia.

The present study has demonstrated that ELISA detects the presence of malarial antibody activity in serum from subjects exposed to malaria, although these antibodies may not necessarily be protective. Recent studies by Wilson et al (1969) and McGregor and Wilson (1971) demonstrated the heterogeneity of malarial antigens. Indeed the observation that some 30 distinct antigens have been found in association with erythrocytic P.falciparum in man, is suggestive of the complexity of development of protective immunity to malaria. However, the sensitivity of ELISA for malarial antibodies observed in the present study, using crude antigen extract, would suggest that the availability of purified species-specific and stage-specific malarial antigens with standardised reference reagents, will further enhance the sensitivity and specificity for the detection of specific malarial antibodies. At the present moment ELISA can be used as an aid to diagnosis.

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Results obtained for the Nigerian population reflect the sero-epidemiological value of the ELISA test where 85% of all babies and 100% of all adults living in endemic areas with no malarial control activities gave positive ELISA values. Sera from the post-control group gave a wider range of ELISA values and only 40% of babies and 98% of adults gave positive ELISA values. The difference in mean ELISA values between the pre-control and post-control sera in both adults and babies was shown to be statistically significant. Malaria ELISA therefore quickly reflects changes in endemicity resulting from malaria control activities.

All the reagents used in this study can be produced in large quantities and can be made stable. They can be used under field conditions (Voller et al, 1975) without the need for elaborate equipment. Very little antigen is required and only 10 µl of serum or finger-prick blood is required; results are quantifiable. ELISA therefore can be used as a substitute for IFA test, as a routine laboratory test. It also lends itself to epidemiology, and its greatest value will be in seroepidemiology and the evaluation of control programmes to provide information on malaria endemicity rates.

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THE STUDY OF MALARIA IN THE GHANAIAN SAMPLE POPULATIONS

THE PREVALENCE OF MALARIA IN GHANAIAN RURAL AND URBAN POPULATION

Three groups of sera were tested. These comprised:

1. Sera selected from Group A (see Materials and Methods, pp.79) a total of 2,077 (1,063 from rural population and 1,014 from urban population).

2. 122 cord sera from healthy urban mothers were also selected from Group A.

3. 534 sera from Group E (rural children aged 24 months. They comprised 250 sera from age group 3 - 8 months, 178 from age group 9 - 14 months and 106 from age group 15 - 24 months).

All sera were tested as described above for malarial antibodies by ELISA and IFA. Results were evaluated and where possible comparison of the parasitological and serological variables have been made.

In order to make ELISA results easier to analyse, ELISA values were divided arbitrarily into 7 defined groups as follows: the first 0 - 0.2 (0.2) represented negative test values. The positive values ranged from 0.2 - 0.5 (<0.5); 0.5 - 0.8 (<0.8); 0.8 - 1.2 (<1.2); 1.2 - 1.5 (<1.5); 1.5 - 2.0 (<2.0) and >2.0. These groups were used for all age groups throughout the study.

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Antibodies in rural and urban populations The overall prevalence of malaria antibodies in the two populations is shown in Table 19, together with mean ELISA values in each age group. Figure 25 shows the distribution of antibody levels in the two populations. There was a higher proportion of positive results in the rural than the urban population. The statistical significance of this difference was determined by the Kendall tau test (analysis of variance) which showed significance at the one per cent level.

The seropositivity rate was analysed by age groups (Figure 26). This showed that the significant difference in antibody levels occurred in the young children, differences in older groups were not statistically significant. Analysis by sex did not show any significant different between sexes in either population.

The results in Table 19 also show that the number seropositive, and mean ELISA values increased with age in both populations. In the rural population all sera were positive by 3 years whereas 100% seropositivity did not occur at all in the urban population, although higher rates (87%) were reached by 6 - 11 years and 99% by >15 years.

Antibody detection by ELISA in cord sera (urban) The proportions positive and the mean cord ELISA (0.64) was slightly lower than the urban adult mean value of 0.79, but the difference was not statistically significant.

Antibody detection by ELISA in rural children The distribution of ELISA values in children under 24 months of age is shown in Figure 27. The distribution shows ELISA values in the early age groups 3 - 8 and 9 - 14 months are similar, but higher levels increase after 15 months. There was no difference in the mean ELISA values obtained for 3 age groups, 0.56; 0.50

TABLE 19 Frequency distribution of malarial antibodies by ELISA from rural (R) and urban (U) populations in Ghana

ELISA values E405	Age group in years and survey populations									
	0 - 2		3 - 5		6 - 10		11 - 15		>15	
	R	U	R	U	R	U	R	U	R	U
0 - 0.2	100	161	0	42	0	8	4	2	0	5
0.2 - 0.5	161	65	16	36	10	17	19	10	41	177
0.5 - 0.8	212	12	15	10	6	13	24	5	84	161
0.8 - 1.2	119	11	15	16	4	9	27	6	82	105
1.2 - 1.5	18	7	3	5	3	3	11	3	32	58
1.5 - 2.0	9	3	2	0	2	2	4	3	27	53
2.0	2	0	0	0	1	0	1	0	9	5
Total	621	259	51	109	26	52	90	29	275	565
Mean ± SD	0.52* ± 0.38	0.29 ± 0.31	0.63* ± 0.37	0.43 ± 0.35	0.63 ± 0.06	0.61 ± 0.47	0.83 ± 0.05	0.76 ± 0.09	0.95* ± 0.49	0.79 ± 0.50
% positives	84%	48%	100%	61%	100%	85%	96%	93%	100%	99%

* P value indicating statistical significance of difference in mean ELISA between the 2 survey populations at different age groups where $P < 0.05$

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% of population

(a)

60

0

0-0.2 0.2-0.5 0.5-0.8 0.8-1.2 1.2-1.5 1.5-2.0 >2.0

ELISA VALUE E^{405}

(b)

60

0

20 20 80 320 1280 5120

I.F.A. Reciprocal titre

FIG.25: THE FREQUENCY DISTRIBUTION OF MALARIA ANTIBODIES BY ELISA(a) AND IFA TITRES(b) IN RURAL AND URBAN POPULATIONS.

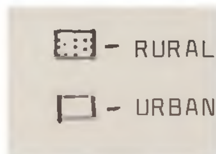

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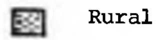
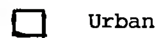
FIGURE 26

A) Frequency distribution of malaria ELISA values in various age groups in rural and urban populations in Ghana

B) Frequency distribution of malaria IFA titres in various age groups in rural and urban populations in Ghana


Rural


Urban

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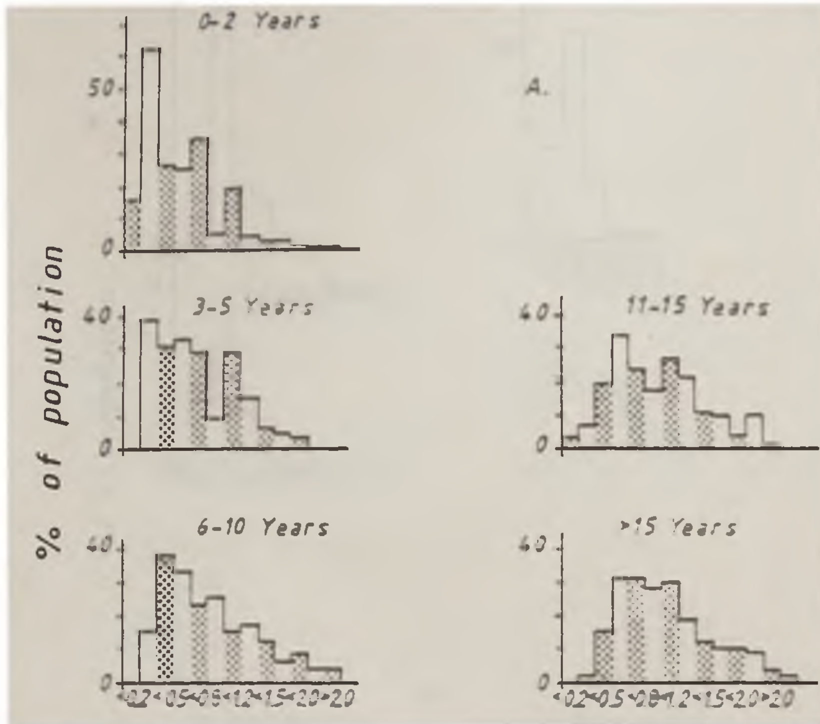
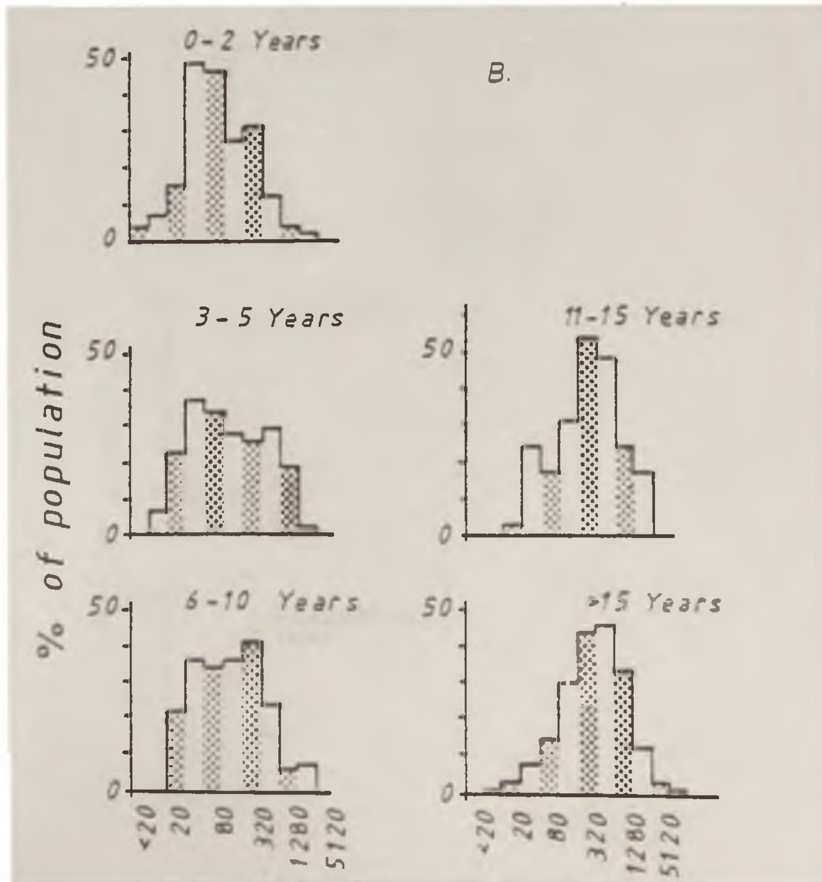


ELISA VALUES E^{405}



I.F.A. Reciprocal titre

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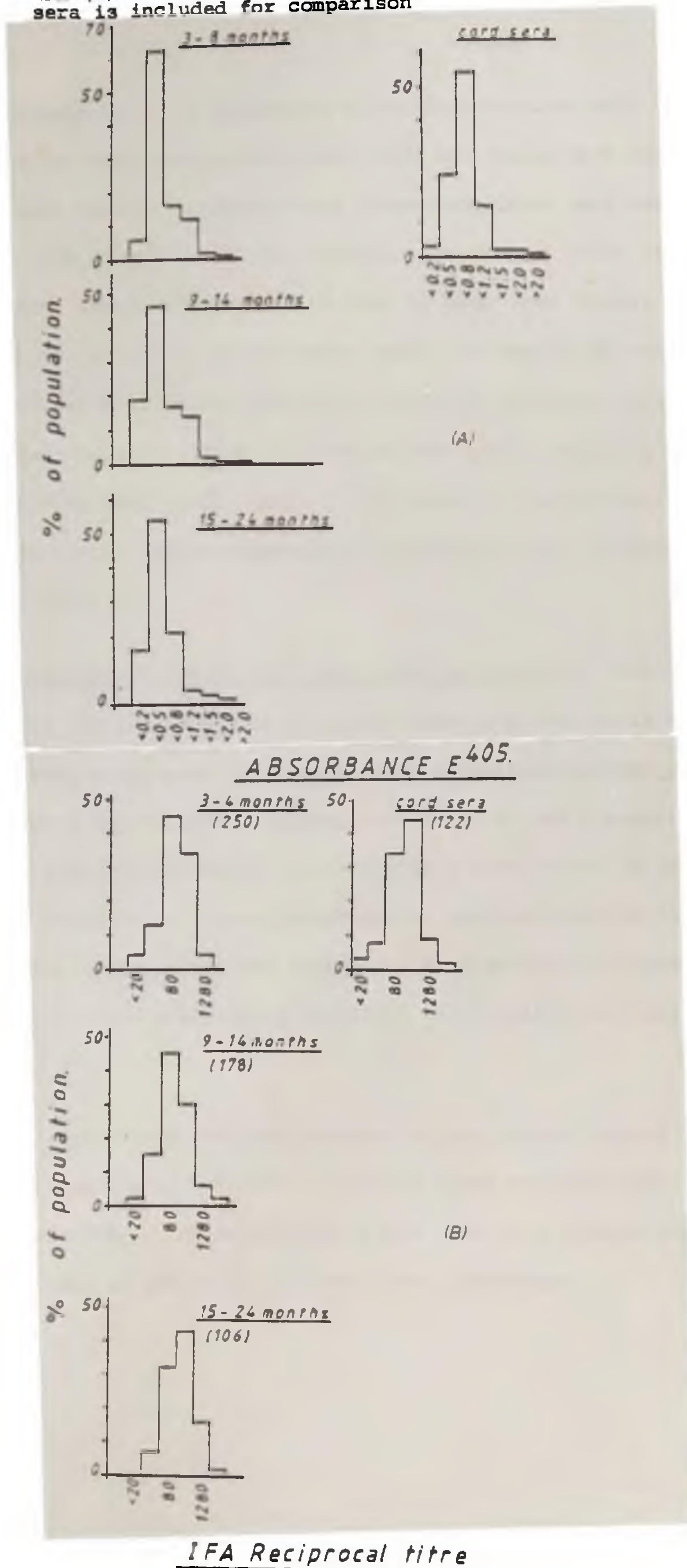
FIGURE 27 Distribution of malarial antibodies determined by ELISA (a) and IFA (b) in rural children aged 24 months. Distribution in cord sera is included for comparison



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and 0.50 respectively. A comparison of results obtained with these rural children groups with results for rural cord sera would have been ideal. However, since results available were those from urban cord sera, these were used. The difference in distribution patterns of ELISA values in the 3 children groups as compared to that in cord sera (Figure 27) was significant ($P < 0.01$). On the other hand, the mean ELISA value for cord sera (0.64) was not significantly different from mean values observed for the rural children. Also the results show an initial drop in mean ELISA value from cord levels until 9 - 14 months; a lag phase occurs from 14 - 24 months, before subsequent progressive rise in older age groups as shown in Table 19.

Antibody detection by IFA in rural and urban populations The overall prevalence of IFA titres in the rural and urban populations is shown in Table 20; results are also given as frequency distribution and as geometric mean reciprocal titres (GMRT) for each age group of the 2 populations studied. The overall distribution of IFA titres in the 2 populations is shown in Figure 25. There was a higher proportion of positive results in the rural than the urban populations, the difference in distribution pattern analysed by Kendall tau test (analysis of variance) was statistically significant ($P < 0.01$).

The seropositivity rate was analysed by age groups (Figure 26). In both populations the proportion of positive tests and GMRT shows an increase according to the rising age groups, and both indices were considerably higher in the rural than the urban population.

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TABLE 20 Frequency distribution of fluorescent antibody titres of sera from rural (R) and urban (U) populations in Ghana

IFA test titre	Age group in years and survey populations									
	0 - 2		3 - 5		6 - 10		11 - 15		> 15	
	R	U	R	U	R	U	R	U	R	U
20	22	30	0	6	0	0	0	0	0	5
20	100	125	11	40	9	11	3	7	7	47
80	282	70	17	31	9	17	16	9	41	167
320	189	30	13	30	6	21	49	11	122	261
1280	25	4	9	2	2	3	22	2	94	75
5120	3	0	1	0	0	0	0	0	11	10
20480	0	0	0	0	0	0	0	0	0	0
Total	621	259	51	109	26	52	90	29	275	565
GMRT	246*	109	290*	168	283 NS	220	546*	275	686*	394
% positives	96%	88%	100%	94%	100%	100%	100%	100%	100%	99%

* INdicates significance between rural and urban GMRT at different age groups

P < 0.05 NS = Not significant

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The difference in GMRT between all age groups in the 2 populations was statistically significant except for the age group 6 - 10 years. The GMRT for the different age groups in the 2 populations shows a progressive rise throughout childhood into adulthood.

The results in Table 20 also show that in the rural population, all sera were positive ($\geq$20 reciprocal titre) by age group 3 years but in the urban group, not until 6 - 10 years. Analysis by sex did not show any significant differences between sexes in either population.

<u>Antibody detection by IFA in cord sera (urban)</u> The proportion of sera positive and the GMRT (350) were comparable to those observed for urban adults; GMRT 394.

<u>Antibody detection by IFA in rural children</u> The distribution of IFA titres in children 24 months of age is shown in Figure 27. Again titres and GMRT increased with age. GMRT obtained for age groups 3 - 8 months, 9 - 14 months and 15 - 24 months are 112, 115 and 195 respectively.

The IFA titre distribution for the rural children as compared to that for cord sera was significantly different ($P < 0.01$) (Figure 27). A comparison of the GMRT in the 3 age groups (for the rural children) with GMRT for cord sera (350) were statistically different ($P < 0.01$). The results indicate that cord titres, representing neonate antibody levels and a measure of passively acquired immunity transmitted placentally, decreased rapidly in the first 3 months to a minimum and then gradually increased until adult levels were reached.

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Comparison of serological and parasitological results rural and urban populations Figure 28 shows that antibody levels rise with increasing parasitaemia in young children until they reach a peak parasitaemia when parasite levels decline but antibody remains high. This shows the very strong positive correlation between antibody and parasitaemia in the young age groups and a conversion of a positive to a negative correlation is seen to occur at a lower antibody level in the urban than rural population. Parallel results were obtained with IFA (Figure 28). However, when the percent seropositivity of the populations was analysed ELISA showed a bigger difference in seropositive rates between the 2 populations (Figure 29).

Cord sera (urban) As described in Chapter Five cord sera and their placentae were parasitologically negative. The distribution of antibody was similar by ELISA and IFA and reflected maternal levels.

Rural children Antibody for rural children 24 months of age had been compared with cord mean ELISA values (Figure 30). As previously explained cord sera were obtained from urban mothers (cord levels reflect maternal values, and had cord sera been obtainable from the rural mothers they would probably have had somewhat higher values). ELISA values and IFA GMRT dropped although parasite rates and densities were increasing showing a loss of maternal antibodies before the individual's own antibodies began to increase. Antibodies detected by IFA rose more quickly than that detected by ELISA. The difference probably reflects the fact that IFA detects antibodies to surface antigens whereas ELISA antibody detects reaction to soluble antigen.

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FIGURE 28 Relationship between mean antibody levels, parasite rates and parasite densities by age groups in the rural and urban populations (ELISA results are shown in (A) and those for IFA are shown in (B)

GMRT or mean ELISA value (rural)
GMRT or mean ELISA value (urban)
Parasite rate rural
Parasite rate urban
Mean parasite density rural
Mean parasite density urban

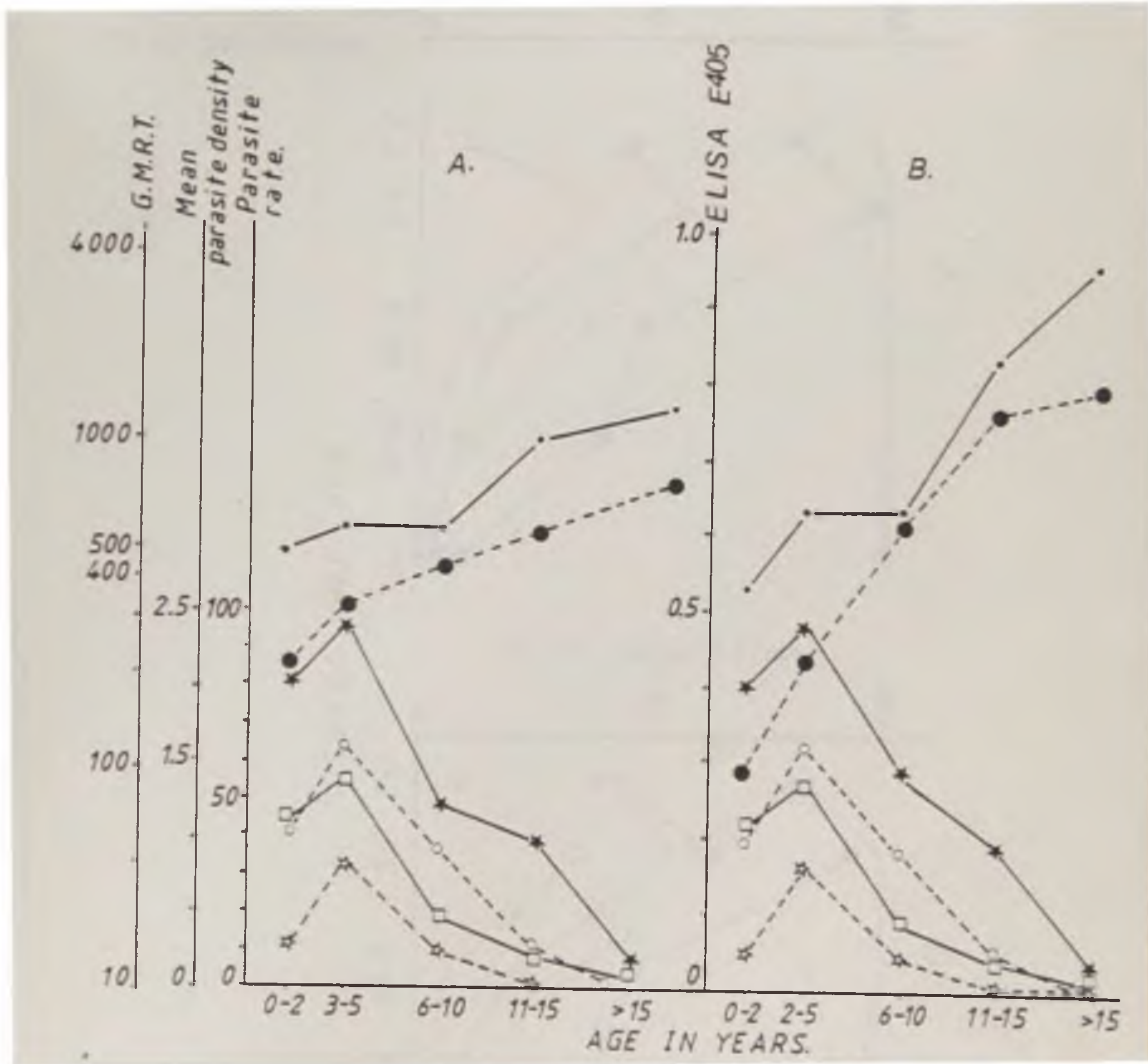

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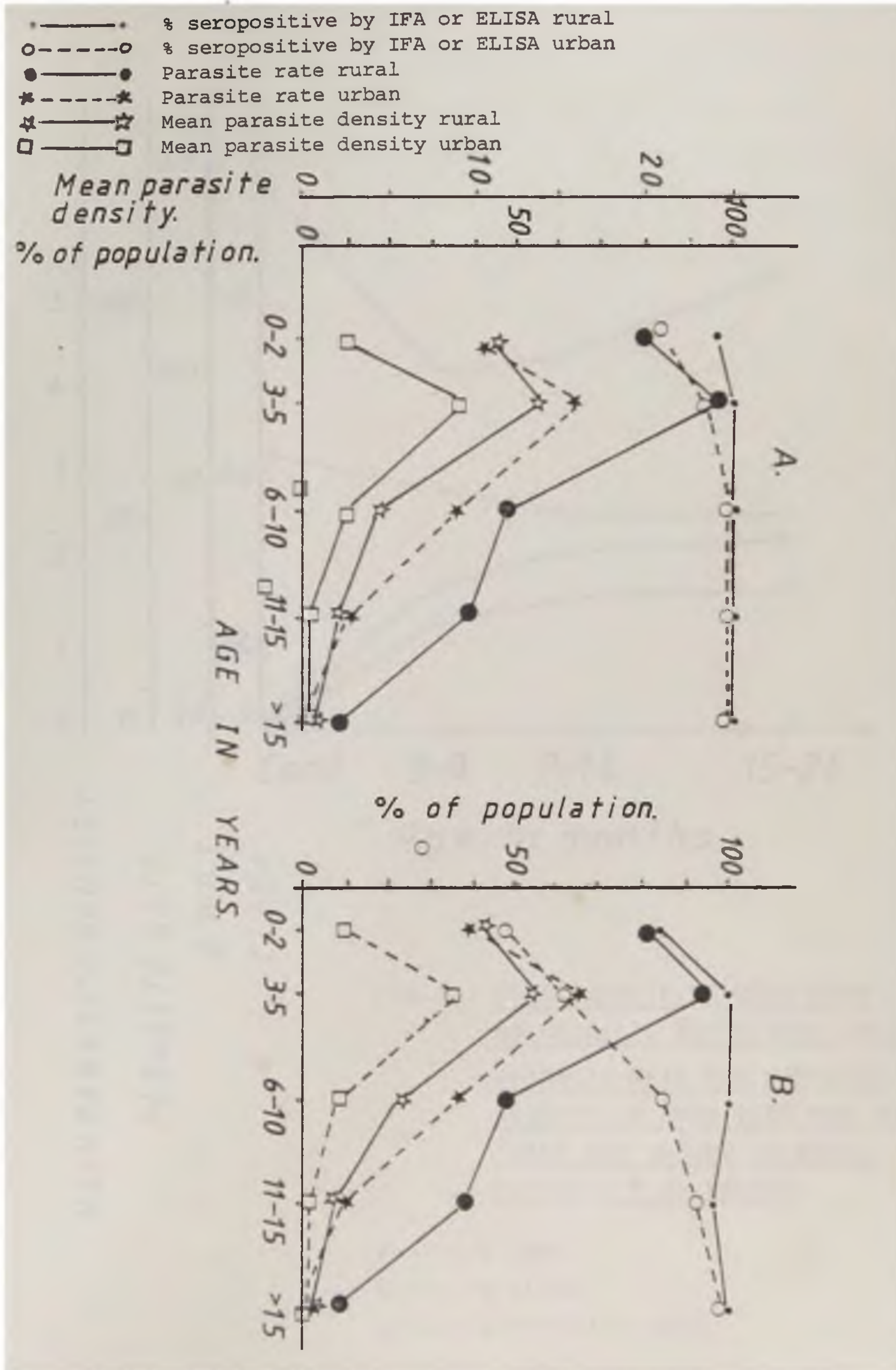
FIGURE 29 Relationship between % seropositivity of malaria antibodies by ELISA (A) and IFA (B) with parasite rates and parasite densities in rural and urban populations by age groups



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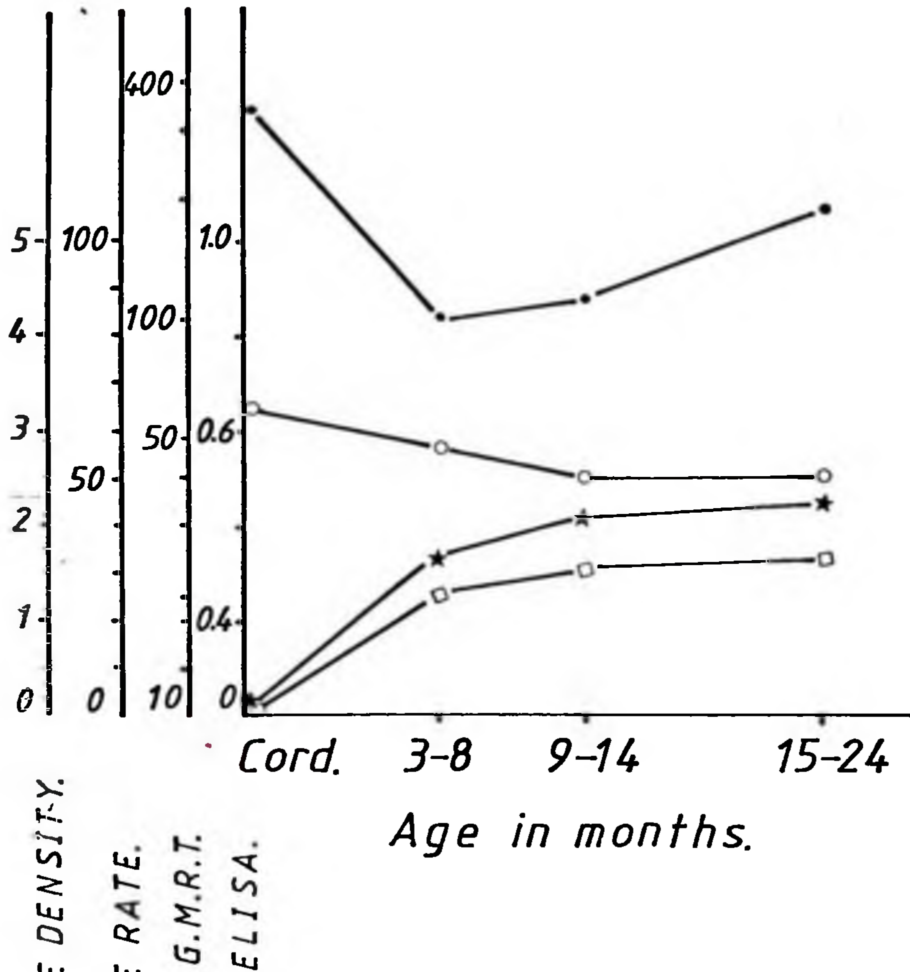

FIG.30: RELATIONSHIP BETWEEN MEAN MALARIA(IFA AND ELISA) ANTIBODY PARASITE RATE AND PARASITE DENSITY IN CORD SERA AND IN THE THREE AGE GROUPS IN RURAL CHILDREN ≤ 24 MONTHS.

■———■ GMRT
O———O ELISA
×———× PARASITE RATE
□———□ MEAN PARASITE DENSITY

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Figure 31 shows distribution of antibody in the rural children according to parasitaemia. There was an apparent difference in distribution of ELISA values in all 3 groups according to the presence or absence of parasitaemia and the differences were significant (Kendall tau test $P < 0.02$). Similar results were observed for antibody distribution by IFA titres (Figure 31). However, the mean ELISA values observed for the 3 groups and the GMRT were not significantly different.

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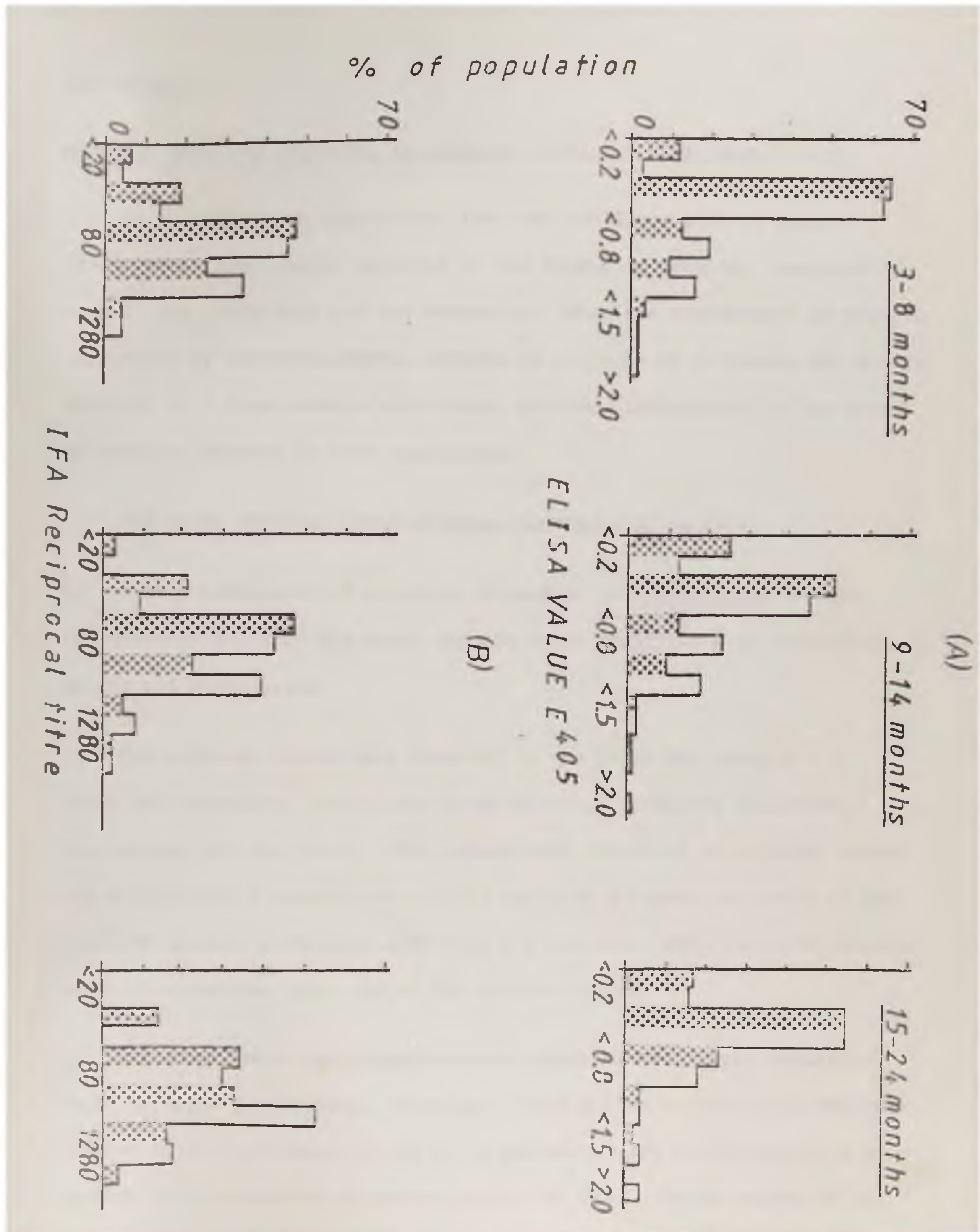

FIGURE 31 Distribution of antibodies by ELISA (A) and IFA (B) in rural children aged 24 months according to parasitaemia

▒ = parasitologically negative

☐ = parasitologically positive

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DISCUSSION

Malarial antibody responses in Ghanaian rural and urban populations

It has long been appreciated that the manifestations of malaria (Ross, 1910) are largely governed by the degree of immunity possessed both by the individual and the community. Thus the measurement of malaria antibodies by immunodiagnostic methods in subjects of different age groups resident in a hyperendemic environment provides information on the extent of immunity enjoyed in that population.

The study provided the following immunological profile.

1. A high prevalence of malarial antibodies to P.falciparum antigen was observed for both the rural and the urban populations as determined by IFA and ELISA tests.

2. Low antibody levels were observed in the early age group 0 - 2 years and thereafter levels rose progressively throughout childhood, adolescence and adulthood. This age-related increased in antibody levels was statistically significant. There was also a higher incidence of low antibody levels in children less than 5 years old. Adult antibody levels tended towards the upper end of the positive scale.

3. Antibody levels were significantly higher in the rural populations than the urban populations. With both tests a 100% seropositive rate was reached by age 3-5 years in the rural population and by the age 6-10 years in the urban population as determined by IFA test. ELISA values in the urban population did not ever achieve the 100% ceiling although high positive rates were reached 87% by age 6-10 years.

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4. High positive rates of malaria antibodies were also obtained in urban cord sera, and the 97% seropositivity was shown by both IFA and ELISA tests. The antibody levels observed were comparable to urban adult levels.

5. Results obtained with rural children aged 3-24 months further emphasised that antibody levels remain relatively low until the end of the first year of life and thereafter significant increases were observed in age group 15-24 months as determined by IFA tests. ELISA malaria antibody levels however showed a progressive decline in early age from 0-14 months. The low ELISA levels prevailed until after the age of 2 years when rapid increases were observed until adulthood. This slow ELISA response may be due to a differential immunocompetence to particulate and soluble antigens in children and not related to a lack of sensitivity or specificity of the ELISA test.

This differential behaviour of IFA and ELISA test observed in the present study by age and by population is important, and should be taken into account when antibody responses to malaria are being considered. Cornille Brogger et al (1978) used several serological tests to study humoral immune responses to malaria in Northern Nigeria, and similarly observed that different tests followed somewhat different patterns. They showed that IFA - P.falciparum test reached a plateau by 1-4 years, IFA - P.malariae test reached a plateau by 5-8 years, IFA - P.falciparum reached a plateau by 19-28 years. The number of bands in the ouchterlony P.falciparum test increased throughout life, on the other hand when results of the precipitin tests were expressed as the percentage positive (i.e. having at least one band) then the 100% ceiling was practically reached by

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5-8 years. They suggested from these findings that the IFA and IHA titres level off to a plateau if the number of molecules of immunoglobulin available at high dilutions of an initially small volume of plasma or serum falls below critical threshold. However, since an individual's immune response to malaria is dependent on several factors, age, immunocompetence, cumulative exposure to malarial antigens, and the kind and amount of specific therapy which he has received (Draper et al, 1972b), the use of different serological methods which employ different fractions of antigenic preparations will undoubtedly determine different aspects and different levels of the individual's immune response. Thus the use of different serological tests at the same time to provide information on different aspects of an individual's humoral response to malaria will contribute to a better prediction of state of protection than the use of a single test for antibodies.

The results of the present survey show a plateau of high IFA titre and ELISA values occurring early in children in the rural population but seen in adults in the urban population, would suggest that the high antibody levels bear some relation to the level of malarial transmission in the 2 populations, and may also be related to the level of functional immunity.

Malaria is characteristically a dynamic infection and therefore conclusions drawn from an isolated cross-sectional study as pointed out by McGregor et al (1965) must be made with caution. But despite the many shortcomings of cross-sectional surveys, the information this one has provided is valuable point prevalence data on an individuals and the rural and urban populations cumulative experience to malaria.

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Wilson, Garnham and Swellengrebel (1950) and McGregor (1960) defined the classical epidemiological effects of acquired immunity to malaria in man, where a consistent pattern of susceptibility to malaria infection is seen, especially in populations resident in hyperendemic regions. These workers reported that infants born in such areas are relatively resistant during the first 3 months of life and thereafter all children suffer severe and recurrent attacks of the disease. Clinical malaria becomes comparatively infrequent in later childhood and among adults the disease is rarely seen in acute form.

The parasitological results obtained for the 2 populations discussed previously, in conjunction with results of the present study would suggest that a state of stable malaria as described by McGregor (1960) exists in Ghana. The present results therefore conform generally to the classical concept of the acquisition of malarial immunity and thus is in accordance with results of similar field studies carried out in endemic malarious regions of Africa, i.e. in Liberia (Voller and Bray, 1962), Gambia (McGregor et al, 1965; Haverson et al, 1968), Senegal (Coudert et al, 1966), Northern Nigeria (Voller and Bruce-Chwatt, 1968; Cornille Brogger et al, 1978) and Tanzania (Voller et al, 1971; Draper et al, 1972b).

Specificity and sensitivity of IFA and ELISA in this study

The specificity of a test is the proportion of true negatives it detects,(Grab and Pull, 1974) and the sensitivity, the proportion of true positive it detects. Kuvin et al (1962) demonstrated the specificity of the fluorescent antibody test in malaria and since then the sensitivity and specificity has been often validated. The specificity and sensitivity

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of ELISA has been evaluated and validated for malaria as discussed above. Using the operational definitions of serological positivity to malaria set in this study (i.e. $\gtrsim$20 reciprocal titre for IFA and $\gtrsim$0.20 absorption at E_{405} for ELISA), both tests showed specificity of 100% since individuals never exposed to malaria infection were consistently negative in the test systems. Also ELISA test would appear as sensitive as IFA in determining exposure to malaria in the rural population, as by age group 3-5 years everyone was positive by both tests. However, IFA test was more sensitive than ELISA in determination of positive rates in the urban population. On the other hand, high sensitivity of a test system can sometimes be obtained at the cost of low specificity. However, it is suggested here that the difference in sensitivity between IFA and ELISA observed in the urban population represents true difference which may be related to the type of antibody detected by each test system, and possibly dependent on the maturity of immunocompetence of the individual. The age-related increase in antibody levels, faster by IFA in children, reaching early plateau and subsequent slow increase in adults, compared to the slow ELISA antibody decay between age group 0-14 months, a period of consolidation of antibody concentration (14-24 months) and a very fast increase with age until adulthood would confirm the above suggestion. It is also possible that the type of antibody determined by ELISA develop more slowly than those measured by IFA test. The differences may also be related to the fact that IFA test determines antibody concentration whereas ELISA results reflect the affinity as well as the concentration of antibodies (Butler et al, 1978) and may further suggest that production of high affinity antibodies may be age-related.

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The lack of difference in sensitivity of the 2 tests in the rural population implies that factors prevailing in the rural population may mask the difference between the 2 tests. Since a high titre probably bears some relationship to the combination of density and duration of parasitaemia (Voller and Bruce-Chwatt, 1968) and to the time since the last bout of parasitaemia., the difference in patterns shown by IFA and ELISA test between the 2 populations may therefore be related to differences in malaria parasite transmission rates observed,(i.e. a prolonged antigenic stimulation in the rural population) and it is possible that the upper limit of IFA test sensitivity may mask the difference.

Relationship between serology and parasitology

As previously suggested the serological differences between the urban and rural populations can be explained by the corresponding parasitological differences. However, at the overall population level, there was a positive correlation between parasite rate and antibody levels from age 3 months to 5 years (as determined by IFA) and from 14 months to less than 5 years (as determined by ELISA). From 5 years onwards (with both tests) the relationship became negative until adulthood. The absence of parasitaemia in cord sera and children aged 0-3 months, but showing positive levels of malaria antibody would suggest a brief negative association in early childhood, probably associated with maternal antibodies. It is suggested that the fall of parasite rate and parasite density occurring progressively and concurrently with a rise in antibody levels from late childhood to adulthood, suggests a direct correlation between increasing IFA titres, ELISA values and degree of effective immunity acquired. The rural population showed more malaria, higher test results and thus a suggestion of an

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associated higher level of immunity in that population. However, it has been repeatedly stressed (Cohen et al, 1961; McGregor, 1967) that higher antibody levels do not necessarily represent the concentration of the specific protective antibody. It has further been suggested by Voller and Bruce-Chwatt (1968) that the pattern of the progressive rise of immunoglobulin G and M reported by McGregor (1967) and studied in detail by Rowe et al (1968) may be reflected by the immunofluorescent test and in certain circumstances may give a measure of functional immunity in populations exposed to a significant amount of transmission. The relationship between serum immunoglobulin level and malaria antibodies in Ghanaian sera will be discussed in subsequent chapters.

Haverson et al (1968) carried out a similar survey in Gambia and they showed good correlation between parasite rate and IFA tests in the rural areas when the transmission was at a high level. In the urban area the relationship was closer in the younger age groups but not in adults who showed a low parasite rate with a relatively high proportion of positive titres by IFA test. In the present study IFA titres in the younger age groups of both populations were strongly correlated with the presence of parasites but this association changed to negative earlier in the urban population than the rural population despite the higher levels of antibody observed in the rural population. Thus this low parasite inoculation level, early recovery, and apparent immunity in the urban Ghanaian population as expressed by the relatively high antibody titres is analogous to the Gambian study (Haverson et al, 1968) and suggests that differences between the rural and urban results may also be related to effects of drug usage in the urban areas.

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Results of the present study would imply that the two tests used were not perfect indications of protection from infection but they do reflect the level of contact and partial immunity to malaria in the populations tested.

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<u>Some evidence for passive immunity to malaria in babies, malarial antibodies and functional immunity</u>

The presence of malaria antibodies as measured by both tests in the urban cord sera confirms similar reports of malarial antibody presence in cord sera of Liberian mothers (Voller and Bray, 1962) and in Nigerian cord sera (Edozien et al, 1961; Gilles et al, 1969; Williams and McFarlane, 1969; Seitz, 1971). Edozien et al (1962) further demonstrated the antiparasitic effect of pooled cord sera when administered to a 12 month old child with a severe *P.falciparum* infection. Thus suggesting a protective value of cord malaria antibody and therefore support for the idea that immunity to malaria in the neonate in endemic regions is placentally transmitted. Also the observed decline of antibodies during the first few months of life further supports the view that, in man, antibody transfer from mother to child is solely pre-natal and transplacental (Brambell, 1958; Bangham, 1960).

Indirect evidence of passive immunity to human malaria has been widely documented. Koch (1898) suggested that the African child was immune at birth because he inherited a certain degree of immunity from his ancestors who were already immune to malaria. He further surmised that during infancy the child may contract the disease in a mild form, and so become completely immune. Stephens and Christophers (1900) studied malaria in West African children and reported that the mildness of malaria infections in West African children was due to immunity transmitted from the parent to the offspring. Clark (1915) working in Panama reported the absence of malarial infections in newborns despite heavy concentrations of *P.falciparum* placental infections. He further noted that children less

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than 2 years rarely died of malaria. Clark (1937) further suggested that the reduced early infant mortality to malaria was due to protection afforded by the mother. This observed phenomena of early neonatal resistance to malaria in hyperendemic regions has been summarily confirmed in different parts of Africa. In Sierra Leone Blacklock and Gordon (1925) in a study of 800 children under the age of 2½ years found only one child with positive parasitaemia during the first month of life, from then on they observed a rise in parasitaemia until the age of 1½ years when the incidence curve flattened. They offered 2 explanations for their observations; they suggested that transferred factors from mother to offspring steadily diminished until the age of 1½ years, and also, that effective exposure to infection steadily increased as the child grew until age 1½ years. Garnham (1949) working in Kenya, and Bruce-Chwatt (1952a) working in Nigeria both found a parasite rate of about 10% in children less than 3 months of age. This observation was at variance with the theoretical parasite rate for infants below the age of 2 months (55.6%) based on the sporozoite rate and the biting rate of the vector, established by Macdonald (1957). The low parasite rate and parasite densities found by both workers in different parts of hyperendemic Africa in very young infants, thus constituted clear evidence of a factor of protection in these children. Further evidence of the importance of the maternal immunity is provided by the observations made by Davies (1954) in Kampala (Uganda) where neonata malaria was reported to be common in children born of mothers from malaria transmission free areas, but rare among children born to mothers from endemic malaria areas.

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Other protective factors operating during the first months may be responsible for this neonatal resistance to malaria. In Ghana a child less than 2 months is physically shielded from contact with people and all other environmental factors, thus a reduction in exposure may be one explanation. A selective feeding habit has been described in certain species of anopheles, which avoid young infants (Muirhead-Thompson, 1951). The exclusive milk diet, deficient in p-aminobenzoic acid may interfere with the normal development of parasites (Maegraith et al, 1952; Hawking, 1953). Allison (1954) further suggested that the presence of high proportions of foetal haemoglobulin may contribute to the suppression of malaria infection during early life.

The present study shows evidence of absence of parasitaemia in cord sera, and in children 0-3 months old. This observation coupled with the high antibodies in cord sera, and a decrease in antibody level in the early month of life confirms the above suggestion of passive transmission of acquired immunity to malaria. Bruce-Chwatt (1963) reviewed the incidence for passive transmission of immunity to malaria and made equal reference to the passive transmission of immunity to bacterial, viral and other parasitological diseases.

The serological responses detected by IFA and ELISA tests for the 2 populations show that the neonate is relatively free from malaria infection, probably due to passively acquired maternal antibody. This passive protection decays within a year in the urban adult child and between 3-8 months in the rural child and serologically detected antibodies similarly decline. A phase of susceptibility to malaria prevails with little immunity between ages 1-3 years. By 5 years of age parasitaemia

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declines with increasing immunity until adulthood, when absence of parasites correlates with high antibody levels. Both tests are useful indicators of malaria endemicity, but it is possible that IFA is a better indicator of antiplasmodial activity and ELISA may correlate better with protection by virtue of the fact that it detects soluble antibodies, antibody concentration, and antibody affinity.

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EFFECT OF PREGNANCY ON MALARIAL ANTIBODY LEVELS AND MALARIAL IMMUNITY

Serological responses to malaria in 250 urban pregnant and 100 rural pregnant women were compared to those of 107 urban female age-matched non-pregnant control sera. These sera form part of Group B of the survey population (see Chapter Four, pp. 79).

All sera were tested for malarial antibodies by ELISA and IFA tests. Results were evaluated and where appropriate serological responses have been compared with parasitological data.

The relationship of the relative frequency distribution of malarial antibodies obtained with ELISA and IFA tests, in the 3 groups of sera tested, are shown in Figure 32. The mean and 95% confidence limits obtained with both tests for the 3 groups of sera tested are shown in Tables 21 and 22. Although the mean values of the groups were not different, the 3 groups of sera did show different patterns of distribution. An analysis of variance of the ELISA results showed that there was significant variability in all the groups and between any paired association ($P < 0.02$). Similar analysis was carried out with the IFA results. This showed that variance between urban non-pregnant control and urban pregnant was not significant, but between non-pregnant and rural pregnant, and urban pregnant and rural pregnant, the variation in antibody distribution pattern was significant ($P < 0.05$). The difference between the mean ELISA values of the groups was not statistically significant. However, the difference in IFA GMRT observed between the following paired groups: urban pregnant/rural pregnant; and non-pregnant/rural pregnant was statistically significant ($P < 0.001$), but not significant for the non-pregnant/urban pregnant pair.

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FIG.32: THE RELATIONSHIP BETWEEN THE FREQUENCY DISTRIBUTION OF MALARIAL ANTIBODIES OBTAINED WITH ELISA (a) AND IFA(b) IN URBAN PREGNANT RURAL PREGNANT AND URBAN FEMALE CONTROL SERA.

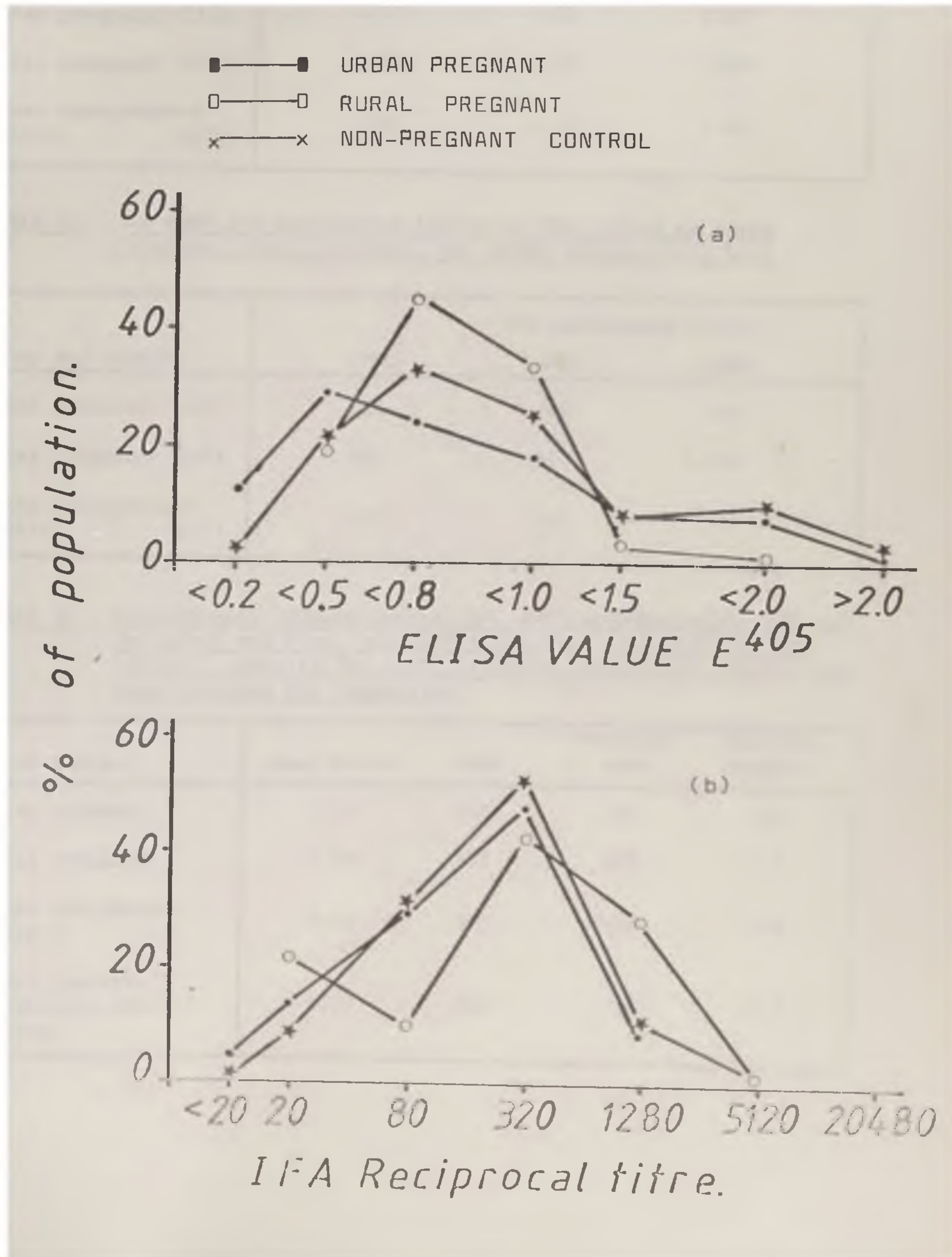

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TABLE 21 Mean and 95% confidence limits of ELISA values in urban pregnant, rural pregnant, and urban non-pregnant sera

Group and number	Mean	95% confidence limits	
		Lower	Upper
Urban pregnant (250)	0.70	0.03	1.37
Rural pregnant (100)	0.84	0.33	1.35
Urban non-pregnant control (107)	0.81	0.21	1.42

TABLE 22 IFA GMRT, 95% confidence limits of IFA titres in urban pregnant, rural pregnant, and urban non-pregnant sera

Group and number	GMRT	95% confidence limits	
		Lower	Upper
Urban pregnant (250)	299	240	410
Rural pregnant (100)	907	510	1,300
Urban non-pregnant control (107)	370	300	440

TABLE 23 Relationship between serological and parasitological results for urban and rural pregnant women and urban non-pregnant control. Results for rural (general population) adults have been included for comparison

Group tested	Mean ELISA	GMRT	Parasite rate	Parasite density
Urban pregnant	0.70	299	-ve	-ve
Rural pregnant	0.84	907	25%	1.2
Urban non-pregnant control	0.81	370	-ve	-ve
Rural (general population adults) control	0.95	686	8%	0.9

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The rural pregnant sera showed a 100% seropositive rate for both tests, but urban pregnant sera showed 98% seropositivity. On the average, the urban pregnant sera showed slightly lowered antibody titres and ELISA values than their non-pregnant controls.

A complete absence of parasitaemia was observed for the non-pregnant controls and the urban pregnant women. The rural pregnant women, on the other hand, showed a parasite rate of 25% indicating a level of parasitaemia higher than (previously discussed 8%) observed for rural adult population. The relationship between mean ELISA, GMRT, parasite rate and mean parasite density in the urban pregnant, rural pregnant and urban non-pregnant control is shown in Table 23. Results for rural adult population previously discussed have been included here for comparison.

DISCUSSION

The results indicate the malaria antibody levels tend to be lower in pregnancy, and results obtained with both IFA and ELISA tests reflect this in the urban population, although the differences observed were not marked. Since proper controls were not available for the rural population, it is impossible to draw firm conclusions from antibody values obtained. However results obtained with rural adults (in the general population) have been used for comparison. ELISA values were lower in the rural pregnant group than in the rural adult population, but the IFA GMRT value was higher.

The most striking observation in the present study was the high parasite rate and mean parasite density in the rural pregnant group, well above the levels observed in the rural population in general. This is consistent with similar observations made by Gilles et al (1969), Otieno et al (1971) and Kortmann (1972), who showed that in pregnancy the frequency of

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malarial parasitaemia and the density of infection are higher in pregnant women than either the same individuals before pregnancy or in a non-pregnant control group of the same age. In all these cases, P.falciparum was the infecting species. The reduction of resistance to malaria in pregnancy has also been reported to be higher among the primiparous than in multiparous women (McGregor and Smith, 1952; Schofield et al, 1964; Lelijveld et al, 1971). The observed increase in parasite rate and density in the rural pregnant group may therefore be related to their primiparity and the stage of pregnancy at which samples were collected. Both groups of pregnant sera (rural and urban) were primiparous and samples were obtained at 30 weeks gestation. The highest rate of parasitaemia in pregnancy has been found during the second trimester but decreased during the last trimester (Pingoud, 1969). However, others have reported that the increased second trimester parasitaemia is maintained up to the time of delivery (Hung, 1951; Hamilton et al, 1974). Results presented here are from a cross-sectional study and therefore it is not possible to substantiate any of the above observations. It is nevertheless possible that the high rural pregnant parasitaemia may be related to the stage of pregnancy at which samples were collected.

The absence of parasitaemia in the urban pregnant women is difficult to explain and may be due to several reasons, a lowered parasite transmission in the urban areas (previously discussed), a wider use of malaria chemoprophylaxis and/or to a better standard of living and health care enjoyed by the urban group. It is also possible that the highly active reticulo-endothelial system that develops in the placenta around the second trimester (Garnham, 1938) may have limited malaria infection in the urban pregnant group. Unfortunately, the nature of this study only provides a point prevalence data, and does not provide information from which the related events of pregnancy can be determined.

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Reports from a number of studies would suggest that the increase of malaria during pregnancy may well be due to a decrease of the acquired immunity to malaria during pregnancy (Bruce-Chwatt, 1952b; McGregor and Smith, 1952; Cohen and McGregor, 1963; Gilles, 1967; Gilles et al, 1969). Malaria serological indices have therefore been used in recent times to determine and/or confirm the suggested lapse of immunity in pregnancy.

McGregor et al (1965) detected substantial differences in IFA titres between parasitised pregnant women and their non-pregnant controls in the Gambia and suggested that immunofluorescence might well be used with profit in the study of the immunological changes that attend pregnancy. Several workers have since shown that despite the fact that there is some loss of protective immunity to malaria during pregnancy, no corresponding change in IFA titre can be detected (Gilles et al, 1969; Kortmann, 1972). In the present study, although malaria antibodies (by ELISA) were significantly lowered in the pregnant sera, no appreciable difference was observed in IFA titres in the urban group, and in the rural pregnant group IFA GMRT was even higher than that for the rural non-pregnant adults. These results seriously question the value of ELISA results and IFA titres as indicators of acquired protective immunity against malaria.

Endemicity studies in malaria endemic areas (Voller and Bray, 1962; McGregor et al, 1965; Voller and Bruce-Chwatt, 1968; Lelijveld, 1971; Draper et al, 1972a; Cornille Brogger et al, 1978) and the findings in this thesis suggest that both IFA titres and ELISA values may be used as practical parameters of acquired collective immunity to malaria. However the lack of any marked decrease in ELISA values and the increased IFA titres in rural pregnant group implies a lack of correlation between antibody

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levels and parasitaemia, and would further suggest that correlation between protective antibody and serologically demonstrable antibodies are also limited. According to Voller and Bruce-Chwatt (1968) IFA titre does not necessarily reflect the level of functional immunity, but is a reliable indicator of past exposure. These workers further showed that IFA titres bear some relationship to both density and duration of parasitaemia, and to the time elapsed since the last bout of parasitaemia.

The information provided by this study as previously pointed out is limited and the antibody results are difficult to interpret. It is suggested therefore that future studies of a longitudinal nature, following urban and rural mothers during and after pregnancy, to observe for changes in parasitology, immunoglobulin and malaria antibody levels (by ELISA and IFA) may provide more useful information. Such a study may provide an explanation of the reduced resistance to malaria during pregnancy observed in the rural pregnant women, and also that of the absence of parasitaemia in the urban pregnant group. It may also help to clarify the relationship between IFA titres, ELISA values and the level of protective immunity.

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MALARIAL ANTIBODY LEVELS IN DIFFERENTIATING BETWEEN ACUTE AND CHRONIC MALARIAL ASSOCIATED DISEASE SYNDROMES

This section is concerned with making a comparison of serological responses to malaria in hospitalised patients with acute malaria, patients with chronic malarial associated disease syndromes as well as those resident in areas endemic for malaria and suffering from other parasitic diseases. The possibility that differential levels of malarial antibodies could be used as a diagnostic aid will be discussed.

Sera for this study were selected from the clinical cases and their matched controls from Group F of the survey population (Chapter Four, p.81) which consisted of the following: 1) Malaria associated disease groups made up of 43 cases of acute malaria, 44 with nephrotic syndrome, 20 with tropical splenomegaly (TSS) and 28 with Burkitt's lymphoma (BL). 2) Other parasitic disease groups comprising 50 with chronic tuberculosis (TB), 56 with schistosomiasis and 30 with onchocerciasis, all resident in areas chronically endemic for malaria. 3) Hospital controls, i.e. control groups age matched for the individual disease groups (see Chapter Four, Group F, pp. 81) and, giving similar antibody distribution patterns and levels. The sera of each control group were pooled. All the patient groups were resident in either semi-rural or rural parts of Ghana. Of all the groups tested only the acute malaria group, one nephrotic syndrome and 3 BL patients had parasitaemia. *P.falciparum* was the infecting species in all cases.

Sera were tested for malarial antibodies by ELISA and IFA tests. Class specificity of malarial antibodies was also determined using IgG and IgM class specific antisera conjugates. Antibody levels observed in the malaria associated disease and other parasitic disease groups have been compared with levels in hospital controls. Where appropriate serological and parasitological results have been compared.

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The relative frequency distribution of malaria antibodies by ELISA and IFA in the disease groups compared with levels in the hospital controls is shown in Figure 33. The range, mean and 95% confidence intervals of malarial antibody levels as determined by the 2 tests, for all groups of sera are shown in Tables 24 and 25. Malarial antibody tested by both ELISA and IFA was present in all sera except for one nephrotic syndrome case. Results obtained by both tests in all groups showed statistically significant correlation (Spearman Rank Correlation Coefficient $r = 0.91$ with $P < 0.001$).

Malaria associated diseases Antibody distribution patterns (by ELISA values) in the acute malaria, nephrotic sera, and hospital controls were similar, although levels in nephrotic syndrome sera were lower. At the extreme ends of the distribution curve 29% of nephrotic sera had values at the lower end of the positive scale and 42% of acute malaria subjects had values at the upper end. The distribution patterns of ELISA values of TSS and BL were significantly different from that of the hospital controls (Kendall Tau test analysis of variance $P < 0.001$). A large proportion of TSS had high ELISA values and more than 60% of BL sera gave lower ELISA positive values. The mean ELISA values for acute malaria and TSS were not significantly different from the hospital control mean value. However, mean ELISA values in nephrotic syndrome and BL were significantly lower than mean levels in acute malaria, TSS and hospital controls ($P < 0.01$).

The distribution pattern of IFA titres in all the malaria associated disease groups was similar to levels in hospital controls except for the distribution pattern in BL which was significantly different and skewed towards the lower end of the positive scale. The IFA GMRT in all groups was not significantly different from GMRT of hospital controls.

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FIGURE 33 Frequency distribution patterns of malarial antibodies by ELISA (A) and IFA (B) observed for malarial associated diseases and other parasitic diseases

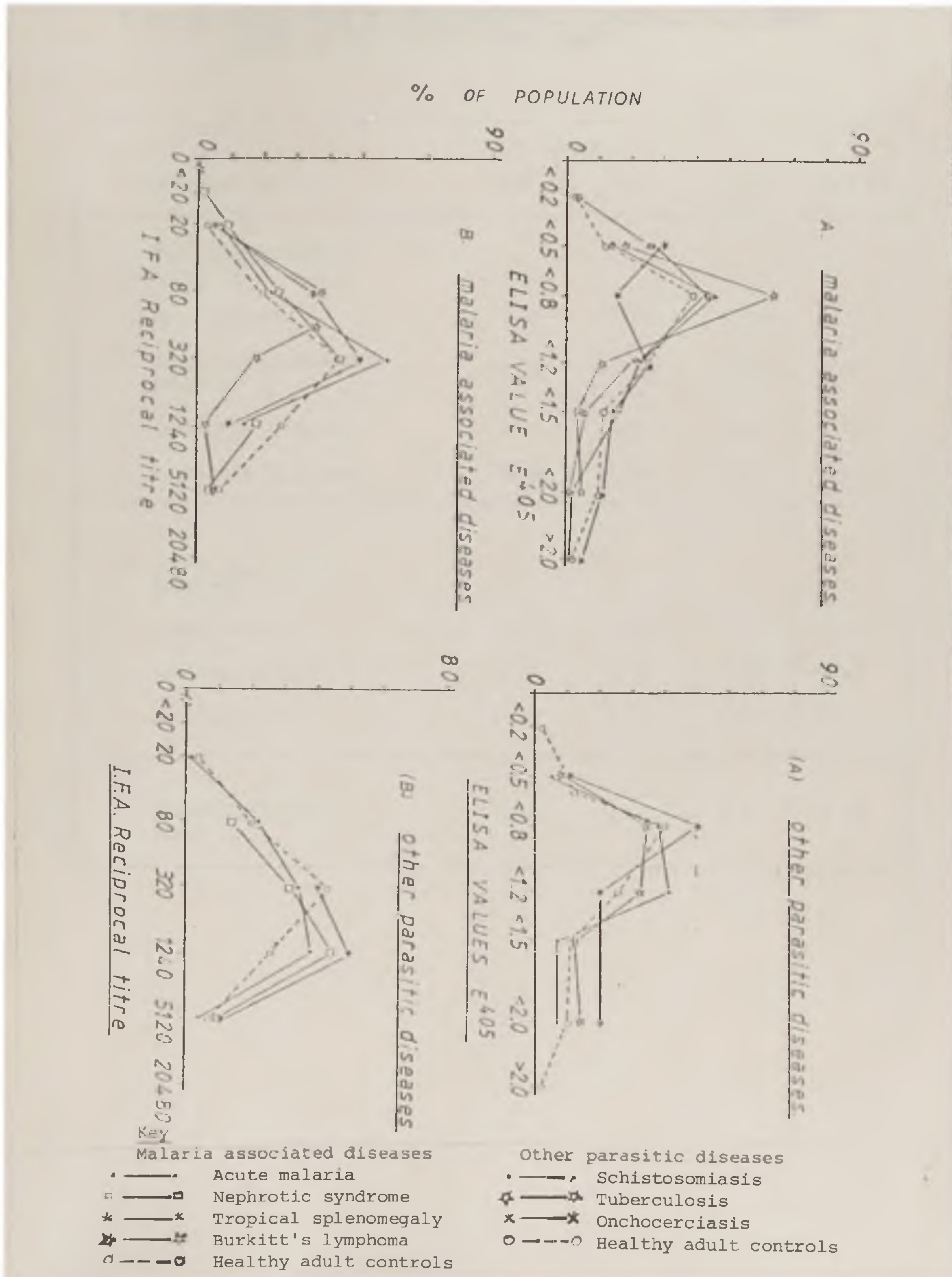

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TABLE 24 Range, mean and 95% confidence interval (CI) of ELISA values in the groups of sera tested

Populations tested	Range			
	Min	Max	Mean	95% CI
1. Malaria associated diseases				
Acute malaria	0.40	2.00	0.84	± 0.11
Tropical splenomegaly syndrome	0.34	2.24	0.99	± 0.27
Nephrotic syndrome	0.20	1.60	0.69	± 0.09
Burkitt's lymphoma	0.37	1.69	0.67	± 0.12
2. Other parasitic diseases				
Tuberculosis	0.31	1.95	0.96	± 0.12
Schistosomiasis	0.32	1.85	0.89	± 0.09
Onchocerciasis	0.50	1.74	0.90	± 0.31
3. Hospital control group	0.06	2.14	0.91	± 0.06

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TABLE 25 Range, GMRT and 95% confidence interval (CI) of IFA titres in the groups of sera tested

Populations tested	Range		GMRT	95% CI
	Min	Max		
1. Malaria associated diseases				
Acute malaria	20	1280	253	± 70
Tropical splenomegaly syndrome	20	1280	297	± 76
Nephrotic syndrome	0	5120	235	± 78
Burkitt's lymphoma	20	5120	231	± 168
2. Other parasitic diseases				
Tuberculosis	20	5120	422	± 126
Schistosomiasis	80	5120	624	± 169
Onchocerciasis	320	5120	845	± 412
3. Hospital control group	20	5120	390	± 78

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Other parasitic disease groups The antibody distribution patterns for all the other parasitic disease groups were significantly different from the distribution in the hospital controls, both by ELISA and IFA (Kendall tau test $P < 0.02$). The distribution curves obtained with these sera, i.e. TB, schistosomiasis and onchocerciasis, were all shifted to the upper end of the scale. High mean ELISA values and IFA GMRT were observed in all the groups, and were statistically different from mean levels observed in the hospital controls ($P < 0.02$).

Immunoglobulin class specificity of malarial antibodies Malaria antibody levels determined in all groups of sera were predominantly IgG, and the levels observed are as shown in Tables 24 and 25 and Figure 33. Positive levels of IgM antibodies were present at very low levels in all groups of sera except TSS sera, in which low, but significantly increased levels were observed. The mean IgM ELISA value and GMRT were significantly higher in TSS than in any of the other groups of sera ($P < 0.05$). The range, mean and seropositive rates of IgM antibody, in all groups are shown in Tables 26 and 27.

Comparison of serological and parasitological results Further analysis of results for acute malaria, nephrotic syndrome, and Burkitt's lymphoma groups were carried out to see if any relationship exists between high antibody levels, parasite rate and parasite density. All the acute malaria patients had slide-proven parasitaemia. Parasite density ranged from 0.1 to 10% with a mean density of 4.5%. Although the group showed a 100% seropositive rate and high antibody levels there was no direct correlation between antibody levels and parasite density. Serum from the one nephrotic syndrome and 3 BL patients with patent parasitaemia did not show the highest, nor indeed the lowest, antibody levels in their respective groups.

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TABLE 26 Range, mean ± 1SD and seropositive rate of IgM malarial antibodies by ELISA in all groups of sera

Group	Range Min	Max	Mean	± 1SD	Seropositive rate (%)
1. Malaria associated diseases					
Acute malaria	0.03	0.30	0.19	± 0.15	10
Nephrotic syndrome	0.01	0.33	0.25	± 0.16	16
Tropical spleno-megaly syndrome	0.04	0.55	0.45	± 0.19	55
Burkitt's lymphoma	0.03	0.35	0.17	± 0.09	9
2. Other parasitic diseases					
Tuberculosis	0.05	0.35	0.20	± 0.15	11
Schistosomiasis	0.10	0.40	0.20	± 0.16	6
Onchocerciasis	0.10	0.35	0.19	± 0.18	12
3. Hospital control group	0.03	0.44	0.23	± 0.20	13

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TABLE 27 **Range, GMRT and seropositive rate of IgM IFA titre in all groups**

Group	Min	Max	GMRT	Seropositive rate (%)
1. Malaria associated diseases				
Acute malaria	20	80	38	13
Nephrotic syndrome	20	80	49	12
Tropical spleno-megaly syndrome	20	320	99	60
Burkitt's lymphoma	20	80	25	9
2. Other parasitic diseases				
Tuberculosis	20	80	49	15
Schistosomiasis	20	80	65	10
Onchocerciasis	20	80	50	10
3. Hospital control group	20	80	40	15

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DISCUSSION

The above results show that some differences exist between antibody levels in acute malaria and in malaria associated chronic disease syndromes. The high correlation coefficient observed for the 2 tests suggests that they both measure specific immunological responses to malarial infection and therefore results by both tests are comparable.

Malaria antibodies in acute malaria All sera showed positive levels of malarial antibodies by both tests. These results are in concordance with the clinical and parasitological status of these patients. High antibody levels were generally found in this group than in their hospital matched controls with no detectable parasitaemia, although the difference observed between levels in the 2 groups was not statistically significant. These results are consistent with those observed by Rey et al (1967) in a study of hospitalised cases of malaria in Senegal. It is possible that the higher antibody levels in acute malaria cases bear some relationship to a combination of parasite density and duration of parasitaemia. The point can also be made from these results that malaria serological responses can only be properly assessed in conjunction with the subject's clinical and parasitological status. Without a knowledge of the prevailing epidemiological conditions, results are only indicative of past or recent exposure to malaria infection, and not of a subject's current clinical status.

The singular observation of patent parasitaemia and clinical illness concurrent with high levels of malaria antibodies in adults living in an endemic malarious region suggests a limited protective role for these antibodies and seriously questions the role of malaria antibodies, determined by IFA and ELISA tests in functional immunity to malaria.

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In an area of high malarial endemicity survival to adult life can usually be considered adequate evidence for the presence of malarial immunity. The correlation between age-related rise in malarial antibodies accompanied by reduction in parasitaemia and clinical activity observed in endemicity studies (Voller and Bray, 1962; McGregor et al, 1965; Voller and Bruce- Chwatt, 1968; Cornille Brogger et al, 1978) has been used as another practical parameter of acquired immunity. Several workers, however, using the immunofluorescent test have shown that IFA titres of subjects with patent parasitaemias are no different from titres of individuals with negative blood smears (Coudert et al, 1966; Marsden et al, 1967; Lelijveld, 1971). Further observations by Bray et al (1962) and Bruce-Chwatt (1963) that inoculation of individuals living in endemic areas of P.falciparum infection resulted in patent parasitaemia in over half the subjects, and were also accompanied with clinical symptoms in 15% to 25% of these subjects is further affirmation that correlation between protective antibody and serologically demonstrable antibody (by IFA and ELISA) is probably limited.

Another interesting observation which questions the extent to which serologically demonstrable antibody can be used as an indicator of protective immunity, is the observation, that the decrease in malaria (IFA) antibodies observed after prophylactic administration of antimalarial drugs to individuals in endemic areas, does not result in a corresponding decrease in protective immunity (Voller and Wilson, 1964). The effect of chemoprophylaxis, the absence from reinfection from malaria on the decay of malaria antibodies in Ghanaian subjects, and the susceptibility of these subjects to reinfection after re-exposure will be discussed in subsequent

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parts of this chapter. The point that can be made from the present finding, is that serological examination of blood from individuals resident in an endemic region at a single point in time, provides information regarding the individual's past or recent past exposure to malaria and is not indicative of the individual's clinical status or immunity to malaria, as parasite density is not directly related to antibody levels.

Malarial antibodies in nephrotic syndrome Antibody distribution in nephrotic syndrome sera showed a similar distribution pattern to that of the hospital control group, although there was a preponderance of lower values with the nephrotic syndrome sera. The GMRT and ELISA value were significantly lower than in controls ($P < 0.01$). These findings are at variance with observations made by Kibukamusoke et al (1967) in a study of malaria antibody levels (by IFA) in patients with nephrotic syndrome. They found a significant increase in titres among nephrotic syndrome patients compared with their matched controls. They further observed that the significant malarial antibodies were maintained despite considerable loss of identifiable malarial antibodies in the urine of their nephrotic syndrome patients. A further suggestion was made by these workers that without such loss, antibody concentrations would differ even more from controls. However, Powell et al (1977) in a study of nephrotic syndrome in New Guinea, showed results which are consistent with those observed in this study. The mean IFA titre in their nephrotic syndrome cases was significantly lower than in their matched controls.

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The present findings, of lowered malaria antibodies compared with levels in controls, which were predominantly P.falciparum IgG malarial antibody, and the absence of significant positive levels of IgM malarial antibodies, coupled with other serological features of Ghanaian nephrotic syndrome (low serum IgG, normal IgM, circulating soluble malaria antigen antibody immune complexes, antinuclear antibodies and high prevalence of anti-single stranded DNA antibodies, which will be discussed in subsequent sections) all point to a causal association with malaria infection. When P.malariae antigens were used as substrate in the IFA test, the antibodies detected were 2 to 4 fold titres lower than when P.falciparum antigen was used. Malaria parasitology in the Ghanaian nephrotic syndrome patients was largely negative. In all patients, multiple thick and thin films were examined for the presence of malaria parasites and only one patient was positive and the infecting species was P.falciparum. Also it has been previously established in this study that P.malaria occurs predominantly in the age groups 1-5 years at a rate of 3%, but all the nephrotics studied here were older than 5 years. However, there is evidence that P.malariae can exist subpatently in the blood of exposed individuals and relapse after 36 years (Spitler, 1948) and 53 years (Garnham, 1970).

Immunological evidence supporting the causal association between P.malariae and nephrotic syndrome was provided by Dixon (1966), Houba et al (1970; 1971) and Houba and Lambert (1974), but this has yet to be confirmed. The possibility that the utilisation of complement by malaria antigen-antibody complexes may be responsible for the kidney lesion in nephrotic syndrome seems an attractive concept. However, the finding by Wing et al (1971) that Ugandan nephrotic syndrome patients with immuno-fluorescent demonstration of IgG, IgM, IgA and C3 deposited along the

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glomerular membrane had glomerular nephritis resembled that of post-streptococcal rather than that of the quartan malaria is relevant here. This would suggest that immune complexes from any source, whether malarial, helminthic or bacterial, may be responsible for the nephrotic syndrome seen in populations in developing countries.

The Ghanaian nephrotic syndrome in the absence of possible viral (negative HBs antigenaemia, Cayzer,personal communication) streptococcal or other parasitic infection (Adu et al, in preparation) and with available serological data to be subsequently discussed, all suggest a malarial aetiology. The lowered malaria antibody levels when compared to controls may be due to renal loss of IgG due to renal damage that may result from the pathogenic effects of malaria antigen-antibody complexes deposited in renal gomeruli. Malaria serology can therefore be useful in evaluating the related events in nephrotic syndrome.

Malaria antibodies in tropical splenomegaly syndrome The contribution of clinical data and serological responses observed for this group of sera is consistent with the diagnosis of TSS. Serological features observed for TSS are serum IgG above the local normal mean, raised serum IgM, 2 to 3 times above local normal mean, raised levels of circulating immune complexes, auto-antibodies and evidence of in vivo activation of complement. The occurrence of these serological parameters and their relationship to the pathogenesis of TSS will be discussed in further sections. However the present finding of high levels of malarial P.falciparum specific antibodies, and low but significant levels of IgM specific antibody does fit with the recognised criteria for a diagnosis of TSS (Marsden and Crane, 1976) and is compatible with similar observations made by Ziegler et al (1973) and Crane et al (1977) on the incidence of malarial antibodies in TSS in Uganda and Papua New Guinea.

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The findings presented in this study are consistent with the following characteristics observed in TSS. Crane et al (1977) noted that the high IgG titres in TSS do not necessarily imply a greater exposure to malaria in the past. Indeed this lack of direct relationship between parasitaemia and antibody titre has been studied by Marsden et al (1967). These workers carried out a serological study of 2 villages in New Guinea where a high incidence of splenomegaly and hepatic sinusoidal infiltration were associated with endemic malaria. They reported that the group with the highest incidence of parasitaemia had antibody titres within the range of 1:320 to 1:640 while in the group with titres of 1:5120 only 44% had parasites in the blood. However, high titres of fluorescent antibody were found consequent to a sustained malarial challenge. The absence of parasites in the TSS group of sera studied here is therefore not at variance with the diagnostic criteria.

Malaria antibodies in Burkitt's lymphoma The epidemiological evidence incriminating malaria as a contributory factor in the aetiology of Burkitt's lymphoma is now beyond dispute (Lancet, 1970), but the frequency of parasitaemia, the type of parasite,and the immunological response to malaria in patients with BL remains unclear.

Epstein Barr Virus (EBV) is the other factor implicated in the aetiology of BL, and work in Uganda by de The et al (1978) provided convincing evidence of the causal association between EBV and BL. However the nature of the interaction between virus, malaria and the lymphoid tissues is not known. The simplest view (Lancet, 1970) is that lymphoid tissues which are exposed to the severe universal, persistent and relentless stimulus and stress exerted by malaria are somehow rendered more

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susceptible to neoplastic transformation in the presence of EBV. It is suggested here that if the BL-malaria hypothesis was true then this would be reflected in the amount of antibody to malaria present in the sera of Burkitt's lymphoma patients.

In this study, the results demonstrate that differences in antibody responses to malaria between BL and controls are not marked. Malaria antibody values in BL sera are much lower than in adult controls but when compared to their age-matched rural group the difference is not significant. These results are compatible with those reported by Feorino and Mathews (1974) who studied malaria antibody levels in Ugandan patients with BL and their age-matched healthy controls, by IFA and IHA tests. The studies failed to show marked differences between results from patients and controls. Similarly Nkrumah et al (1979) failed to show a difference in malaria IgG antibodies to P.falciparum in Ghanaian BL patients and their nearest neighbour age-matched controls. However these workers detected a difference in the malarial IgM antibodies, and the lowered titres in BL patients were significantly different from controls. In the study by Nkrumah et al (1979) positive levels of IgM specific malarial antibodies were only observed in 9% of cases. In the present study IgM antibody detected in both BL patients and controls was quite often below the baseline level of positivity <20 reciprocal titre and <0.20 for ELISA. It was interesting to note that Nkrumah et al set their positive baseline for IgM malarial antibody IFA titre at 1:4 reciprocal titre. The difference in reported results can therefore be attributed to the interpretation of the fluorescent antibody test which lacks inter-laboratory comparability and objectivity.

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Although the present results failed to show marked antibody increases in sera from BL patients by IFA and ELISA tests with P.falciparum they do not exclude malaria infection as a predisposing factor. Despite the rather low parasitological data of the group, i.e. only 3 out of 28 patients had parasitaemia with P.falciparum as the infecting species, it is quite possible that all the 4 human species of malaria are quite capable of inducing BL lymphoma, but in a country where the predominant infecting species is P.falciparum (98%) a causal link between P.falciparum and BL is most likely.

Other parasitic diseases Sera from patients with chronic tuberculosis, schistosomiasis and onchocerciasis were all from adult rural population. The distribution patterns observed with IFA titres and ELISA values are comparable with levels obtained for the rural adult population. All 3 groups showed very high GMRT and mean ELISA values and further suggests that all patients have developed some immunity to malaria.

Polyparasitism is predominantly a rural phenomena; Buck et al (1978a) carried out a series of studies relating the occurrence, frequency, and distribution of multiple infections in 13 villages of ecologically contrasting areas in 3 countries. In these villages, it was found that multiple infections with 2 or more parasites and with other infectious agents were the rule rather than the exception.

Virtually nothing is known about the dynamics of multiple infections, whether they develop together or independently (Buck et al, 1978b), whether some parasites may predispose the human host to certain intercurrent diseases or whether they may alter each other's symptomatic stages. The prevalence

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of polyparasitism in the urban population will be discussed in the next chapter. However it was noted in the present study that malarial antibody levels in all the 3 chronic parasite diseases tended to be higher than in healthy adults. This may reflect the higher parasite transmission observed in the rural areas, or may be enhancement due to concurrent chronic infection. Also none of these patients showed patent parasitaemia or clinical symptoms of malarial infection. The possibility that the enhanced antibody titres and ELISA values in these 3 groups may be due to antibodies against substances antigenically related to plasmodia present in TB, onchocercal and schistosome parasites should not be overlooked although it is unlikely.

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EFFECT OF MALARIA CHEMOPROPHYLAXIS ON MALARIAL ANTIBODY LEVELS

A decrease in IFA titres as a result of prophylactic administration of anti-malarial drugs to individuals in endemic malaria areas (Voller and Wilson, 1964) has been reported and variously substantiated (McGregor et al, 1956; Mattern et al, 1967). The purpose of this study is to verify this observed effect of malaria chemoprophylaxis (chloroquine) on serologically detected malarial antibodies (detected by ELISA and IFA tests) in 3 Ghanaian population sample groups, i.e. pregnant women, healthy adults and children. The results are discussed in terms of the effects of such prolonged chemoprophylaxis on acquired protective immunity, and on the importance of this effect in interpreting the results of serological surveys.

Sera from the 3 following groups were studied:

1. Pregnant women Blood films and blood samples were taken from 200 urban pregnant women on regular antimalarial prophylaxis throughout 30 weeks gestation, and a similar group of 250 urban pregnant women who had not been on regular anti-malarial prophylaxis. These form part of Group C of the survey population (see pp.79).

2. Healthy adults 200 Army officers resident in urban areas of Accra (see Group D of the survey population pp.81). They were divided into study and control groups, each comprising 100 individuals. The study group was given malarial chemoprophylaxis weekly for a period of 8 months. The control group received vitamin substitutes for the same period of time. Blood films and blood samples were taken before and after 8 months. Unfortunately at the second sampling only 57 of the study group (protected) and 63 of the control group (unprotected were available for sampling.

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3. Children 500 rural children, 250 aged 3-8 months and 250 aged 9-14 months were used. (These form part of Group E of the main survey population) Half the children in each age group were placed into the control group and the rest were placed into the study group. Weekly chloroquine sulphate syrup 75 mg base was administered to the subject group. The control group received a weekly vitamin syrup preparation. Unfortunately the study had to be terminated after 2 months. Blood samples and blood films were taken before and after 2 months of prophylaxis. At the follow-up survey 2 months later out of the 250 aged 3-8 months only 107 of the study group (protected) and 106 of the control group (unprotected) were available for sampling. For the 9-14 month old children 103 of the protected and 105 of the unprotected were available for sampling.

Blood films were examined for malaria parasitaemia and sera were tested for malarial antibodies by ELISA and IFA tests.

Urban pregnant women All subjects both protected and unprotected, were parasitologically negative. The relative frequency distributions of malaria (ELISA and IFA antibodies) are shown in Figure 34. The range, mean and 95% confidence intervals of ELISA values and IFA titres are shown in Tables 28 and 29. The difference in the distribution patterns in the 2 groups was not statistically significant. A higher proportion of the protected group, however, tended to have lower values than the unprotected group. The overall seropositivities were not different in the 2 groups using both tests. Values obtained were 95% and 98% for protected and for unprotected groups respectively by ELISA. Similarly IFA tests gave a 98% seropositivity for protected and 99% for unprotected groups. The mean ELISA value and IFA GMRT obtained for both groups were not significantly different.

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FIGURE 34 THE RELATIVE FREQUENCY DISTRIBUTION OF MALARIA (ELISA (a) and IFA (b) ANTIBODIES IN PROTECTED AND UNPROTECTED URBAN PREGNANT WOMEN

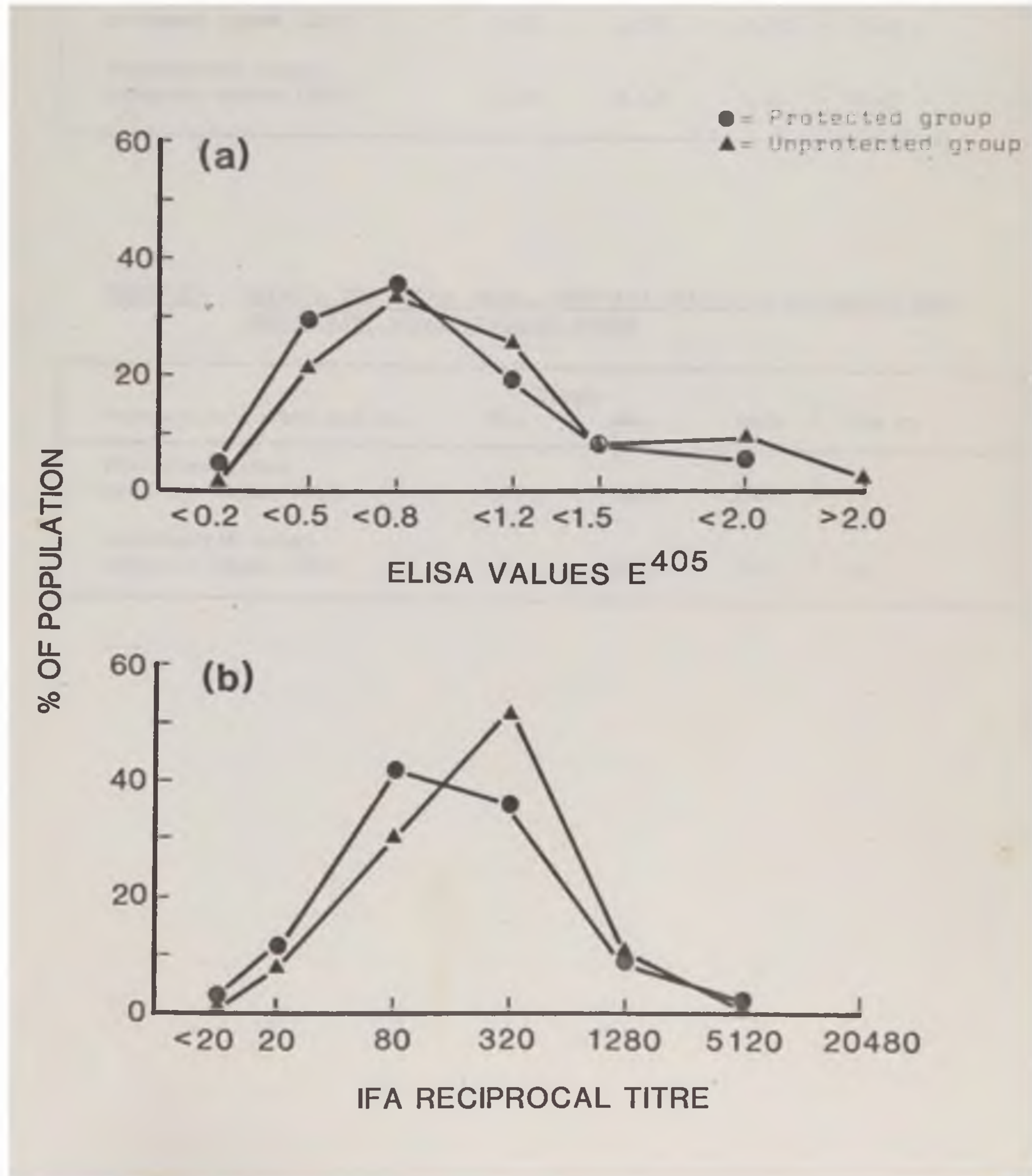

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TABLE 28 **Malaria ELISA antiobdy range, mean and 95% CI in protected and unprotected urban pregnant women**

Population tested and no.	Range Min	Max	Mean ELISA	±	95% CI
Protected urban pregnant women (200)	0.12	1.97	0.70	±	0.06
Unprotected urban pregnant women (250)	0.15	2.12	0.81	±	0.07

TABLE 29 **Malaria IFA titre range, GMRT and 95% CI in protected and unprotected urban pregnant women**

Population tested and no.	Range Min	Max	GMRT	±	95% CI
Protected urban pregnant women (200)	0	5120	299	±	73
Unprotected urban pregnant women (250)	0	5120	370	±	65

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Healthy adults All subjects were parasitologically negative before and after 8 months prophylaxis. The change in antibody distribution resulting from prolonged chemoprophylaxis was assessed by comparing the distribution in the protected group against its baseline control values and similarly for the unprotected group. The frequency distribution of antibody levels of the 2 groups expressed against their baseline distribution by both tests is shown in Figure 35. The range, mean and 95% confidence intervals in protected and unprotected groups with their corresponding baseline levels are shown in Table 30 for ELISA values and Table 31 for IFA titres.

The distribution patterns shown by the protected group and its baseline control were not significantly different using either test, but the protected group had lower antibody levels than the baseline control. A decline in ELISA and IFA antibodies was observed more frequently with sera which had high antibody levels initially (ELISA values of ≥ 1.20 and IFA titres of ≥ 1.320). Lower initial antibody values did not show appreciable decreases. The difference between the mean ELISA values for the protected group and its baseline control was not significant, but the GMRT was significantly different at the one percent level.

The unprotected group showed similar distribution patterns compared to its baseline control by both tests. Mean ELISA values for the 2 groups were not significantly different, although the difference in GMRT between the 2 groups was significant ($P < 0.01$). There were no significant antibody increases or decreases in individual sera with respect to time.

The antibody distribution levels for the baseline of the protected group were compared with the unprotected group and its baseline control. There were no appreciable differences in distribution patterns observed with both tests.

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FIGURE 35 THE RELATIVE DISTRIBUTION OF MALARIA (ELISA (a) and IFA (b)) in HEALTHY PROTECTED AND UNPROTECTED ADULTS AGAINST THEIR BASELINE VALUES

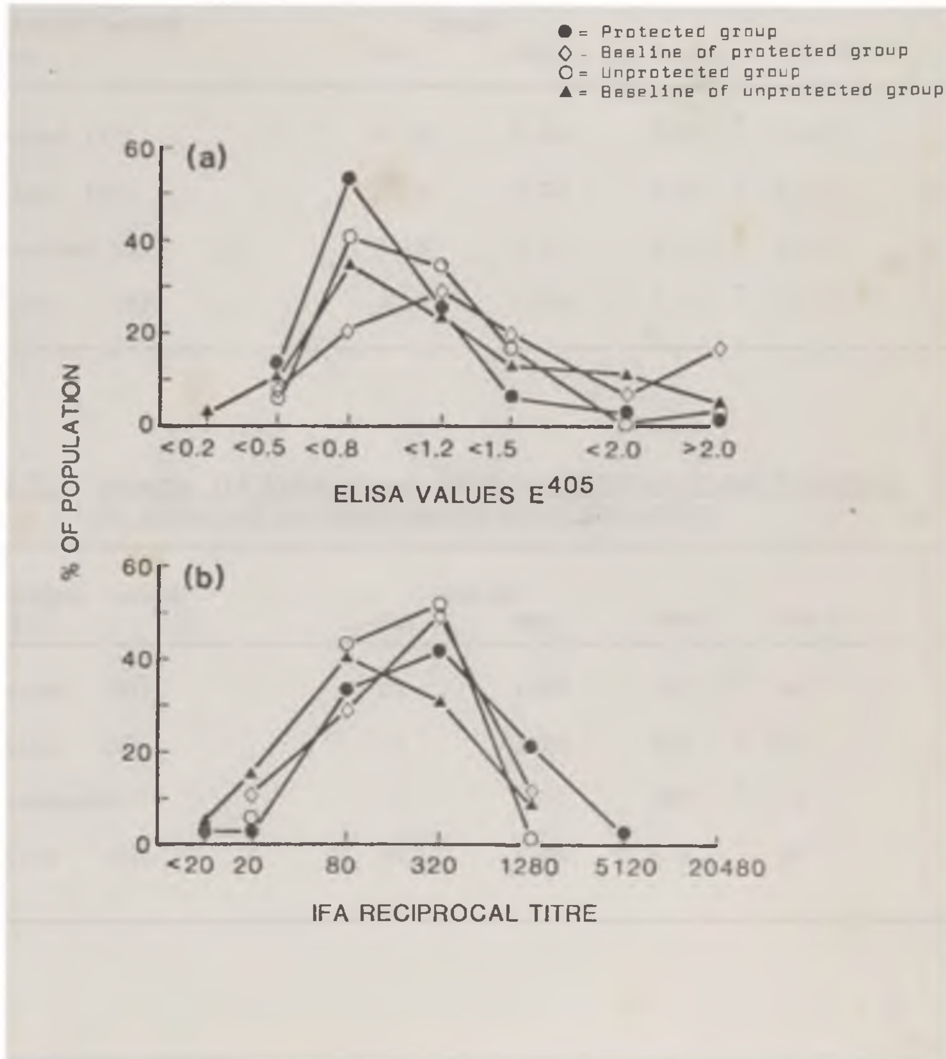

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TABLE 30 Malaria ELISA antibody range, mean and 95% confidence interval of protected and unprotected adult population with the corresponding baseline control values

Population tested and no.	Range Min	Max	Mean	95% CI
Protected (57)	0.10	1.96	0.60	± 0.13
Baseline (57)	0.13	2.24	0.63	± 0.18
Unprotected (63)	0.13	2.14	0.64	± 0.13
Baseline (63)	0.12	1.76	0.77	± 0.17

TABLE 31 Malaria IFA titre range, GMRT and 95% confidence intervals of protected and unprotected adult population

Population tested and no.	Range Min	Max	GMRT	95% CI
Protected (57)	20	1280	307	± 46
Baseline (57)	0	5120	602	± 107
Unprotected (63)	0	1280	347	± 79
Baseline (63)	20	1280	538	± 78

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The protected group was then compared with the unprotected group. The protected group had lower ELISA values than the unprotected group, but the IFA differences between the 2 groups were not marked. The differences in mean ELISA values and GMRT between the 2 groups were not significant.

Rural children Parasitological results for this group are shown in Table 32. The unprotected baseline control group showed the highest parasite rate and density. However 2 months chemoprophylaxis in protected children did not lead to significant decrease in parasite rate and parasite density. Only results from children who were available at the follow-up survey were analysed. These were analysed as follows: The baseline antibody distribution levels for both the protected and unprotected groups were similar, these were pooled to provide a single baseline control group for comparison with the protected and unprotected groups.

It was appreciated that antibody results of an age group, whether protected or unprotected, are affected by variations due to individuals changing between age groups on ageing (seroconversion with age). The antibody distribution levels in the unprotected children were compared with those for the baseline control group in order to observe for the effect of ageing. To observe for the effect of prophylaxis, the distribution pattern of the protected group was compared with the unprotected group and also with the baseline control. The distribution patterns for protected, unprotected and baseline controls for both age groups are shown in Figure 36. The range, mean and 95% confidence intervals in the 3 groups are shown in Table 33 (ELISA values) and Table 34 (IFA titres).

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TABLE 32 Parasite rate and mean parasite density observed for protected and unprotected rural children and their baseline controls for age groups 3-8 months and 9-14 months

Population group	Parasite range (%)	Mean positive parasite density
3-8 months		
Protected	21.1	1.04
Unprotected	25.5	1.60
Baseline	26.0	1.65
9-14 months		
Protected	25.5	2.11
Unprotected	28.6	2.10
Baseline	43.0	2.21

TABLE 33 Malaria ELISA antibody range,mean and 95% CI for rural children protected and unprotected groups. Baseline control has been added for comparison

Population tested and no.	Range Min	Range Max	Mean	95% CI
5-10 months Protected (107)	0.11	1.19	0.43	± 0.06
5-10 months Unprotected (106)	0.11	1.24	0.44	± 0.04
3-8 months Baseline control (213)	0.13	1.90	0.56	± 0.02
11-16 months Protected (103)	0.12	1.29	0.45	± 0.04
11-16 months Unprotected (105)	0.12	1.70	0.53	± 0.06
9-14 months Baseline control (208)	0.11	1.57	0.50	± 0.02

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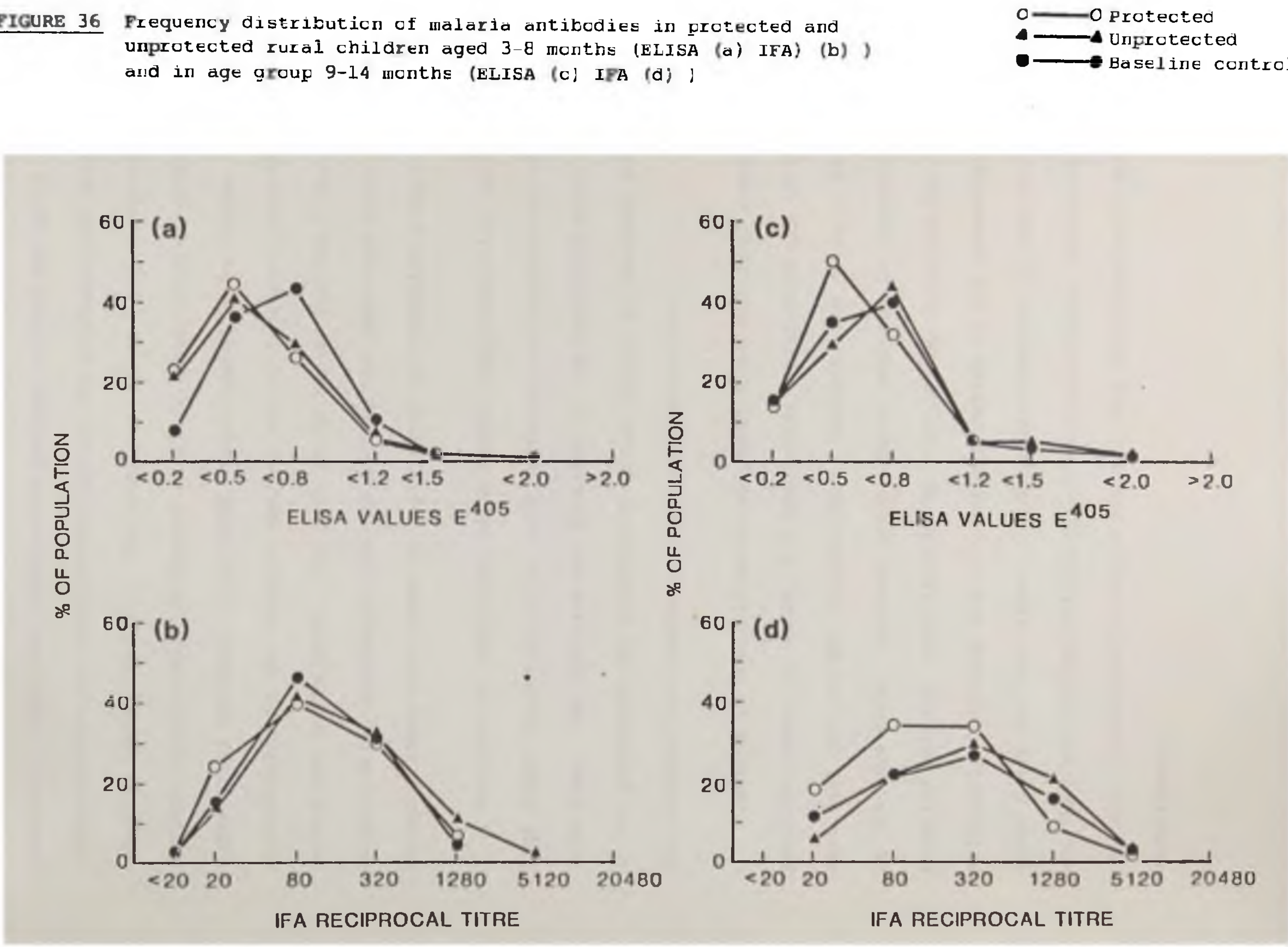
FIGURE 36 Frequency distribution of malaria antibodies in protected and unprotected rural children aged 3-8 months (ELISA (a) IFA) (b)) and in age group 9-14 months (ELISA (c) IFA (d))



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In the 3-8 month age group seropositivity rates by ELISA were 78%, 79% and 92% for protected, unprotected and the baseline control respectively. By IFA, the seropositive rate for the 3 groups were 97%, 99% and 98% respectively. When the distribution pattern in this age group was analysed, little difference was observed between the protected and unprotected groups (by both ELISA and IFA). The baseline control however showed a distribution pattern significantly different ($P < 0.02$) from those of protected and unprotected children, also ELISA values tended to be higher. The IFA titre distribution in the baseline control was no different from those of protected and unprotected. The mean ELISA values for the 3 groups were not significantly different, but the GMRT in the protected group was significantly lower than those in unprotected and baseline groups.

Analysis on individual bases showed that sera which initially had high ELISA values (>1.2) in both protected and unprotected groups declined with time. The decrease was greatest in the protected sera. IFA titres in the protected and unprotected groups showed an increase with time, but the increase was greatest in the unprotected sera.

Results in the 9-14 month age group were similarly analysed, seropositive rates, by ELISA, for protected, unprotected and baseline control were 84%, 86% and 84% respectively. Seropositive rates in all 3 groups by IFA were 100%. Antibody distribution patterns in unprotected and baseline control were similar but that in the protected group was significantly different and a higher proportion of the protected group had lower ELISA and IFA titres. Differences observed in mean ELISA values and GMRT between the following paired associations, protected/baseline control, unprotected/baseline control, and protected/unprotected were not significant.

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TABLE 34 Malaria IFA titre range, GMRT and 95% CI for rural children protected and unprotected groups. Values for baseline controls are also shown

Population tested and (no)	Range Min	Max	GMRT	95% CI
5-10 months Protected (107)	20	1280	98	± 34
5-10 months Unprotected (106)	20	5120	148	± 36
3-8 months Baseline control (213)	0	1280	112	± 29
11-16 months Protected (103)	20	5120	151	± 43
11-16 months Unprotected (105)	20	5120	200	± 59
9-14 months Baseline control (208)	0	5120	195	± 39

TABLE 35 Relationship between serological and parasitological results in rural protected and unprotected children

Population tested age group and (no)	GMRT	Mean ELISA	Parasite rate %	Mean Parasite density
5-10 months Protected (107)	98	0.43	21.1	1.04
5-10 months Unprotected (106)	148	0.44	25.5	1.60
3-8 months Baseline control (213)	112	0.56	26.0	1.65
11-16 months Protected (103)	151	0.45	25.5	2.11
11-16 months Unprotected (105)	200	0.53	28.6	2.10
9-14 months Baseline control (208)	195	0.50	43.0	2.21

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Analysis on an individual basis showed that a significant decrease in antibody levels occurred in protected sera which initially had high antibody levels ($\geq$1.20 by ELISA and $\geq$1:320 IFA titre) 20 to 40% decrease in ELISA value and 2-4 fold IFA titre decreases were observed.

Relationship between serological and parasitological results in rural children Table 35 shows the combined serological and parasitological results. In the 3-8 month age group the initial negative correlation between ELISA values and parasitaemia in the early age group (previously observed and discussed in a previous section of this chapter, pp.81 was enhanced after the period of prophylaxis. The positive correlation between IFA titre and parasitaemia observed previously in this age group was reduced after the period of prophylaxis. Similarly for the 9-14 month age group the positive correlation which initially existed between malarial (ELISA and IFA) antibody and parasitaemia, was decreased after the period of protection.

DISCUSSION

Protection of populations against malaria in endemic areas can be obtained by various methods of vector control (Draper, 1960; Kortmann, 1972, Bruce-Chwatt et al, 1973). However individual protection can also be achieved by prophylactic use of antimalarial drugs. Both methods have been used in the past in order to study the effects of malaria on health.

The effective use of mass chemoprophylaxis in reducing the overall prevalence of malaria, infant morbidity and mortality rates have been numerously verified (Archibald, 1951; Massequin and Polinacci, 1953; Colbourne, 1955; McGregor et al, 1956; Hamid and Omer, 1978). On the contrary studies on the effect of such mass chemoprophylaxis on the

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acquired collective immunity have produced variable results which are difficult to interpret. Some workers have shown a decrease in IFA titres following treatment (Kuvin et al, 1963; Tobie and Coatney, 1964; Sadun et al, 1969; Wilson et al, 1970) but others have not observed any detectable differences (Collins et al, 1964; Lupascu et al, 1967; Lupascu et al, 1969; Lelijveld, 1971). Voller and Wilson (1964) observed a marked decrease of IFA titres, in sera from Gambian mothers and their infants, after a year of regular chemoprophylaxis. This observation was confirmed and extended by Mattern et al (1967) and similar observations were made by Kortmann (1972), Draper et al (1972a)and Cornille Brogger et al (1978).

In this study, marked decreases in malarial (ELISA and IFA) antibody levels were not observed after prolonged chemoprophylaxis. However the overall evaluation of the results would suggest that high antibody levels are maintained by the antigenic stimulation due to the persistence of malaria parasites in the blood. Suppression of parasitaemia by chemoprophylaxis would therefore limit antigenic stimulation and subsequently lead to a lowering of antibody levels.

Very little difference existed in seropositive rates and mean antibody levels between protected and unprotected urban pregnant women, urban healthy adult and rural children. Differences observed in malarial (ELISA and IFA) antibodies in all protected and unprotected groups were mostly associated with the pattern of antibody distribution. Furthermore, in both adult and child groups, decrease in antibody levels after the period of protection was mostly observed with sera which initially contained high levels of antibodies. Low antibody levels were hardly affected by chemoprophylaxis and were more frequent in the protected than the unprotected sera. In none of the groups studied was there a clear cut

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difference in mean ELISA values and GMRT between the test and control groups. The significant difference in GMRT observed between the adult protected and unprotected groups showed only a 2-fold difference which could be attributed to factors other than the effect of suppressive malarial drugs. However, the present study has certain shortcomings. For instance, the marked reduction of participants in the follow-up surveys limits any conclusions which could be drawn in a comparative study of this nature. In the pregnant and adult groups, it would have been preferable to have obtained at least monthly or 2 monthly blood samples during the period of prophylaxis so that the true suppressive effect of chloroquine could be assessed alongside the monthly or 2 monthly variations in mean antibody levels. Such serial sampling would have brought to light the 1 to 3 fold antibody differences encountered in individual patient's sera as a result of periodic examination. Also the difference observed in mean antibody levels after a single follow-up survey could be attributed to the factors, environmental, humoral and cellular prevailing at the time of sampling and not necessarily related to a true suppressive effect of antimalarial drugs. Finally the period of prophylaxis in the rural children was less than optimum for any marked effect on both their parasitological and immune status.

In spite of these limitations the present findings may be assessed in relation to the following factors: 1) the suppressive drug employed; 2) parasite sensitivity to the drug; 3) the intensity of parasite transmission in the areas where study population were resident; and 4) the implications of prolonged chemoprophylaxis on the acquired immunity to malaria.

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In this study the drug used for weekly prophylaxis was chloroquine 300 mg base in adults and 75 mg in children. The monitoring of the weekly administration in the urban pregnant women and the Army officers was most satisfactory. These 2 groups were very co-operative and adequate supervision was ensured. The rural population were not as co-operative. Weekly distribution of drugs in rural pregnant women proved unsatisfactory and there were many defaulters causing this study to be abandoned. The 2 months regular prophylaxis in the rural children was more satisfactory, regular supervision being maintained by trained health workers. However even such dedicated workers could not continue regular supervision of the rural children for more than 2 months, hence the termination of the project after this period. These observations and the difficulties encountered in maintaining uninterrupted protection show that although chloroquine is an effective antimalarial drug with so far no drug resistant strains reported in Ghana (Beausouleil , 1968), the use of mass chemoprophylaxis alone cannot be recommended at the present time. Any control methods should incorporate the systematic use of anti-mosquito measures as well as chemoprophylaxis.

The epidemiological characteristics of malaria in the rural and urban population in Ghana has been established earlier (pp.183-210) A high rate of malarial transmission accompanied by a high acquired collective immunity were observed for the rural population. In the urban population very low parasite rate and parasite density were accompanied by a relatively high acquired immunity, although positive serological tests were higher in the rural than urban sera. The difference in serological responses between the 2 populations was ascribed to a lowered intensity of malarial transmission in the urban area (Colbourne and Wright, 1955) and also to

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irregular and indiscriminate use of malaria suppressive drugs in the urban area. It is suggested from the above that the failure of the present study to show any marked antibody differences in the protected and unprotected urban sera may be related to the lowered level of malaria transmission and an absence of antigenic stimuli, but not to the ineffective action of the anti-malarial drug used.

Another point that needs further emphasis is the predominant effect of anti-malarial drugs on high ELISA values and IFA titres with little or no effect on the lower antibody values. Kortmann (1972) reported similar observations in a study of chemoprophylactic protection of pregnant women in Tanzania. He noted that maximum decrease in IFA titres occurred in patients who initially had the highest titre indices, and that the decrease in titre indices after a year of protection never reached the baseline of positivity. Similar observations were made by Cornille Brogger et al (1978) that after 1½ years of active malaria control activities in Northern Nigeria, decreases observed in serologically positive tests never fell below the baseline. The full significance of these observations with regard to functional immunity is not clear.

Cohen and McGregor (1963) showed that Gambian women protected from infection by drugs for only 2 years, exhibited a marked fall in gamma-globulin levels. They suggested that this may be reflecting a loss of immunity to malaria during that period. Bray et al (1962) observed that the IFA titres of Liberian adults in areas protected by DDT spraying for 3 years were rather higher than might be expected. In their study only one subject out of a group of 20 showed patent parasitaemia. These workers concluded that 3 years of protection did not greatly lower the immunity of the subjects.

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Cornille Brogger et al (1978) also reported that the active control measures in Northern Nigeria were effective in reducing the antigenic stimulus and resulted in a decline of immunological parameters, such as serum IgG, IgM and malarial antibodies (precipitating, IFA and IHA). However in the absence of systemic protection (post-intervention phase) parasite prevalence increased even though the entomological factors or transmission were still below baseline. They suggested that this re-establishment of high levels of parasitaemia was probably caused by a lowered level of immunity as a result of control.

Further observations made by Cornille Brogger et al (1978) in their malarial endemicity study suggested that all the serological tests currently used in malaria, at the total population level, indicate both contact with and partial immunity to malaria. However, as already shown in this study(pp.218-234) at the individual level in endemic areas, serologically demonstrable (ELISA and IFA) antibodies bear little relationship to current or clinical malaria status or indeed have any bearing on immunity to malaria. This limited correlation between serologically detectable antibodies and protective immunity may largely be due to the present lack of knowledge concerning the rate of decay of malarial antibodies. The variously reported persistence of malarial antibodies in the serum of originally immune populations after long periods of absence from exposure (Kuvin and Voller, 1963; Bruce-Chwatt et al, 1972; Fasan et al, 1976) raises a pertinent question, as to whether protective immunity to malaria may persist as long. The persistence of malarial antibodies and the susceptibility of individuals to infection after re-exposure in Ghanaian students has been studied, and the data will be presented.

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There is little doubt that the influence of malaria control activities on the result of immunological surveys (recently demonstrated by Cornille Brogger et al, 1978; Molineaux et al, 1978) is considerable. However current knowledge of the immunology of malaria is still fragmentary. Improvements in the sensitivity and specificity of current serological tests for use in the study of malaria are necessary. Only then can the relationship between serologically detectable malarial antibodies and their significance with respect fo functional immunity be properly evaluated.

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PERSISTENCE OF MALARIA ANTIBODY AND SUSCEPTIBILITY TO INFECTION AFTER RE-EXPOSURE IN GHANAIANS PREVIOUSLY EXPOSED TO MALARIA

Two groups of Ghanaian students previously exposed to malaria and now residing in England and Germany were studied.

Group 1 These comprised 48 students resident in Britain for varying lengths of time (from less than 1 year to 20 years). They were all healthy at the time of sampling, and had not shown any recognisable signs of clinical malaria since leaving home. Their ages ranged from 18 to 55 years.

Group 2 These comprised 10 healthy Ghanaian adults aged 20 to 45 years, resident in England and Germany for 2-5 years. They had paid brief visits to Ghana over periods ranging from 6 weeks to 3 months. During the visits none of them took any prophylactic measures against malaria with the exception of 3 students who occasionally took daraprim to relieve symptoms of fever. On return to Britain all subjects developed malaise after a week to a fortnight. Blood samples revealed a patent parasitaemia with parasite densities ranging from 0.5 - 2%. Despite this there were no signs of clinical illness and temperatures ranged from 98.4 - 99.8^{o}F. After a week of chloroquine therapy parasitological cure was effected.

Blood films and samples were taken from all subjects from Group 1 and Group 2. Samples from Group 2 were taken before and after the visit to Ghana. Blood films were examined for malaria parasites and serum samples were tested for malarial antibodies by ELISA and IFA tests. In order to detect any association between antibody levels and the length of absence from exposure students were divided into 6 defined groups of absence from exposure: 1 year; 1-2 years; 2-5 years; 5-10 years; 10-15 years and 15-20 years.

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<u>Group 1</u> Malaria parasites were absent in all groups studied. Table 36 shows the results of malarial antibody levels obtained for all 48 students. ELISA values ranged from 2.50 to 0.08 and IFA titres ranged from 1280 to <20. The variability of antibody levels and the steady decline in antibody values for each period of absence is shown in Figure 37. The mean ELISA value and GMRT were analysed for each period of absence (Table 36). This varied from 0.83 for the less than one year's group to 0.14 for the 15-20 year's group. IFA showed a similar variation with GMRT of 138 and 6 for the less than 1 year group and 15-20 year group respectively. In fact the 2 students in the 15-20 year period of absence have been in Britain for 18 and 20 years, both were negative by ELISA, but an IFA titre of 1:20 after 20 years absence from exposure was an interesting finding. Mean values for both ELISA and IFA showed a steady but gradual fall as the period of absence from the endemic area increased. The rate of fall slowed with time, the highest rate of antibody decrease occurring during the first year of absence from the malarious area. Throughout the study results obtained by ELISA paralleled those by IFA. However, ELISA values were detected only at baseline level up to 15 years of absence, but IFA titres at baseline were present after 20 years absence from exposure. The difference observed between the mean ELISA values for the less than 1 year and the above one year of absence groups was significant above the 5% level. Similarly the difference noted between the GMRT of subjects absent for less than 1 year to those above 1 year was statistically significant above the 1% level.

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FIGURE 37 Effect of absence from exposure on malarial ELISA (a) and IFA (b) antibody levels

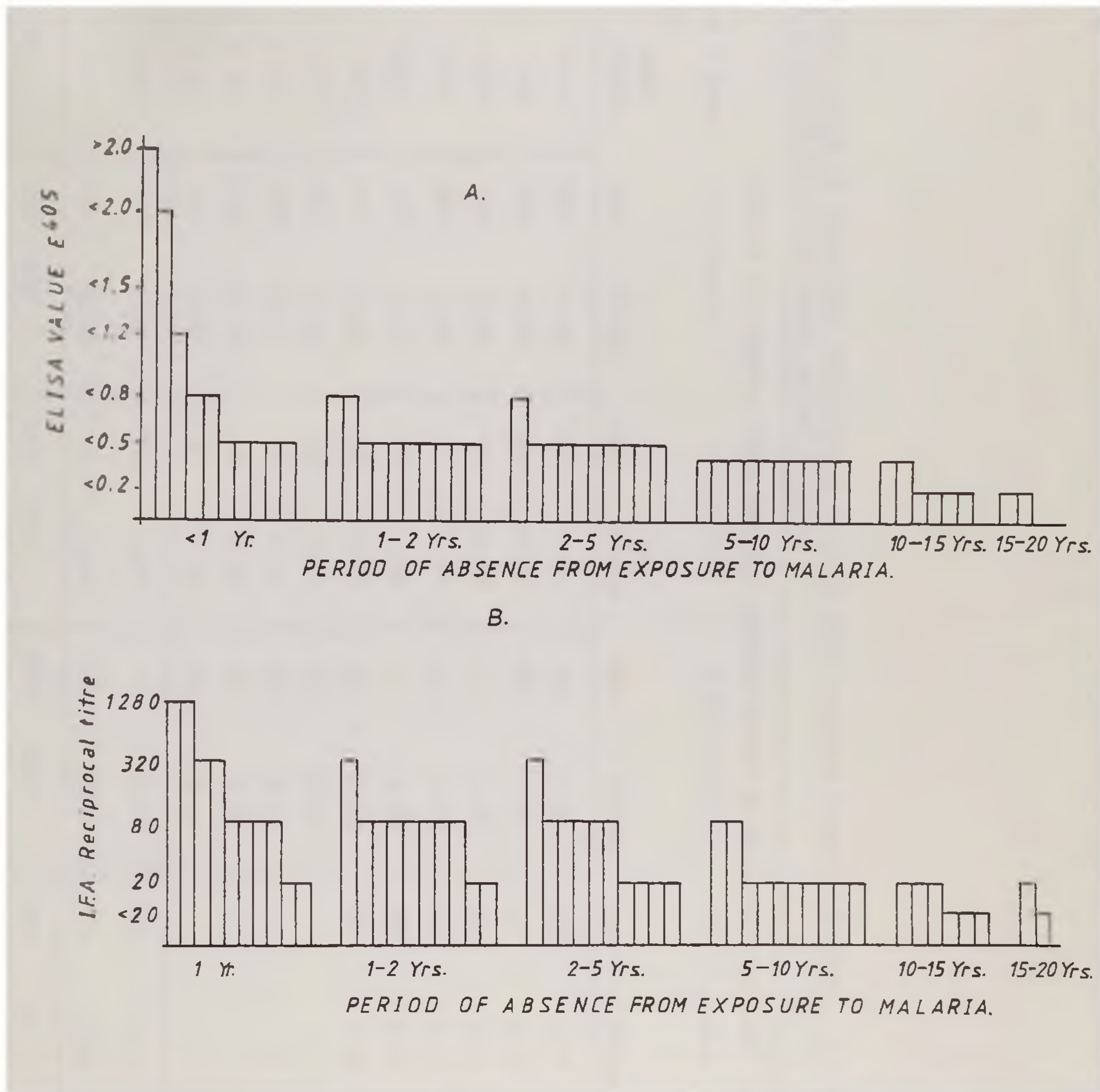

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TABLE 36 IFA and ELISA values of Ghanaians resident in Britain over varying lengths of time. IFA titres are shown in increasing order for each period studied

	Effect of absence from malaria endemic areas on malarial antibody levels											
	Less than 1 year		1-2 years		2-5 years		5-10 years		10-15 years		15-20 years	
Subjects	Reciprocal titre IFA	E405 ELISA	IFA	ELISA	IFA	ELISA	IFA	ELISA	IFA	ELISA	IFA	ELISA
1	1280	2.50	320	0.61	320	0.47	80	0.43	20	0.43	20	0.18
2	1280	1.95	80	0.65	80	0.54	80	0.36	20	0.22	<20	0.09
3	320	0.84	80	0.50	80	0.45	20	0.38	20	0.14		
4	320	0.67	80	0.49	80	0.45	20	0.32	20	0.18		
5	80	0.56	80	0.42	80	0.40	20	0.30	20	0.16		
6	80	0.50	80	0.40	80	0.27	20	0.28	20	0.08		
7	80	0.38	80	0.36	20	0.38	20	0.25				
8	80	0.36	80	0.35	20	0.34	20	0.25				
9	20	0.29	20	0.23	20	0.29	20	0.21				
10	20	0.25	20	0.20	20	0.29	20	0.20				
IFA GMRT / mean ELISA values	138	0.83	69	0.42	52	0.39	24	0.30	14	0.17	6	0.14
	44	0.77	22	0.15	25	0.09	19	0.08	14	0.06	4	0.06
% positive	100	100	100	100	100	100	100	100	50	67		

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Group 2 All the 10 students in this group had patent parasitaemia, and *P.falciparum* was the infecting parasite species. Malaria parasite density, antibody levels, were measured in all subjects before and after visiting Ghana and results are shown in Table 37. The increase in malarial antibody levels as a result of re-exposure is demonstrated in Figure 38. ELISA values were significantly increased in 8 out of 10 sera tested, and 7 out of 10 showed significant increases in IFA titres. These individual antibody increases were reflected in the mean ELISA value and the GMRT. There was a puzzling lack of any apparent increase in antibody levels in 2 sera, by ELISA, and in 3 sera, by IFA, despite patent parasitaemia. Also one subject with a parasitaemia of 1% showed only a marginal increase in antibody levels by ELISA (from 0.20 before the visit to 0.23 after the visit) whereas a much more significant increase in the IFA titre was observed. Another subject showed an increase in ELISA value from 0.23 to 0.25 with a parasitaemia of 0.5%. However, in this subject the IFA titre remained the same after exposure. In all subjects studied parasitaemia was not accompanied by any severe clinical illness except for general malaise and tiredness.

DISCUSSION

The persistence of malarial antibodies in the absence of re-exposure has been the object of many studies in both immune and non-immune individuals. Several authors have shown that malarial antibodies remain demonstrable for a long time after the infection. However there is some confusion in reported studies as to the length of time that antibodies are retained in the absence of exposure.

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TABLE 37 Malaria antibody levels by FIA and ELISA for Ghanaian students resident in Britain and Germany 2 years and less than 5 years before and after visit to Ghana for periods ranging from 6 weeks to 3 months

Subject	IFA		Length of stay in months	% Parasitaemia	ELISA		
	Before visit	After visit			Before visit	After visit	
1	80	320	1½	0.1	0.25	0.45	
2	80	80	2	0.5	0.35	0.64	
3	80	320	3	1.2	0.38	0.41	
4	20	320	2½	0.9	0.42	0.62	
5	80	1280	2	1.0	0.20	0.23	
6	320	320	3	1.0	0.40	1.00	
7	20	1280	4	2.0	0.20	0.95	
8	20	5120	3½	1.5	0.21	1.37	
9	20	1280	4½	1.2	0.28	0.91	
10	20	20	3	0.5	0.23	0.25	
GMRT	46	170	12	0.99	$\bar{x}$=0.29	0.68	mean ELISA
				± 0.54	± 0.09	± 0.37	1SD

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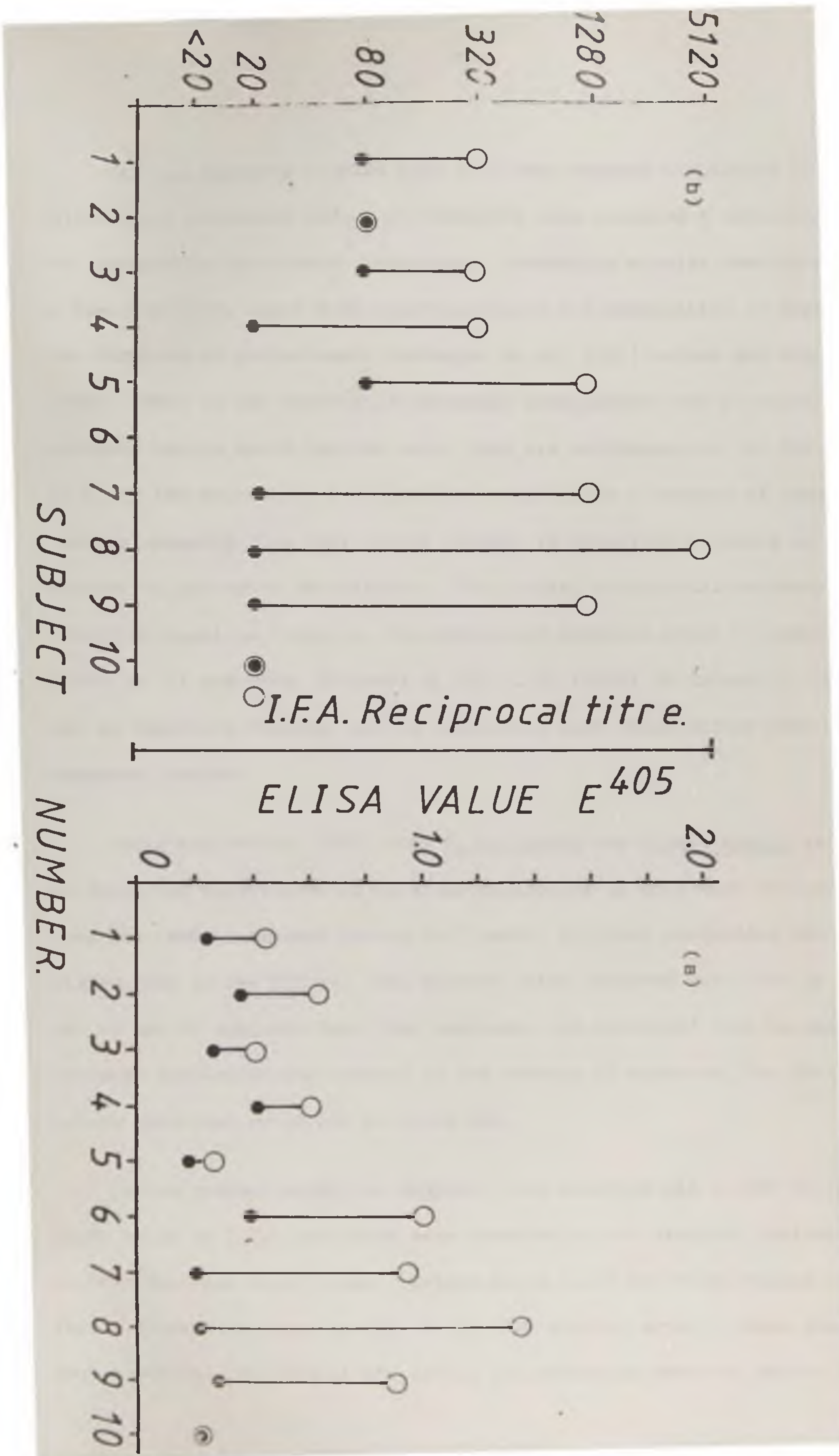

FIGURE 38 Malarial antibody levels before and after re-exposure to malaria. ELISA values are shown in (a) and IFA titres in (b)

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All the students studied here have been exposed to malaria from birth until adulthood and would therefore have acquired a certain level of immunity to the natural infection. Endemicity studies have shown that a high IFA titre bears some relationship to the combination of density and duration of parasitaemia (McGregor et al, 1965; Voller and Bruce-Chwatt, 1968). Thus in the absence of antigenic stimulation over a period of time antibody levels would decline until they are undetectable. On the other hand, if the antibodies stimulated by malaria are a measure of long lasting immunity then they should persist in significant levels in the absence of antigenic stimulation. The finding of malarial antibody positivity at baseline levels in the absence of exposure after 15 years by ELISA (0.23) and after 20 years by IFA (1:20 titre) is indeed an interesting and an important finding, and is consistent with observations from other reported studies.

Kuvin and Voller (1963) used P.falciparum and P.bastianelli as antigens to study the persistence of malarial antibodies in some West Africans after they had been in England for up to 7 years, malarial antibodies had persisted, but in low titres. The highest titre observed was 1:200 in one of the 26 subjects that they examined, and concluded that malarial antibody production was reduced in the absence of exposure, but that subpatent infection could not be ruled out.

In the present study the highest titre observed was 1:1280 by IFA and an ELISA value of 2.50, and these were observed in the students resident in Britain for less than 1 year. Titres above 1:320 and ELISA values of more than 0.50 were not seen in any of the sera studied after 6 years absence from exposure. In general the levels of antibodies observed showed a steady

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but slow fall over many years and low antibody titres correlated with the length of absence. The highest fall in ELISA values and GMRT was observed between absence of less than 1 year and 1-2 years, and was reflected in the twofold drop in both mean ELISA and GMRT. This apparent difference in mean values between less than 1 year absence of exposure as compared to all the other defined periods of absence was statistically significant at the 1% level.

Fasan et al (1976) in a similar study carried out on 52 healthy Nigerian students resident in the U.S.A. for varying periods (2 days to 10 years) confirmed that the levels of malarial antibody as determined by IFA and IHA using P.falciparum and P.knowlesi respectively, were detectable 10 years from last exposure. They further observed a correlation between IFA titres and the periods of absence from the endemic area,and also noted that the fall in malarial antibodies was slow, over many years. On the other hand, they observed much higher titres to P.falciparum of 1:4096 in 23% of subjects studied, and the GMRT for students who had been away for less than 1 year was 1120, and GMRT after 4 or more years after last exposure was 300.

It is suggested here that the difference in maximum titres observed in the present study and those by Kuvin and Voller (1963) and Fasan et al (1976) for homologous antigen may be merely reflecting inter-laboratory differences and the subjectiveness in the interpretation of IFA tests, and does not detract from the general view that the highest fall in malaria antibody level occurs within the first year of absence from exposure and that subsequent falls over several years in antibody levels are steady and slow. In the present study this was true for both antibodies measured by ELISA and IFA tests.

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Indeed the present difficulties of direct comparison between inter-laboratory IFA results are due not only to the variation in the basic technique but other factors such as antigen used, unavailability of standardised reagents and personal preferences in the choice of microscopes and filters. The point is emphasised by Voller and Bruce-Chwatt (1968) that there is no particular virtue in obtaining very high titres, and that it is the degree of reproducibility which is more important.

Dranga et al (1969) also observed that in areas endemic or formerly endemic for P.malariae, specific antibodies were detected even after 20 years after transmission of malaria had been interrupted, and this persistence was marked when the same strain was used as antigen.

Collins et al (1968) found antibodies in patients even 10-15 years after P.malariae, and further noted that after P.falciparum infections antibodies were invariably still present 6-18 months after the infection (Collins et al, 1964; Lunn et al, 1966; Sadun et al, 1969).

Bruce-Chwatt et al (1972) using P.falciparum and P.vivax antigen in the IFA test detected malarial antibody in low titres in veterans of the Second World War with a history of malaria infection then and without subsequent re-exposure. On the other hand, it has been reported that radical cure of one single infection resulted in serological negativity after a period of 6-12 months (Wilson et al, 1970), or 1-2 years (Lupascu et al, 1969) after the infection. This would suggest that the persistence in the absence of exposure in an individual previously exposed to malaria may be dependent on his cumulative malarial experience before the period of absence. The individual previously resident in an area, possibly rural

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with a very high parasite transmission rate will have a high cumulative experience and thus higher malarial antibodies than the individual resident in the urban areas of endemic regions where, as previously shown, malaria transmission is much lower.

The variability in the persistence of ELISA values and IFA titres observed in the present study would therefore reflect different malarial experience of individuals studied. On the other hand, it may suggest the persistence of subpatent infection.

The duration of malaria infection in man without re-infection varies between wide limits. Generally P.falciparum infections are relatively short-lived. With many strains the infection lasts not more than 10 months but longer durations have been recorded; up to 503 days in an artificially inoculated Panamanian strain (Jeffrey and Eyles, 1954). Ciuca et al (1955) reported the duration of parasitaemia without re-infection after 27 months in induced P.falciparum infections. Garnham (1970) detailed the survival time of the 4 human malaria parasites in the erythrocytic or in the exo-erythrocytic forms: P.falciparum 4 years; P.ovale 5 years; P.vivax 8 years and P.malariae 53 years.

Malaria parasites were not detected in any of the blood films examined for the 48 students (Group 1). The predominance of P.falciparum as the infecting species in Ghana has been reported. Plasmodium ovale and P.vivax were not seen. Plasmodium malariae was predominantly observed in children under 5 years of age. However the possibility that P.malariae infections contracted in childhood may persist throughout life should not be overlooked; and it is possible that in the absence of P.falciparum,

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P.malariae may become re-activated. However the inherent limitations of parasitological examination of peripheral blood have been well documented. Grab and Pull (1974) showed that the probability of missing a positive case is more than 5% when the probable parasite density per mm^3 is less than 15. Therefore the persistence of scanty levels of P.malariae will go unnoticed.

From the above considerations, the persistence of malaria antibody observed in the present study may not be attributable to possible sub-patent P.falciparum infections, and may be related to the fact that ELISA and the IFA test indicate an immune response of the host to the infection, and not necessarily only the response of the parasite to the host (Bruce-Chwatt et al, 1972). If that be the case then does persistence of anti-bodies indicate a persistence of immunity to re-infection? The present finding of persistence of detectable antibody levels by ELISA after 15 years and IFA after 20 years exposure would imply the persistence of B cell memory responsible for the continued production of the malarial anti-bodies. Bruce-Chwatt et al (1972) in a sero-epidemiological study on population groups previously exposed to malaria, reported the presence of detectable levels of antibodies after 20 years, and also noted that the frequency of positive (≥16 titre) sera did not decline with increasing absence from exposure. This would confirm the presence of a memory retaining mechanism for continued baseline antibody presence.

Another observation of interest from the present study is the resurgence of parasitaemia and a corresponding increase in antibody levels following re-exposure to malaria after 2 years and 5 years absence from endemic malarious areas. This observation implies that adult students returning home after a period of absence in non-malarious areas are just

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as susceptible to, and can develop clinical malaria on re-exposure. The increases in antibody levels were variable, ranging from 1 to 4-fold increases. This differential increase in antibody may be related to the density and duration of the infection in each individual.

The susceptibility to re-exposure after a period of absence from endemic areas, despite the presence of detectable antibodies, suggests that antibody presence as measured by ELISA and IFA test does not correlate with protective immunity. There is therefore a need for better tests which would measure functional immunity.

The lack of correlation between antibody presence and protective immunity or even to resistance to infection observed here gains support from the work of Bray et al (1962) in Liberia and Bruce-Chwatt (1963) in Nigeria. These workers reported that inoculation of individuals living in endemic areas of P.falciparum infection resulted in patent parasitaemia in more than half of the subjects, and even clinical symptoms developed in 15% to 25% of these subjects. These findings and those of the present study confirm that the mechanism of immunity to malaria is complex possibly involving an interaction between protective immunity and cellular immunity. If cellular immune mechanisms play a role in the protective immunity to malaria, then these would not be detected by serological tests.

The present finding of susceptibility to infection after re-exposure and the relatively poor development in immunity to malaria even in natural infections indicate that other mechanisms operate to prevent the development of immunity which is fully protective.

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The present finding that malaria antibody can persist for more than 10 years and as long as 20 years does not mean that immunity to malaria can last as long. Also the IFA test appears to detect antibody for longer periods than the ELISA test. This suggests that soluble antibodies detected by ELISA may not persist as long as antibodies measured by the IFA test. Also higher antibody levels indicate recent infection or the presence of a metabolising parasite. Furthermore the findings infer that at least resistance to severe clinical activity may last as long as 5 years, and in that respect persistence of baseline antibody levels indicates not only evidence of past exposure to malaria, but also suggests a certain level of acquired resistance to severe clinical infection. Further work will be required to substantiate this finding.

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CHAPTER SEVEN

INVESTIGATION OF POLYPARASITISM IN AN URBAN GHANAIAN POPULATION BY THE USE OF IMMUNODIAGNOSTIC TESTS

Data are presented from a preliminary study on the frequency of specific antibodies to Mycobacterium tuberculosis, Vibrio cholerae, Treponema pallidum, Entamoeba histolytica, Toxoplasma gondii, Trypanosoma gambiense and Trypanosoma rhodesiense, Schistosoma mansoni and Toxocara canis by a rapid indirect immunofluorescent antibody (IFA) test. The study includes the application of the enzyme linked immunosorbent assay for the detection of antibodies in onchocerciasis, using Onchocerca gutturosa antigen, as well as the prevalence of antibodies to Epstein Barr viral capsid antigen (VCA) and hepatitis B surface antigenaemia (HBsAg). The study was carried out in a sample urban Ghanaian population.

It would have been preferable to determine the prevalence of antibodies to the above-named antigens in the rural population as parasitic diseases have been shown to be predominantly rural diseases (Colbourne et al, 1950; Wenlock, 1978; Buck et al, 1978a), and to have compared results with prevalence rates in the urban population. Unfortunately sera from rural populations could only be obtained from blood collected by finger prick in heparinised microcapillary tubes. These proved insufficient for the numerous serological tests applied in this study. Large quantities of sera were easier to obtain from the urban Ghanaian groups, and also from clinical groups with confirmed diagnoses. The study was therefore carried out on available and well classified urban sera. Clinical sera from patients with confirmed tuberculosis, schistosomiasis, onchocerciasis and Burkitt's lymphoma (all from rural areas of Ghana) have been included

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for comparison. Data presented here provide preliminary information on the prevalence of some parasitic diseases of major health importance in the urban environment. These would be useful for comparison with similar future studies on rural populations.

The frequency of antibodies to the different parasite antigens observed in the present study is discussed with regard to the types of antigen employed, the reliability of results and the sensitivity and specificity of the tests. The way in which these affected the interpretation of serological results is also considered. In addition the prevalence of specific anti-parasitic antibodies detected are briefly discussed together with some aspects of polyparasitism.

180 sera from the urban population and 128 clinical sera with confirmed diagnosis were used.

Urban sera These were selected from Group A (see survey population pp.81) They comprised 40 cord sera, 55 sera from schoolchildren aged 6-11 years and 85 adult sera (40 males and 45 females, aged 20-45 years). The adult population was especially selected to comprise nursing, medical and hospital ancillary personnel as they represented a migratory group who had lived in both the rural and urban areas of Ghana.

Clinical sera These were selected from Group F. The source of sera and details have been given (see survey population pp. 81). They comprised 50 sera from patients with chronic tuberculosis, 20 with schistosomiasis, 30 with onchocerciasis and 28 with Burkitt's lymphoma. As previously pointed out, these sera were obtained from patients resident in the rural areas of Ghana. They were included for comparison because

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they represented a well classified group of positive sera, useful for assessing the sensitivity and specificity of the serological tests used. They were also useful for evaluating the reliability of test results obtained with sera from apparently healthy urban individuals showing neither clinical nor parasitological evidence of infection.

Rapid IFA test All sera were tested for antibodies to the following antigens: M.tuberculosis, V.cholerae, T.pallidum, E.histolytica, T.gondii, T.gambiense and T.rhodesiense, S.mansoni (cercariae) and T.canis (larvae) by IFA(as described pp.99-102). A small preliminary control test was carried out on 50 blood donors from South East England, and 20 sera from laboratory staff of Wellcome Research Laboratories before testing the Ghanaian sera. This group represented individuals resident in Britain from birth until adulthood. The rapid IFA test was carried out using M.tuberculosis, V.cholerae, T.pallidum (unabsorbed), E.histolytica, T.gondii, T.gambiense, T.rhodesiense, S.mansoni (cercariae) and T.canis (larvae) antigens and a serum dilution of 1:10. Sera from Caucasian patients with proved infections (see survey population pp.86) were also tested. Of the 70 British samples tested the following antibody frequencies were observed: 77% had antibodies to M.tuberculosis, 24% showed antibodies to V.cholerae, 26% to T.pallidum (unabsorbed), 4% to E.histolytica, 15% to T.gondii, and 3% to T.canis larvae. No antibodies were observed for Trypanosomes nor S.mansoni cercariae antigen. However all sera which were positive at a titre of 1:10, were negative when tested at 1:100 and 1:1000. Titres obtained for clinical sera with infections were consistently equal to or above 1:100. The significant diagnostic titre for each of the 8 parasitic infections in the rapid IFA test was therefore considered as 1:100 and above. It was also possible that titres at 1:10 were non-specific and represented cross-reactions with related antibodies.

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The above initial analysis of antibody profile results in blood donors and laboratory staff, also showed that no sex association existed between the antiparasitic antibodies investigated. Positive and negative results for all the parasite systems tested were reproducible from duplicate tests on the same sample.

On the basis of these preliminary study on healthy Caucasians and Caucasians with confirmed parasitic disease, Ghanaian sera were screened at 3 dilutions 1:10, 1:100 and 1:1000 against the parasite antigens tested above. It was further confirmed that as all clinical sera from patients with a confirmed diagnosis tested by the rapid IFA test constantly showed IFA titres of at least 1:100 but titres above 1:10 were not observed in negative Caucasian sera, thus all Ghanaian sera showing antibody titres of 1:100 to any of the 8 antigens tested, may represent the presence of specific anti-parasitic antibodies.

ELISA for onchocercal antibodies All sera were tested for onchocercal antibodies by ELISA using *O.gutturosa* antigen (method as described in Chapter Four).

IFA test for Epstein Barr Virus capsid antigen (VCA) Sera from children, adults and Burkitt's lymphoma patients were screened for antibodies to VCA by the method of Henle and Henle (1966).

Passive haemagglutination test for HBsAg Sera from children and adults were screened for HBsAg by HA test using the Wellcome Hepatest Screening and Confirmatory test kit.

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ELISA and IFA tests for malarial antibodies Malarial antibody levels in all urban and clinical sera were determined by ELISA and IFA tests (described in Chapter Four).

The prevalence of antiparasitic antibodies to the 9 parasite antigens, antibodies to VCA and seropositivity for HBsAg in the Ghanaian sera tested is shown in Tables 38 - 45. Analysis of the antibody profiles shows much higher anti-parasitic antibody frequency rates than were noted for Caucasian sera, even at IFA titres of 1:100.

In order to interpret results obtained in this preliminary study, criteria for positivity had to be set for each parasite system studied. This was done taking into consideration the results obtained for Caucasians with and without confirmed parasitic diseases in the initial control tests cited.

Rapid IFA test

Positive results observed for *M.tuberculosis*, *V.cholerae*, *T.pallidum*, *E.histolytica*, *T.gondii*, *T.gambiense* and *T.rhodesiense*, *S.mansoni* (cercariae) and *T.canis* (larvae) were interpreted on the basis of the following criteria: Seropositivity at an IFA titre of only 1:10 was considered to be largely non-specific, whereas an IFA titre of 1:100 was considered as a significant diagnostic titre and therefore specific for the parasite antigen. However an IFA titre of 1:1000 was highly specific for the parasite antigen and indicative of active infection but it is appreciated that the serologically positive reactions observed were not necessarily species-specific. Further analysis of the antibody profile for frequency correlations between

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the different anti-parasitic antibodies detected (chi-square tests) showed an absence of correlation between positive rates observed for the 8 antigens, even at a dilution of 1:10. Therefore antibodies to the 8 antigens described in this study may represent independent variables.

Antibodies to M.tuberculosis Table 38 shows the number and frequency rates of sera showing non-specific and specific reaction to this parasite antigen. Reactions in cord sera were all non-specific. Sera from all children (55) were seropositive at 1:10 titre, only 40% (22) of these were positive at 1:100 serum dilution, but none showed seropositivity at a titre of 1:1000. A large proportion of adults tested 85% (72) showed positive test reactions at 1:10, 65% of these gave specific reactions but only 6% showed seropositivity at 1:1000. All the 50 sera from the Ghanaian chronic TB sera gave highly specific reactions showing seropositivity of 100% at 1:1000 IFA titre. The high prevalence of specific antibodies observed in children and adults indicates a high level of exposure to mycobacteria in the urban Ghanaian population. The higher specific antibody frequency in adults may represent a higher cumulative experience to mycobacteria, and it is of further interest that only 6% of adults attained antibody titres of 1:1000.

Antibodies to Vibrio cholerae Sera exhibiting a positive antibody reaction to V.cholerae are shown in Table 39. Cord sera did not show positive reaction to this parasite antigen. 60% of sera from children were positive at 1:10 titre and only 20% of these showed specific reactions. Adults showed a seropositive reaction rate of 55% at 1:10 titre only but 20% had specific antibodies. The antibody prevalences observed in children and adults suggest a degree of exposure to antigenic stimulus to this antigen or its related species in the urban environment.

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TABLE 38 Distribution of antibodies to *Mycobacterium tuberculosis* by IFA test in Ghanaian sera

Sample population studied and no.	IFA reciprocal titres 10	100	1000
Cord (40)	8 (20)	-ve	-ve
Children (55)	55 (100)	22 (40)	-ve
Adults (85)	72 (85)	55 (65)	5 (6)
Chronic TB (50)	50 (100)	50 (100)	50 (100)
Positive control	+ve	+ve	+ve
Negative control	-ve	-ve	-ve

TABLE 39 Distribution of antibodies to *Vibrio cholerae* by IFA test in Ghanaian sera

Sample population studied and no.	IFA reciprocal titres 10	100	1000
Cord (40)	-ve	-ve	-ve
Children (55)	33 (60)	11 (20)	-ve
Adults (85)	47 (55)	17 (20)	-ve
Positive control	+ve	+ve	+ve
Negative control	-ve	-ve	-ve

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Antibodies to T.pallidum Table 40 shows that cord sera were negative at all dilutions. Sera from children showed 15% seropositivity at 1:10 titre with 10% specific reactors. Adult sera showed 25% had positive reactions at 1:10 titre, of these 15% had specific antibodies. These results were obtained with unabsorbed antigen. When antigens were absorbed before being reacted with sera, positive reactions, both specific and non-specific, were completely abolished.

Antibodies to E.histolytica Positive reactions observed for all sera (Table 41) were positive only at 1:10 titre. The following frequencies were observed for cord sera, 10%, sera from children, 20%,and adult sera 25%.

Antibodies to T.gondii Results are shown in Table 42. Positive rates obtained for cord sera were largely seropositive at 1:10 titre, of the 15% seropositive at 1:10, 5% showed specific antibody reactions. Of the 30% of children with positive tests at 1:10 titre, 20% gave specific antibody reactions. All the adult sera were positive at 1:10 titre, 45% of these showed specific antibody reactions and in 15% the reactions were of a high titre.

Antibodies to T.gambiense and T.rhodesiense All sera gave negative reactions at all dilutions.

Antibodies to S.mansoni (cercariae) All Ghanaian urban sera tested gave negative results with cercariae antigen. Positive fluorescence was largely accompanied by heavy background staining. The morphology of cercariae in the positive cases was not distinct and did not meet with the criteria set for positive staining. However the 20 sera from Ghanaian patients with active chronic Schistosoma haematobium infection all gave antibody titres of 1:1000, with a clear distinction of parasite morphology.

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TABLE 40 Distribution of antibodies to *Treponema pallidum* by IFA test in Ghanaian sera

Sample population studied and no.	IFA reciprocal titres		
	10	100	1000
Cord (40)	-ve	-ve	-ve
Children (55)	8 (15)	5 (10)	-ve
Adults (85)	21 (25)	13 (15)	-ve
Positive control	+ve	+ve	+ve
Negative control	-ve	-ve	-ve

TABLE 41 Distribution of antibodies to *Entamoeba histolytica* by IFA test in Ghanaian sera

Sample population studied and no.	IFA reciprocal titres		
	10	100	1000
Cord (40)	4 (10)	-ve	-ve
Children (55)	11 (20)	-ve	-ve
Adults (85)	21 (25)	-ve	-ve
Positive control	+ve	+ve	+ve
Negative control	-ve	-ve	-ve

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Antibodies to T.canis (larvae) Positive tests for T.canis are shown in Table 43. Of 20% cord sera seropositive at 1:10 titre only 15% had specific antibodies. 36% of sera from children were positive at 1:10 titre, and of these only 30% were specific and 10% gave highly specific reactions. Adult sera showed overall seropositivity of 46%, of these 20% were specific and 6% were highly specific reactions. The relatively high prevalence of specific antibodies to T.canis (larvae) in all groups of sera suggests a significant level of exposure to this worm in the general urban population.

Onchocercal ELISA This test was not specific for onchocerciasis because false positive reactions occurred among people who had never had the infection. The test however, was highly sensitive for a variety of helminthic infections. Antibodies to tissue, blood and intestinal helminths all gave positive reactions in this test. However the clear distinction between ELISA values observed for patients with chronic active onchocerciasis and patients with and without other helminthic infections allows for the following criteria of positivity to be set for the present study: 1) An ELISA value of 0.20 to 0.50 was considered non-specific for onchocerciasis but indicative of exposure to tissue, blood or intestinal helminths; 2) ELISA values above 0.50 in the present study could represent acute or chronic helminthic infections; 3) ELISA values of 1.0 and above represented specific antibodies to onchocercal antigens.

The range and mean ELISA values of antibodies to Onchocerca gutturosa antigen in Ghanaian sera tested are shown in Table 44. ELISA values greater than 0.25 were uncommon in cord sera. All adult sera showed values above 0.30 and in 85% of chronic active onchocerciasis patients, ELISA values were consistently above 1.00

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TABLE 42 **Distribution of antibodies to *Toxoplasma gondii* by IFA test in Ghanaian sera**

Sample population studied and no.	IFA reciprocal titres		
	10	100	1000
Cord (40)	6 (15)	2 (5)	-ve
Children (55)	17 (30)	11 (20)	-ve
Adults (85)	47 (55)	38 (45)	13 (15)
Positive control	+ve	+ve	+ve
Negative control	-ve	-ve	-ve

TABLE 43 **Distribution of antibodies to *Toxocara canis* (larvae) by IFA test in Ghanaian sera**

Sample population studied and no.	IFA reciprocal titres		
	10	100	1000
Cord (40)	8 (20)	6 (15)	-ve
Children (55)	20 (36)	17 (30)	5 (10)
Adults (85)	39 (46)	17 (20)	5 (6)
Positive control	+ve	+ve	+ve
Negative control	-ve	-ve	-ve

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TABLE 44 The range and mean values of antibodies to *Onchocerca gutturosa* antigen by ELISA

Sample population studied and no.	Onchocercal antibodies		
	Range		
	Low	High	Mean $\pm$ 1SD
Cord (40)	0.00	0.25	0.16 $\pm$ 0.11
Adult (85)	0.30	0.50	0.35 $\pm$ 0.19
Onchocerciasis patients proven cases (30)	0.67	2.12	1.25 $\pm$ 0.79

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Antibodies to EBV The lowest specific titre value indicative of seropositivity to EBV antigens was considered as 1:8 (Henle and Henle, 1966; Dr. K.C. Ng, personal communications). The positive tests were further evaluated by the intensity of fluorescence staining where (+) indicated a standard positive and (++++) indicated a very strong positive. All sera tested from children (55) showed 100% seropositivity rate but adult sera (85) showed 95% seropositivity. Sera from Burkitt's lymphoma patients (28) were all positive. However, whereas the intensity of fluorescence in the positive tests and children and adults never exceeded (++) to (+++), the intensive ot staining in Burkitt's lymphoma sera was consistently (++++).

Hepatitis B surface antigen The hepatitis B antigen confirmatory kit, used in combination with the Hepatitis B Antigen Screening Kit reagents, provides a haemagglutination inhibition test system for the confirmation of HBsAg in human blood samples. In this test system, end point titres of not less than 1:32 in the screening test and haemagglutination inhibition at 1:4 and above were indicative of seropositivity for HBsAg (Hepatest Wellcome Reagents).

In the present study HBsAg was present in 20% of the adults (17/85) tested and 9% (5/55) of the child sera.

Malarial antibodies Antibody range and mean values observed for Ghanaian sera tested are shown in Tables 45 and 46. All sera, whether cord, child or adult, showed 100% seropositivity when ELISA value of 0.20 and an IFA titre of 1:20 was considered a positive reaction.

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TABLE 45 **The range and mean levels of malarial antibodies and % positive by IFA test in Ghanaian healthy (urban) and clinical (rural) sera**

Sample population studied and no.	IFA antibody titres			
	Range			
	Low	High	GMRT ± 1SD	% seropositive
Cord (40)	20	1280	365 ± 55	100
Children (55)	20	1280	282 ± 49	100
Adult (85)	20	1280	431 ±105	100
Tuberculosis (50)	20	5120	422 ±126	100
Schistosomiasis (20)	80	5120	624 ±169	100
Onchocerciasis (30)	320	5120	845 ±412	100
Burkitt's lymphoma (28)	20	5120	430 ±168	100

TABLE 46 **The range and mean levels of malarial antibodies and % seropositive rate by ELISA in Ghanaian healthy (urban) and clinical (rural) sera**

Sample population studied and no.	Malaria ELISA values			
	Range			
	Low	High	Mean ± 1SD	% Seropositive
Cord (40)	0.20	2.24	0.70 ± 0.47	100
Children (55)	0.20	1.85	0.61 ± 0.49	100
Adult (85)	0.20	2.34	0.80 ± 0.50	100
Tuberculosis (50)	0.31	1.95	0.96 ± 0.12	100
Schistosomiasis (20)	0.32	1.85	0.89 ± 0.09	100
Onchocerciasis (30)	0.50	1.74	0.90 ± 0.31	100
Burkitt's lymphoma (28)	0.37	1.69	0.67 ± 0.12	100

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DISCUSSION

When an individual is infected with a parasite organism or vaccinated with attenuated parasites, antigenic material from the parasite stimulates the immune system of the host to produce specific immunoglobulin which combines with the antigen. It is a feature of the host's immune system that antibodies may be found many years later following an original contact with an antigen; and immunodiagnostic methods provide a means of determining an individual's past or present exposure to parasites.

The high prevalence of specific anti-parasitic antibodies observed for *M.tuberculosis*, *V.cholerae*, *T.gondii* and *T.canis*, and the high seropositive rates to EBV capsid antigen and HBsAg in urban Ghanaian sera may therefore represent a high level of exposure to antigen stimuli to the above organisms and/or their related species and not necessarily be indicative of current infection.

The reported findings further demonstrate the versatility of serological tests, and the potential of the rapid IFA test, where the number of antigens which can be used on a single microscope slide to detect specific anti-parasitic antibody is almost unlimited. It is also important to note that none of the antigen preparations are pathogenic. However the value of any serological test is in providing data which can be of diagnostic or epidemiological use. This value can only be determined by the test's sensitivity, specificity and reliability. The findings of this study will therefore be discussed with this in mind when considering the technical and biological variables and the interpretation of the serological data.

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Assessment of sensitivity and specificity of serological tests used

Detection of anti-parasitic antibodies by the rapid IFA test The immuno-fluorescent antibody technique (Goldman, 1968) offers a rapid method suitable for a wide range of antibodies. However, the method does not lend itself to simple instrumental quantification, and depends on subjective assessment where the presence or absence of antibodies is expressed in terms of serum dilution or titre. This is clearly an over-simplification as the antibody within any positive sera behaves as a continuous variable. Another major drawback of this test is the difficulty of interpreting weak positive reactions and in this study such weak positives were considered as negative reactions. The criterion of positivity set was a distinct appearance of parasites clearly discernable with a positive staining. Such a strict demarcation of positivity and negativity may have improved the specificity of the test to the parasite species but it may also have decreased the sensitivity possibly increasing the incidence of false negatives.

Many parasite antigens possess fairly strong specificity for the genus but little species specificity. In serological tests therefore cross-reactions occur with related antibodies, and therefore caution is required in the fuller interpretation of positive results. This is illustrated by *Treponema pallidum* which characteristically shows a low specificity where infections with yaws and other related Treponema species may show positivity in this test. However, pre-absorption of *T.pallidum* with Reiters Treponeme antigen, a specially prepared sorbent (Wellcome Reagents Ltd.) allows an estimation of true syphilitic antibodies as pre-absorption eliminates non-specific antibody reactions. The observed

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absence of both specific and non-specific reactors to T.pallidum after absorption with Reiters antigen suggests that positive tests were due to cross-reacting antibodies.

The specificity and sensitivity of the test are to a large extent dependent on the source and type of antigen used, whether it is of human origin and is species specific. The manner in which antigen is prepared is also important, whether it is total or fractionated and whether stability and sensitivity are maintained with storage. Generally although serological tests may not be species-specific (Targett, 1970; Denham et al, 1971; Grab and Pull, 1974; Lobel and Kagan, 1978) specificity and sensitivity of tests are improved when antigens used in the test have genus and species homology for the disease parasite and the host.

The IFA test with whole organisms as a source of antigen has been shown to be one of the most sensitive serodiagnostic tests for parasitic infections (Sadun, 1976). In the present study bacterial and protozoal antigens used were whole organisms and were species-specific resulting in a high test specificity. The absence of positive tests with T.gambiense and T.rhodesiense therefore confirms the absence of trypanosomiasis in the urban population tested, and suggests an absence of exposure to related organisms.

For the helminthic group of parasite the use of whole organism as antigen in IFA is not practical and therefore extracts or sections of adult worm or a developmental larvae stage are used as antigen. In this study fractions of S.mansoni cercariae and T.canis larvae were used. A number of studies have reported that parasitic extracts of the helminthic group are complex antigen mosaics which possess shared antigens even at

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the phylogenetic level (Denham et al, 1971; Kagan, 1974; Ambroise-Thomas and Kien Truong, 1974; Sadun, 1976). Thus serological tests for this group of parasites have a low measure of specificity. Kagan (1974) pointed out that although wide cross-reactions occur within this group, which makes the interpretation of the serological results difficult, test sensitivity can be improved by the use of homologous antigen, and often allows a clear differentiation between infected and uninfected groups.

The absence of positive tests to S.mansoni cercariae in the urban samples tested was therefore surprising. It suggests either a lack of antigenic sensitivity, a low level of exposure to nematodal antigen stimuli or a high test specificity, hence reflecting the absence of S.mansoni in the population sampled. It could also be related to the developmental form of the parasite used as antigen. The larval stages of schistosomes differ significantly from that of the adults and it is possible that the larval antigens may be less reactive with phylogenetically related antibodies than would be the case if adult worms had been used as antigen.

Positive tests were obtained with T.canis larval antigens and significant titres were observed even in cord sera. These results (Table 43) suggest significant exposure to toxocaral infection in the urban population, or exposure to other helminthic parasites. In order to evaluate the validity of T.canis larval antigen for toxocaral antibodies, results were compared with those obtained on the same group of sera tested by toxocaral ELISA (de Savigny et al, 1979). This test utilises secretory antigens released from toxocaral migratory larvae as antigen, and antibodies to these secretory antigens are specific and correlate with disease activity

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(de Savigny et al, 1979). The following positive rates were obtained by ELISA in cord sera 67% negative, 26% had low detectable antibody levels and 10% showed significant antibody concentrations (de Savigny, personal communication). Although the ELISA test uses a different set of criteria for positivity and is objective and sensitive the overall results compare well with results by IFA (Table 43). These findings also confirm that antibody titres at 1:100 in the IFA test may indicate specific antibody response to current or recent-past exposure to toxocaral infections.

The sensitivity and reproducibility of a serological test can be affected by stability of antigens and reagents used. In this study the reconstituted suspensions of antigens were stable for several weeks at +4°C without a loss in the antigenic integrity. The use of well standardised and classified commercial antiglobulin conjugates contributed to reproducibility and comparability of test results carried out on different days.

Detection of antibodies in onchocerciasis by ELISA The ELISA test has many advantages, and it provides an objective estimation of antibodies in sera. It is sensitive, easy to carry out on a large scale and uses very little antigenic material (Voller et al, 1979). However, it suffers the disadvantages common to tests in which soluble antigens are used, in particular the difficulty of standardising the antigenic preparations. Bartlett et al (1975) first applied ELISA in a preliminary study on onchocerciasis utilising antigen derived from O.gutturosa. They showed that the test was even more sensitive than IFA and was able to differentiate clearly between infected and uninfected groups of people. The present

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findings are consistent with the above and clear differences were observed between ELISA values obtained for the urban sera compared to sera from patients with confirmed onchocerciasis. These results clearly justify further work. This could be carried out with isolated and well characterised specific antigen from O.volvulus and other filarial worms in order to assess the serodiagnostic value of ELISA for onchocerciasis and other filarial infections.

Detection of antibodies to EBV capsid antigens (VCA) Immunofluorescent tests have been widely used to determine the presence of EBV-mediated antigens in infected cells (Henle and Henle, 1966) and the presence of specific antibodies to these antigens in sera. EBV capsid antigen appears relatively late in the cycle of virus replication in a productive infection and the presence of specific antibodies even at low titre is suggestive of past or present infection (Epstein and Achong, 1973; Ziegler et al, 1977). The inherent problems associated with IFA tests have been discussed. The present finding of antibodies to VCA, in the urban sera tested, is therefore a significant finding.

Detection of HBsAg in sera by haemagglutination (HA) tests The commercial test kit for HBsAg used here was developed by Cayzer (1974) Wellcome reagents. The HA test itself is simple to carry out, no specialised or highly qualified personnel are needed. It is sensitive, reproducible and rarely gives false positive reactions. Chrystie et al (1974) clinically evaluated the commercial passive haemagglutination test for HBsAg and showed that it was only slightly less sensitive than radioimmunoassay, and considerably more sensitive than counter immunoelectro-osmophoresis.

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Also large numbers of sera can easily be handled in this test. The test reagents, in this case sensitised turkey cells, can be prepared in a central laboratory, freeze-dried and used in many areas in the tropics, thus achieving a considerable degree of comparability. The test as it stands can be valuable in diagnosis and blood donor screening in the tropics, and is eminently suitable for field use. The presence of HBsAg in urban Ghanaian sera at a frequency rate of 9% in children and 20% in adults is a significant finding.

The disadvantages of HA tests are similar to those observed for IFA tests. The visual assessment of presence or absence of specific antibodies by bringing together antigens and sera, with the end point of antibody response being expressed in terms of serum dilution or titre, is too subjective, and limits comparability of results between different laboratories. The origin and type of antigen, the techniques for preparation or extraction of antigens (Meuwissen et al, 1972), and small variations in the test procedure can all drastically affect the end results. However, when antigen preparation and other reagents used are standardised, the sensitivity, specificity and reproducibility of the test is markedly improved and results obtained are then reliable and comparable.

Determination of antibodies to malaria by ELISA and IFA ELISA has been evaluated and validated for malaria in this thesis, and the use of IFA as both a diagnostic and seroepidemiological tool has been well documented (WHO, 1975). The presence of malarial antibodies in all urban sera tested by both ELISA and IFA confirms the finding of malarial endemicity in even the urban areas of Ghana.

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Interpretation of serological results

Results obtained with the above immunological tests may reflect the accumulated experience of the individuals to the range of parasitic antigens used. The persistence of antibodies after a parasitic infection has been terminated is well documented (Englebrecht, 1971; Kagan, 1972; Bruce-Chwatt et al, 1972; Ambroise-Thomas, 1976; McGregor, 1976). In order for the results to be of diagnostic value they should be interpreted with a knowledge of clinical, parasitological and pathological evidence, as well as the nature of any treatment received.

However few of the parasitic diseases in man present as typical and easily recognisable clinical syndromes. Most of the signs and symptoms are non-specific in tropical areas where parasitic diseases are most prevalent. They can also be confused with numerous other pathological conditions and therefore some stage of the parasite must be demonstrated before the presence of the infection can be confirmed. Unfortunately parasites frequently cannot be detected by direct microscopic examination (Sadun, 1976) and thus the need for indirect methods, such as provided by serological tests are necessary to supplement and complement individual diagnosis.

The interpretation of serological data further requires the establishment of operational definitions of 'serological positive' and 'true positive'. These definitions can only be used when the test is used for screening a large series of control sera, i.e. patients with confirmed diagnoses, patients with other related parasitic infections, samples from healthy but exposed individuals, and also samples from healthy unexposed groups. Such control tests allow the setting of positive test limits by

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which true positives and true negatives may be differentiated. It must be borne in mind, however, that the definition of a serological positive reaction is arbitrary as long as the antigenic reactants, involved in the antigen-antibody reaction, have not been isolated (Kaay, 1975).

Distribution of anti-parasitic antibodies in Ghanaian sera The greatest value of serological tests for parasitic diseases is in their applicability for use in epidemiological studies and all the tests used in this study (IFA, ELISA and HA) meet most of the criteria set (Lobel and Kagan, 1978) for the suitability of a test for use in epidemiological studies. These tests can therefore be used in future studies to screen larger sample populations from urban and rural Ghana. The frequency of viral, fungal, bacterial, protozoal and helminthic infections of major public health importance can thus be determined.

Despite the many limitations of this preliminary study and the small size of the sample population tested, a few salient facts can be drawn from the positive rates of specific anti-parasitic antibodies observed. The high prevalence of antibodies to M.tuberculosis. V.cholerae, T.pallidum (unabsorbed), T.gondii, T.canis, EBV and HBsAg indicate a high exposure of the urban population to antigenic stimuli by these parasites or their related species. Antibody levels may therefore represent either recent light infections and/or cumulative antibody responses to the above infections.

Trypanosomiasis, schistosomiasis, onchocerciasis and Burkitt's lymphoma are generally seen in populations living in endemic foci in the rural areas of Ghana. The absence of specific antibodies to T.gambiense, T.rhodesiense, S.mansoni and O.gutturosa antigens confirms the lowered transmission of these parasites in the urban environment.

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The findings of the present study further demonstrate the phenomena of polyparasitism even in the urban Ghanaian environment. All sera studied showed significant levels of malaria antibodies, and at least 20% of them exhibited the possible concurrent presence of, or past exposure to, single or multiple viral, bacterial, protozoal or helminthic infections.

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Some considerations on the phenomena of polyparasitism

Virtually nothing is known about the dynamics of multiple infections. They may develop together or independently and some parasitic infections may predispose the human host to certain intercurrent diseases. This is demonstrated in the case of Burkitt's lymphoma (Lancet, 1970) and in malarial immunosuppression, which leads to enhanced susceptibility to infection with other parasitic diseases (Terry, 1977). The number, frequency and type of concurrent diseases may also influence the morbidity and mortality estimates in a population (Buck et al, 1978d). This may cause confusion in the selection of clinical parameters for case detection of specific diseases. Therefore in populations constantly exposed to polyparasitism, the epidemiologies of parasitic diseases should be studied in context of the entire global epidemiological situation.

Recent achievements in immunodiagnostic methods as shown here make it technically possible to screen large populations for several diseases at the same time, and show that serology has a definite place in epidemiological investigations (Schiller, 1967; Fife, 1971; Denham et al, 1971; Draper et al (1972a&b; Bruce-Chwatt et al, 1973; 1975; Sadun, 1976; Lobel and Kagan, 1978). Therefore polyvalent studies can be carried out at the population level. Such studies in communities exposed to polyparasitism should be undertaken with a knowledge of all the factors prevailing which may affect the tests' sensitivity and specificity. It is also possible that false positives may arise as a result of concomitant infections and in this respect the findings of Buck et al (1978c) are relevant here. These workers, in a recent study of the epidemiology of parasitism, showed that concomitant parasitic infections interfere with immunodiagnostic tests both directly, through cross-reactions with antigens and antibodies, and indirectly through its effects on nutrition and on mechanisms affecting the complement system.

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In tropical Africa malaria remains endemic and uncontrolled (WHO, 1974), so that most of the important parasitic diseases are accompanied by at least malaria and often by other parasitic and non-parasitic infections. The overall picture, which emerges from this work is that polyparasitism does occur in populations in Ghana and the epidemiologies of such parasitoses require further study. However, trypanosomiasis, schistosomiasis and onchocerciasis are basically not prevalent in the urban populations, and sera from patients with proven infections were all rural samples. Malaria, on the other hand, is not limited to rural populations. The endemicity of malaria in Ghana and the chronic exposure of both urban and rural populations is suggested from the seroepidemiological findings in this, as well as other studies. Malaria infection causes both an enhancement and suppression of immune mechanisms (WHO, 1975).

Populations exposed to recurrent attacks of malaria have high serum IgG and IgM (Turner and Voller, 1966; Rowe et al, 1968). Only a small proportion of these immunoglobulins is specific malarial antibody (Curtain et al, 1964), the remainder consisting of antibodies with heterophile and autoimmune reactivity. Malaria is also associated with complement activation, immune complex formation, immunosuppression and impaired immune responses to vaccinations and immunisations. Malaria is further implicated in chronic disease syndromes such as the tropical splenomegaly syndrome, nephrotic syndrome and Burkitt's lymphoma. The exaggerated immune responses associated with malaria were reviewed by WHO (1975; 1977) and Lancet (1978).

In order to understand something of the natural history of the disease, immunological investigations were carried out in sample Ghanaian populations to examine some of the defective immunological responses that are implicated in malaria. Data from these studies are presented in subsequent chapters of this thesis.

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CHAPTER EIGHT

SERUM IMMUNOGLOBULINS IN HEALTH AND MALARIA ASSOCIATED DISEASES IN GHANA

Quantitative estimations of serum immunoglobulin levels are used as diagnostic indicators of disease and to monitor therapy in a variety of hypo- and hypergammaglobulinaemic states. However, many factors including race (Lichtman, et al, 1967; Maddison et al, 1975), age (Stiehm and Fudenberg, 1966; Collins-Williams et al, 1968; Allansmith et al, 1968), sex (Rowe et al, 1968; Grundbacher, 1972) and nutrition (Anderson and Altman, 1951; Reddy and Srikantia, 1964; Keet and Thom, 1969; Aref et al, 1970) affect serum immunoglobulin concentrations. However, the effect of the environment is more profound (Fahey, 1965; McKelvey and Fahey, 1965; Fahey and McKelvey, 1965; Micheaux, 1966) and may influence the final dynamic levels attained in various geographical regions. A comparison of serum immunoglobulin levels from different populations, even, from apparently healthy populations, can only be carried out with a knowledge of at least the prevailing environmental factors. Thus in developing countries, such as Ghana, continuously undergoing socio-economic changes, establishment of local serum immunoglobulin standards is of the utmost importance in order that clinically important levels may be readily recognised. And if possible, such baseline should be established at regular intervals.

Serum protein levels differ significantly between African and European populations (Edozien, 1957; Ezeilo, 1970). The apparent hypergammaglobulinaemia in Africans often associated with hypoalbuminaemia has in part been attributed to malarial infection (Holmes et al, 1955; Deegan

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et al, 1956; McGregor et al, 1956; Gilles and McGregor, 1959; Edozien et al, 1960; Edozien, 1961; 1962) although the general polyparasitic African environment has also been implicated. Turner and Voller (1966), Mohammed et al (1973), Nantulya and Lindqvist (1973) have also shown evidence that one or more of the serum immunoglobulins IgG, IgA and IgM are higher in Africans living in malaria endemic regions.

There is a paucity of baseline immunological data on the healthy Ghanaian, and to the best of my knowledge, the concentration of serum immunoglobulin has not been established in any systematic way. The present study therefore defines the local reference standards for IgG, IgA, IgM, IgE and albumin for a normal urban Ghanaian population. Values obtained from blood donors in South East England are included for comparison. Immunoglobulin IgG, IgA and IgM have also been established in sera from cord blood, pregnant women, and primary school children. The relationship between serum immunoglobulins and malaria antibodies has been determined in the apparently healthy adult population, i.e. the cord, pregnant women, children, in Caucasians with a single acute malarial infection, and in malaria associated chronic diseases (nephrotic syndrome and tropical splenomegaly syndrome).

Study population These comprised 3 main groups, and were assessed as follows:-

Group 1 Comprised of 100 Ghanaian adults, 50 male and 50 female, age range 17-50 years (mean male = 29 years, mean female = 25 years) all lived in the Accra region considered as an urban environment. The male population form part of the Army group (see survey population Group D, pp. 81) and the female population were made up of hospital personnel,

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nurses, medical students, laboratory technicians and office clerks (These form part of survey population Group A, pp.79). These subjects were all classified as relatively healthy since they were clinically normal with no observed parasitaemia either for malarial or filarial infections.

The determination of immunoglobulin concentration in this group was carried out at the Protein Reference Unit, Westminster Hospital, in collaboration with Dr. Riches of that Unit, in order to establish local reference standards for Ghanaian adults. Sensitive immunochemical techniques were used and values obtained were calibrated against the International Federation of Clinical Chemistry (IFCC 74/1) Immunoglobulin Standard. For comparison sera from healthy blood donors aged 18-25 years (63 males and 38 females) from South East England were used for the determination of IgG, IgA and IgM. IgE and albumin were measured in sera from 56 laboratory workers aged 20-45 years (28 males and 28 females) free from any known allergy. The result of this study has been published (Riches et al, 1979).

Group 2 Comprised of 45 cord sera, 40 sera from urban primary school children aged 5-11 years and 31 from urban pregnant women in their mid-pregnancy (all sera were drawn from the main survey population, see Materials and Methods, pp.79-81). Also sera from malaria associated diseases, 26 from nephrotic syndrome and 20 from TSS were studied. Budgetry confines limited the choice of study groups to the very few which will provide some relevant answers to the malaria situation in Ghana.

Group 3 Comprised of 31 Caucasians with *P.falciparum* infection (previously described, see pp. 86) and 50 Caucasian blood donors resident in Britain were obtained for comparison.

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<u>Immunoplates</u> Behring commercial Tri-partigen immunoplates were obtained from Behring (Hounslow) and 60% of the immunoplates used were donated by Professor M. Bottiger (Swedish National Bacteriological Department). These standards have been compared with the WHO International Reference Preparation for IgG, IgA and IgM and conversion factors are given in Table 47 in order that results can also be expressed in international units in accordance with WHO's recommendation (Rowe et al, 1970; 1972; Anderson et al, 1971).

<u>Antisera</u> Antiserum to human albumin was raised in sheep (Seward Laboratories, London, UK). Antisera to human IgG, IgA and IgM raised in goat was obtained from Hallestad Laboratories (Mn 55318, U.S.A.), rabbit anti-human IgE from Behring Diagnostics (Hounslow, Middlesex, U.K.).

<u>Other reagents</u> The agarose used for immunoelectrophoresis was Litex HSA (Glostrup Denmark). Tween 20 was purchased from Koch Light Ltd. Colnbrook, U.K.) and all other chemicals from B.D.H(Poole, Dorset, U.K.).

Albumin and IgG concentrations in the healthy adult populations were assayed by the automated immuno-precipitation method. IgA and IgM were assayed by a modified electro-immunodiffusion method (Laurell, 1966; Slater, 1975). IgE was assayed by a radio-immunoassay method (previously outlined). For the rest of the sera, IgG, IgA and IgM immunoplates from Behring were used (with reference sera supplied with kit). All the methods have been described in Chapter Four.

<u>Standardisation and quality control</u> All working standards used in the determination of IgG, IgA, IgM and albumin in Group 1 were calibrated against IFCC 74/1, a recently established International Reference Standard for immunoglobulins (Whicher et al, 1978). The mass concentrations used

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TABLE 47 Conversion factor used for results in Groups 2 and 3 to convert values in mg/100 ml to iu/ml

IgG	=	.115
IgA	=	.595
IgM	=	1.15

TABLE 48 Weights of immunoglobulins corresponding to one iu/ml used for results in Group 1

	ug/iu
IgG	80.4
IgA	14.2
IgM	8.47

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to express immunoglobulin results were calculated from the assigned International Units per millilitre by the standardised conversion factors (Humphrey and Batty, 1974).(Table 48). IgE values are given in International Units per millilitre and determinations were made with standards calibrated against the WHO standard for IgE (Rowe et al, 1973).

In order to assess the reproducibility and uniformity of immunoglobulin measurements between laboratories, 35 Ghanaian sera comprising 5 cord, 5 children, 10 adult, 10 malarious, 5 nephrotic, 5 tropical splenomegaly syndrome sera were tested independently at 4 main immunology laboratories (The London Hospital, Whitechapel (1), The Bone and Joint Unit (2) and Kettering General Hospital (3) and final confirmation at the Westminster Hospital (4)). The values achieved through these independent assessments using 3 different methods were not statistically different. The modified electroimmunodiffusion method (Slater, 1975) was shown to be more sensitive for the detection of low levels of IgA and IgM in cord sera than Mancini radial immunodiffusion by standard commercial immunoplates from Behrin g, Hyland and Miles.

The coefficients of variation for the assays used for Group 1 were 3.5% for albumin, 5.0% for IgG, 8.0% for IgM and 9.5% for IgE.

Statistical analysis Non-parametric statistical tests were used in this study, this was because samples used were often too small to be able to establish if any of the distribution obeyed a normal Gaussian distribution. Differences of means between paired groups were analysed by the Mann-Whitney test. Intergroup differences in means were analysed by the Kruskal-Wallis one-way analysis of variance by ranks. Fisher's exact probability test was also utilised to observe differences in distribution observed between independent groups. Spearman rank correlation coefficient and Kendall rank correlation were used to measure the relationship between immunoglobulin concentration and malarial antibodies.

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Distribution of serum immunoglobulin concentration

Group 1

The distribution of concentrations of all the proteins studied in this group were analysed independently for the male and female groups, Values for IgG, IgA and IgE were not found to be significantly different between males and females in either the Ghanaian or the European populations. For these proteins, therefore, males and females were analysed as a single group. Significant differences were apparent between males and females, for both IgM and albumin concentration in both populations and these proteins were anslysed separately for males and females.

The distribution patterns for both populations either as a single group or separately as males and females where appropriate, for each of the proteins studied are shown in Figures 39 - 43. Table 49 shows mean immunoglobulin and albumin levels in Europeans and urban Ghanaians and the statistically significant differences between means in the 2 populations are also indicated. Three individuals in the Ghanaian population showed extremely high levels of IgE (10,320, 12,610 and 14,800) and were not included in the analysis.

The concentrations of serum IgG, IgE and IgM both in males and females were significantly higher in the African population population compared with the Europeans, and albumin concentrations were significantly lower in both males and females. Serum IgA was the only protein showing no significant differences between the 2 populations.

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FIGURE 39 Distribution pattern of serum IgG concentrations. Continuous line represents the European population and the histogram the Ghanaian population

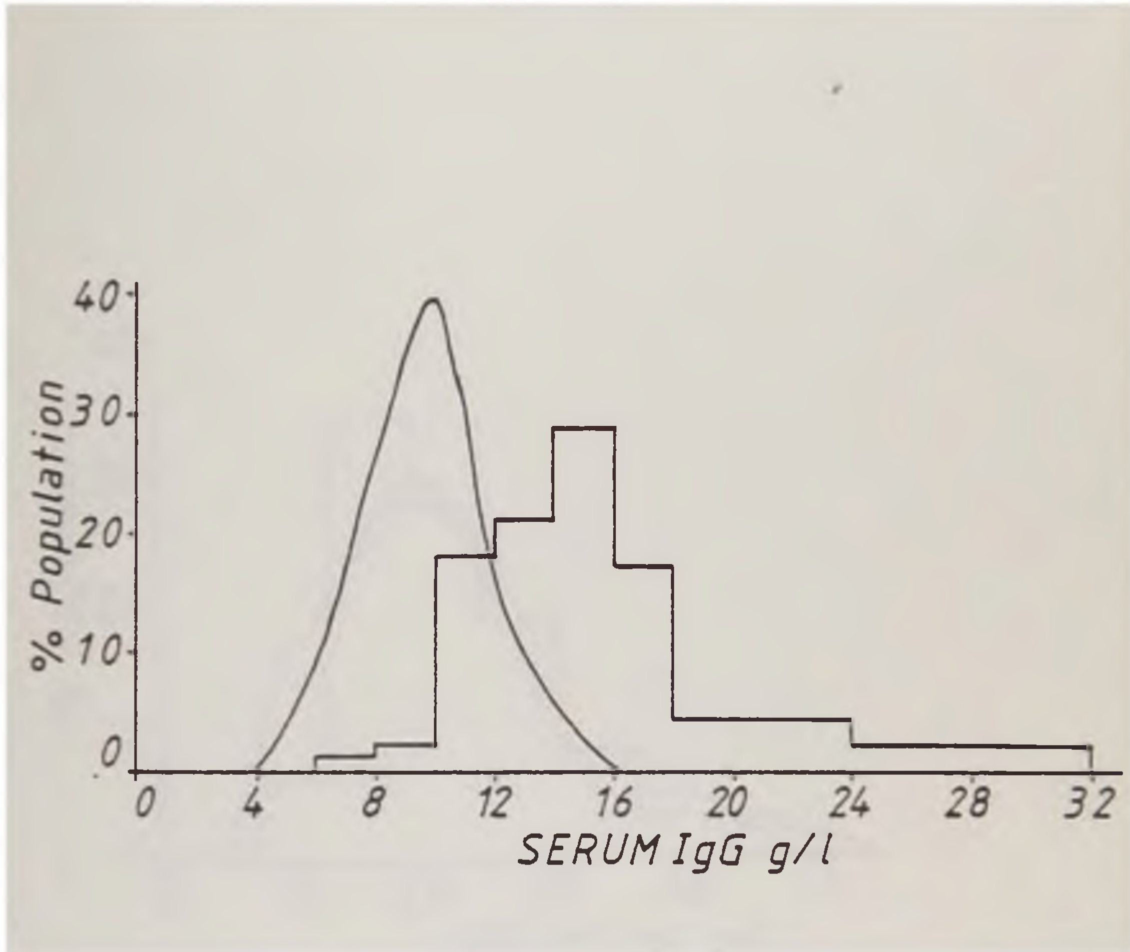

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FIGURE 40 Distribution pattern of serum IgA concentrations. Continuous line represents the European population and the histogram the Ghanaian population

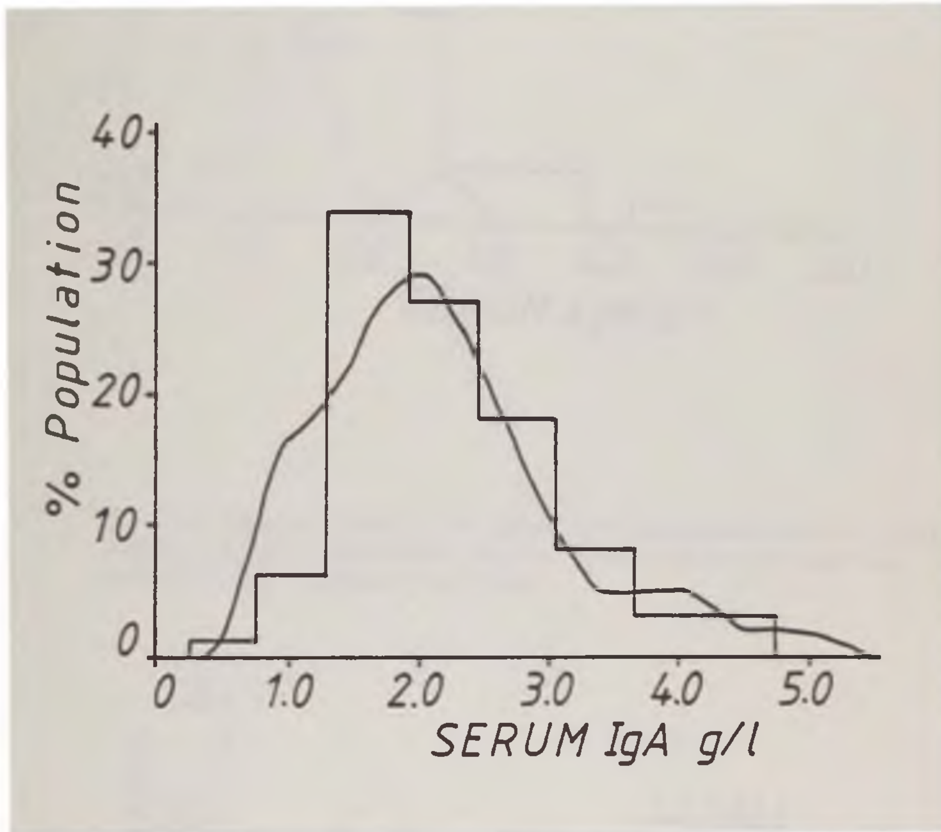

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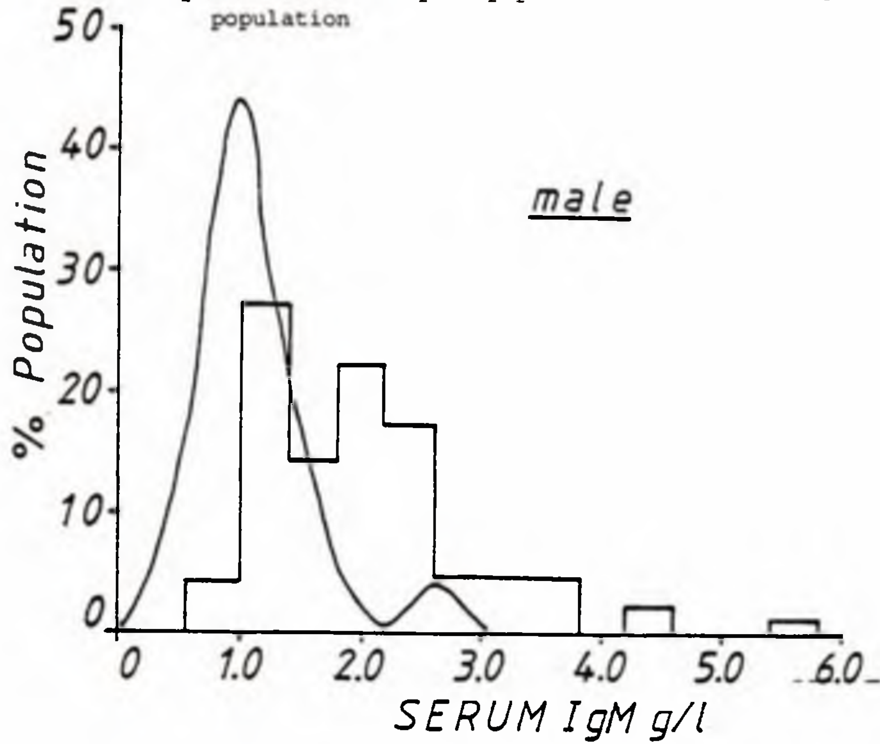
FIGURE 41

a) Distribution pattern of serum IgM concentrations in males. Continuous line represents the European population and the histogram the Ghanaian population



b) Distribution pattern of serum IgM concentrations in females. Continuous line represents the European population and the histogram the Ghanain population

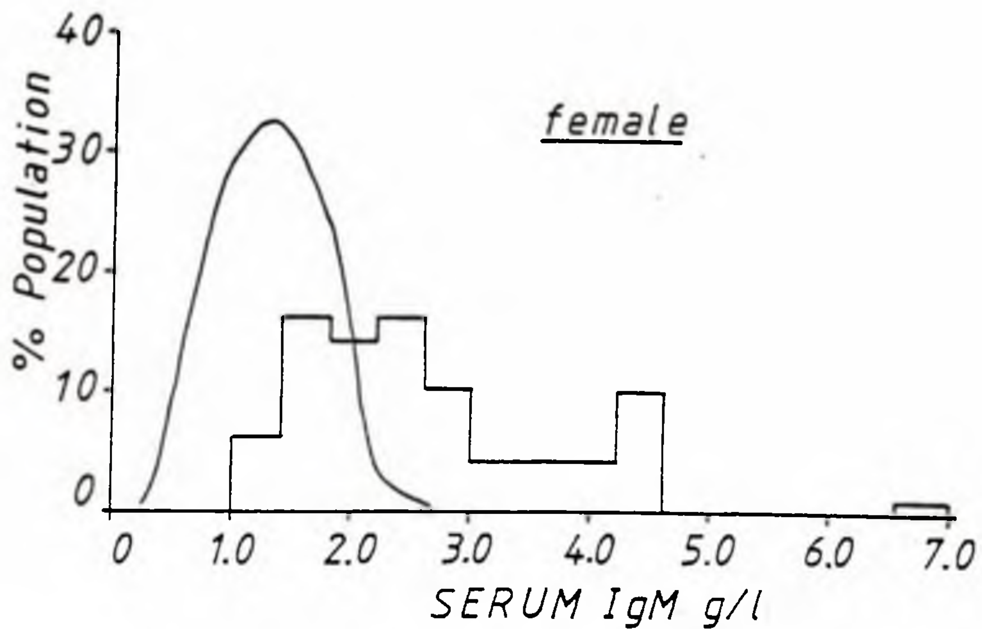


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FIGURE 42 Distribution of serum IgE concentrations. Continuous line represents the European population and the histogram the Ghanaian population

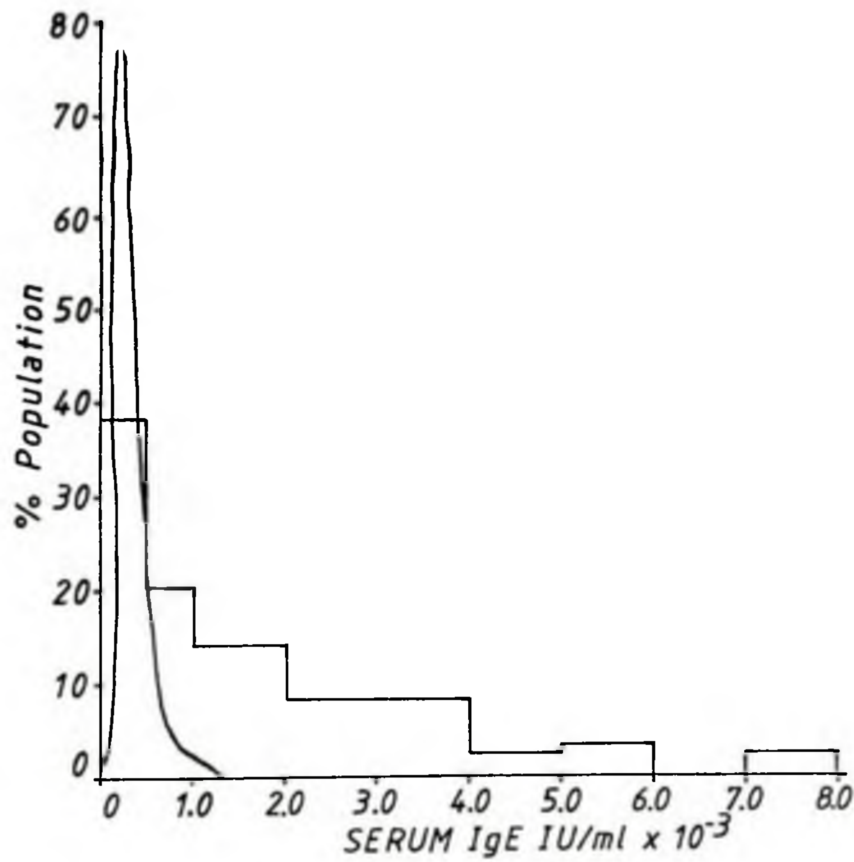

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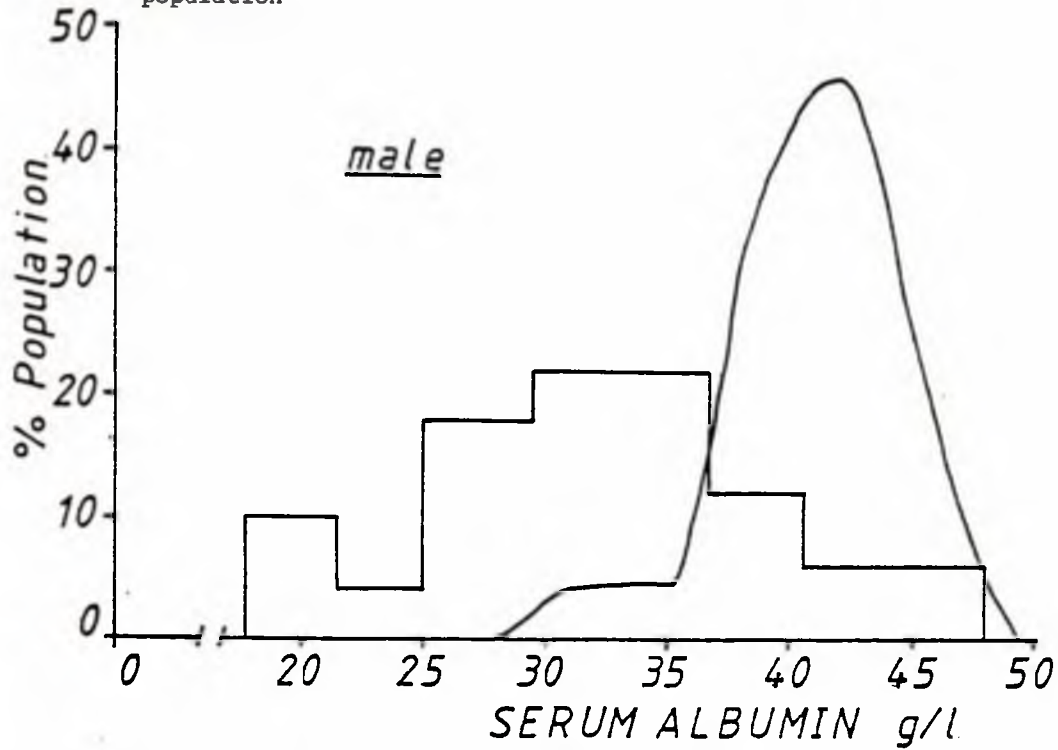
FIGURE 43

a) Distribution pattern of serum albumin concentrations in males. Continuous line represents the European population and the histogram the Ghanaian population



b) Distribution pattern of serum albumin concentrations in females. Continuous line represents the European populations and the histogram the Ghanaian population

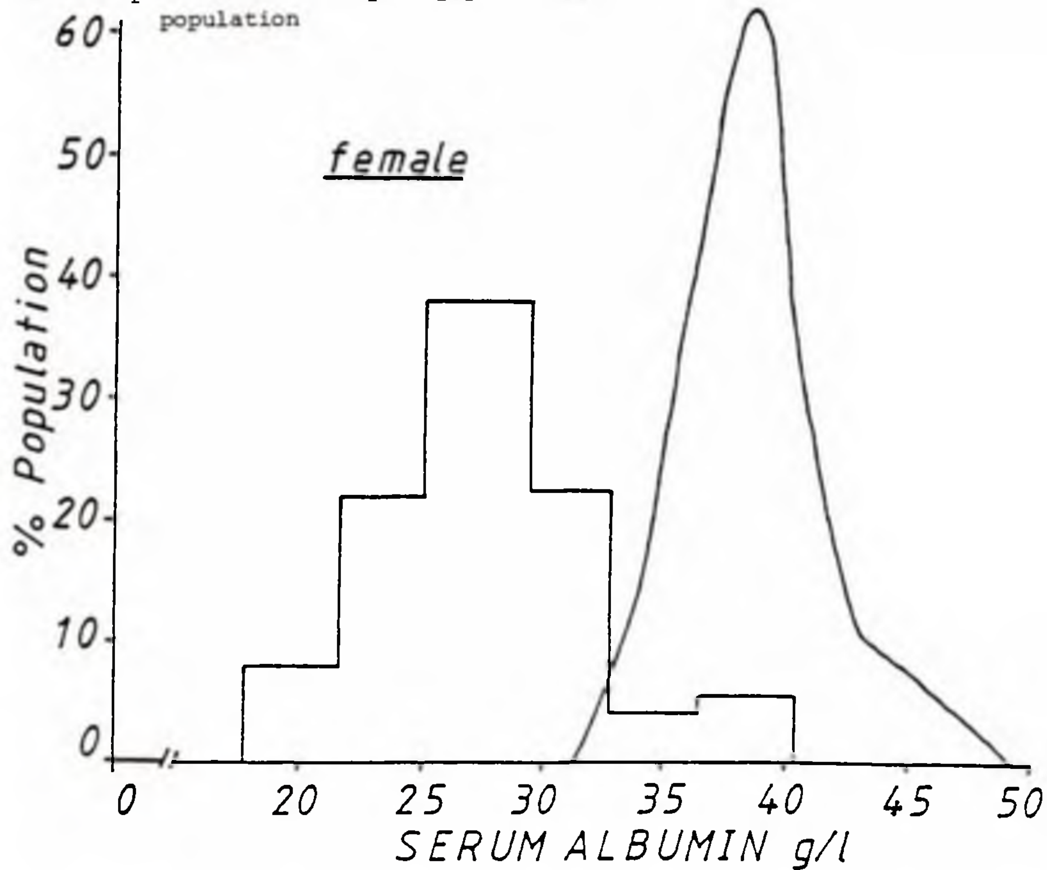


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TABLE 49 A) Mean (g/l) immunoglobulins and albumin levels in Europeans and urban Ghanaians and the statistically significant differences (P) between mean values for the 2 populations

Protein	Mean g/l ± 2SD Europeans	Mean g/l ± 2SD Ghanaians	P
IgG	10.72 ± 2.17	15.61 ± 5.59	<0.001
IgA	2.12 ± 0.93	2.17 ± 0.90	<0.58
IgM male	1.01 ± 0.40	1.73 ± 0.70	<0.001
female	1.25 ± 0.42	2.41 ± 1.10	<0.001
Albumin male	41.0 ± 3.28	31.8 ± 7.15	<0.01
female	38.6 ± 2.97	27.0 ± 4.44	<0.01
IgE range and median values in iu/ml			
Range	0 - 120	0 - 7280	
Median	72	1782	<0.001

TABLE 49 B) Mean (iu/ml) immunoglobulin and albumin levels in Europeans and urban Ghanaians and the statistically significant differences (P) between mean values for the 2 populations

Protein	Mean iu/ml ± 2SD Europeans	Mean iu/ml ± 2SD Ghanaians	P
IgG	133.33 ± 27.00	194.15 ± 69.53	<0.001
IgA	149.30 ± 65.49	152.82 ± 63.38	<0.58
IgM male	119.24 ± 47.23	204.25 ± 82.64	<0.001
female	147.58 ± 49.59	284.53 ±129.87	<0.001
Albumin male	41.0 ± 3.28	31.8 ± 7.15	<0.01
female	38.6 ± 2.97	27.0 ± 4.44	<0.01
IgE range and mean values in iu/ml			
Range	0 - 120	0 - 7280	
Median	72	1780	<0.001

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Group 2

Results are summarised in Table 50. Serum immunoglobulin levels for this group were determined by Mancini's radial immunodiffusion assay using commercial immunoplates, and carried out in a different laboratory from that of Group 1. It is known that serum immunoglobulin levels reported from different laboratories show considerable variation if they are expressed by weight. Therefore, in order to compare values obtained here with the local adult standards established for healthy urban adults in Group 1 (Ghanaian controls), values for both groups have been expressed as iu/ml, (Table 51) using the appropriate conversion factors (see Tables 47 and 48). Also in order to facilitate a better comparison of immunoglobulin concentration in the different subject groups tested, the mean of the local reference standards (mean IgG, IgM and IgA) established for healthy urban Ghanaian adults was assigned a value of 100% and called the mean normal adult value (MNA). Mean serum immunoglobulin values in all groups tested could then be described as a percentage of the MNA.

Cord sera The range and mean values of serum IgG, IgA and IgM for this group were compared with values observed for the healthy Ghanaian adult controls, pregnant sera and the healthy urban Ghanaian children.

The range and mean IgG values were similar to levels in healthy adult controls, evidenced by showing a mean level which is 100% MNA but cord levels showed less variation. Cord IgG concentrations and mean value were also comparable to those of urban pregnant women. Cord IgG levels showed considerable differences when compared to urban children and the difference in means observed for the 2 groups was statistically significant ($P < 0.01$).

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TABLE 50 Range and mean (± SEM) levels of immunoglobulins IgG, IgA and IgM levels (g/l) for Groups 2 and 3 of the study population

Population (No)	Range and mean	IgG Min	IgG Max	IgA Min	IgA Max	IgM Min	IgM Max
Group 2							
Cord (45)	Range	8.50	29.50	0.10	2.65	<0.20	0.89
	Mean	16.95 ±	0.80	0.41 ±	0.03	0.35 ±	0.05
Children (40)	Range	8.40 -	35.00	0.75 -	9.30	0.60 -	7.32
	Mean	21.20 ±	1.03	2.30 ±	0.37	1.09 ±	0.29
Pregnant (31)	Range	8.00 -	35.81	0.60 -	3.40	1.00 -	6.80
	Mean	17.39 ±	1.28	2.01 ±	0.15	2.61 ±	0.24
Nephrotics (26)	Range	3.00 -	48.90	-		0.03 -	5.24
	Mean	13.90 ±	2.34			2.33 ±	0.25
Tropical SS (20)	Range	8.55 -	39.36	0.63 -	9.50	0.86 -	44.00
	Mean	18.23 ±	1.85	2.29 ±	0.42	4.59 ±	2.16
Group 3							
Caucasians with malaria (31)	Range	6.00 -	38.80	0.76 -	5.40	0.80 -	9.00
	Mean	17.60 ±	1.30	3.44 ±	0.21	3.36 ±	0.40
Caucasian blood donors (50)	Range	6.60 -	13.50	0.80 -	4.60	0.50 -	3.60
	Mean	9.11 ±	0.26	1.96 ±	0.11	1.52 ±	0.11

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TABLE 51 Comparison of range and mean (± SEM) levels of immunoglobulins IgG, IgA and IgM (iu/ml) of Group 2 with local reference standards (MNA) established for adult Ghanaians (Group 1)

Population (no.)	Range and Mean	IgG Min		IgG Max	(MNA) % of adult level	IgA Min		IgA Max	(MNA) % of adult level	IgM Min		IgM Max	(MNA) % of adult level	P*
Group 1														
Ghanaian healthy adults (100)	Range	75		647		42		423		71		803		
	Mean	194	±	70	100	153	±	63	100	247	±	19	100	
Group 2														
Cord (45)	Range	98		339		<6		158		<23		102		
	Mean	194	±	9	100	24	±	2	16	40	±	6	16	<0.00001
Children (40)	Range	97		403		45		553		69		842		
	Mean	244	±	12	126	137	±	22	90	125	±	33	51	<0.005
Pregnant women (31)	Range	101		412		36		202		115		782		
	Mean	200	±	15	103	120	±	9	78	248	±	28	100	
Healthy Ghanaian females (50)	Range	109		490		63		310		118		802		
	Mean	190	±	7		158	±	8		285	±	9		
Nephrotic (26)	Range	35		562			-			3		603		
	Mean	160	±	27	82					268	±	29	109	<0.01
Tropical SS (20)	Range	98		453		37		565		99		5060		
	Mean	210	±	21	108	136	±	25	89	528	±	248	214	<0.001

* Statistically significant difference between adult mean and other group means

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The cord IgM levels, on the other hand, were much lower than levels observed in adult controls, urban pregnant and urban children sera, and the mean differences when compared to the other groups were statistically significant ($P < 0.0001$). However, the detection of cord IgM of levels above 24 iu/ml (≥ 20 mg/%) in 27% of cord sera tested is a significant finding.

The IgA levels were also lower than in adult controls, urban pregnant and in children sera. And the difference between mean cord IgA and the other groups was statistically significant ($P < 0.0001$). IgA detectable above 6 iu/ml (the assay limit) was demonstrated in 11% of the cord sera, and these also had corresponding elevated levels of IgM and IgG than did the IgA negative sera.

Pregnant sera Immunoglobulin levels were compared with values determined for healthy adult controls and non-pregnant Ghanaian females. The distribution patterns in the 3 groups were very similar, although 40% of pregnant sera showed lower concentrations when compared to the healthy adult controls and non-pregnant sera.

The mean IgG levels were comparable and only slightly elevated in the pregnant sera.

The IgM and IgA levels were also comparable, but overall, lower levels were seen compared to levels in adult controls and non-pregnant females. Mean pregnant IgM was slightly lowered than mean levels for the non-pregnant female. However, the difference in means was not statistically significant.

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Children sera Immunoglobulin concentrations were compared to levels obtained in the healthy adult controls. The mean IgG level was significantly higher ($P < 0.005$) than that observed in adults, but the form of distribution was less variable in the children's case. Differences existed between the mean IgA levels of children and adult sera but this was not shown to be statistically significant. However, mean IgM between the 2 groups were significantly different ($P < 0.005$) although the frequency of distribution was similar in both groups.

Nephrotic syndrome sera Immunoglobulin concentrations in this group were compared with levels obtained for healthy adult Ghanaians.

The IgG levels and mean IgG were significantly lower ($P < 0.01$) than concentration in healthy adults, and as much as 19% of nephrotics had values lower (<75 iu/ml) than the lowest IgG level observed for the healthy adult control groups.

Serum IgA levels were not determined. Serum IgM levels, however, showed remarkable variations. Levels below 23 iu/ml were observed in 8% of sera tested. But 12% showed levels lower than 69 iu/ml. The rest of the group (88%) showed comparable distribution to the healthy adult groups. In fact the mean IgM of the 88% nephrotics was slightly higher than mean IgM level in healthy adults, but the difference was not statistically significant.

Tropical splenomegaly syndrome (TSS) The IgG levels were significantly greater compared to levels observed for healthy adults, although the high mean IgG level was not significantly different at the 5% level.

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The IgA concentrations in TSS were comparable to those of healthy adults and the differences in means observed was not statistically significant.

The serum IgM concentrations on the other hand, were markedly higher than in adult Ghanaian controls. The increase observed was 214% MNA, well over and above levels in healthy adults, and the difference in means between TSS and healthy adults were highly significant ($P < 0.001$).

Group 3

Results for this group are presented in Table 52.

Caucasians with P.falciparum malaria The serum immunoglobulins concentrations observed in this group were compared with levels in sera from healthy Caucasian blood donors resident in Britain and also with healthy Ghanaian adult levels. The IgG levels were markedly higher than levels observed in normal Caucasians. In 70% of Caucasians with malaria the IgG level was greater than the highest level observed in normal Caucasian controls, and the difference in mean IgG concentration was significant ($P < 0.001$). The IgG levels of the Caucasians with malaria, on the other hand, showed similar distribution frequencies to those of healthy Ghanaian adult controls. The mean IgG level was slightly but not significantly higher than the mean IgG for the adult Ghanaian control.

The IgA concentrations in Caucasians with malaria were also higher than levels in normal Caucasian controls and healthy Ghanaian adult controls.

The difference in means observed were statistically significant ($P < 0.01$).

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TABLE 52 Range and mean (± SEM) levels of immunoglobulins IgG, IgA and IgM (iu/ml) for Caucasians with malaria compared with normal Caucasian values, and also with adult Ghanaian levels (MNA)

Population (no.)	Range and Mean	IgG Min		IgG Max	(MNA) % of adult level	IgA Min		IgA Max	(MNA) % of adult level	IgM Min		IgM Max	(MNA) % of adult level	P*
Group 3														
Caucasians with malaria (31)	Range	69		446		45		321		92		1035		
	Mean	202	± *	15	104	204	± * ⊕	12	133	386	± * ⊕	46	156	* 0.002 ⊕ 0.01
Caucasian blood donors (50)	Range	82		168		53		324		59		425		
	Mean	113	±	3	58	138	±	20	78	179	±	13	72	0.01
Group 1														
Ghanaian healthy adults	Range	75		647		42		423		71		803		
	Mean	194	±	70	100	153	±	63	100	247	±	18	100	

* Statistically significant difference between Caucasians with malaria and normal Caucasians

⊕ Statistically significant difference between Caucasians with malaria and adult Ghanaian controls

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The IgM levels were considerably increased in Caucasians with malaria compared to levels in normal Caucasians. As much as 31% of malarious Caucasians had IgM levels higher than the highest concentration observed for normal Caucasians and the difference in the mean IgM concentrations observed was significant ($P < 0.001$). The increases in serum IgM concentration in Caucasians with malaria were markedly higher than levels in healthy adult Ghanaians and 31% had levels higher than 98% of healthy adult Ghanaian levels. And a significant difference in mean ($P < 0.01$) was observed for the 2 groups.

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Serum immunoglobulin concentrations in relation to malaria antibodies in Ghanaian sera and Caucasians with P.falciparum malaria

Results were further analysed to observe for associations between serum immunoglobulin levels and malaria antibody levels in each subject group studied. Malaria antibody levels by IFA and ELISA (Table 53) showed good correlation in all groups (Spearman Rank correlation) ($P < 0.01$). Correlation coefficients were calculated for malaria antibodies and serum IgG, IgA and IgM in all groups studied.

Healthy urban adult Ghanaians The range and mean levels of malaria antibodies detected by IFA and ELISA are shown in Table 53. There was no evidence of malaria parasitaemia in this group. The IgG, IgA and IgM levels were not correlated with all levels of malaria antibody, although higher concentrations were always associated with higher levels of malaria antibodies usually an IFA titre of above 1:320 and an ELISA value of 0.50. Serum IgM levels interestingly showed good correlation with elevated levels of malaria antibodies ($P < 0.02$).

Cord sera Malaria antibody levels were comparable to those observed in healthy adults, but higher than levels observed in pregnant women. The difference in mean malaria antibody levels observed between cord sera and pregnant sera were however not statistically significant. Malaria antibodies in cord sera on the other hand were significantly higher than malarial antibodies in Ghanaian children ($P < 0.01$).

The cord IgG, IgA and IgM levels were not correlated with positive levels of malaria antibodies. However, in sera where raised levels of malarial antibody occurred, cord IgM was significantly correlated with the raised levels of malarial antibodies ($P < 0.02$).

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TABLE 53 **Malaria antibodies determined by IFA and ELISA for all subject groups**

Population (no)	Range GMRT ± SEM	IFA (reciprocal titres) Min	Max	P values	ELISA (Absorbance value) at E_{405} Min	Max
Group 1						
Ghanaian adults (100)	Range	80	5120		0.22	1.76
	Mean GMRT	319 ±	2.10		0.75 ±	0.03
Group 2						
Cord (45)	Range	20	5120		0.45	1.61
	Mean GMRT	219 ±	3.60		0.71 ±	0.05
Children (4)	Range	20	1280	<0.0001	0.26	0.88
	Mean GMRT	98.5 ± *	3.9		0.47 ± *	0.03
Pregnant women (31)	Range	0	1280		0.25	1.68
	Mean GMRT	271 ±	1.1		0.68 ±	0.06
Non-pregnant women (50)	Range	20	5120		0.20	2.01
	Mean GMRT	305 ±	2.9		0.70 ±	0.04
Nephrotics (26)	Range	0	1280		0.50	1.48
	Mean GMRT	220 ±	3.4		0.72 ±	0.05
Tropical splenomegaly syndrome (20)	Range	20	1280		0.35	2.24
	Mean GMRT	298 ±	2.8		0.98 ±	0.13
Group 3						
Caucasians with malaria (31)	Range	0	5120		0.09	2.34
	Mean GMRT	99.5 ±	5.9	<0.0001	0.58 ±	0.10
Caucasian blood donors (50)	Range	0	0		0.03	0.11
	Mean	0 *		<0.00001	0.01 ±	0.002

* Statistical significant of difference in means observed between healthy adult mean and other group mean levels

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Children sera Malaria antibody levels in this group were significantly lower than levels in healthy adult Ghanaian sera, and the difference in mean values was statistically significant ($P < 0.001$).

Serum IgG showed positive correlation with all levels of malarial antibodies and most significantly with higher levels than with lower values ($P < 0.02$).

No correlation was shown between IgA and malarial antibody levels. IgM, on the other hand, showed significant correlation only with raised malarial antibody levels ($P < 0.02$).

Pregnant sera IFA and ELISA malaria antibody levels were lower in this group compared to levels in healthy adults but differences in means were only statistically significant between IFA mean antibody titres of the 2 groups. Malarial antibody levels were much lower in pregnant sera compared to levels in non-pregnant females, but there was no significant difference in the means observed.

High serum IgG, IgA and IgM levels in pregnant sera were associated with high levels of maternal antibodies, however, significant correlation ($P < 0.03$) was only observed between IgG and high malarial antibody levels.

Nephrotic sera Malarial antibody levels were comparatively lower than those observed in the healthy Ghanaian adults but the difference in means was not statistically significant. All but one patient were negative for *P.falciparum* infection.

Serum IgG showed good correlation with any level of malarial antibody at the 3% probability level, but correlated more significantly with higher levels of malarial antibody at the 1% level.

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Serum IgM levels were not correlated with malarial antibody at any level.

<u>Tropical splenomegaly syndrome (TSS)</u> Malarial IgG antibodies in this group were similar to those of healthy adult Ghanaians. A remarkable finding in TSS was the presence of detectable levels of IgM specific malarial antibodies. The differences in mean IgG malarial antibody values observed between TSS and healthy adult Ghanaians were not statistically significant. But the fact that IgM specific malaria antibody is virtually absent in normal controls made this a significant finding, even though they occurred at low levels (IFA 320; ELISA 0.55).

Serum IgG and IgA were not correlated with malarial antibodies at any level. But IgM correlated with all levels of malarial IgG and IgM antibody ($P < 0.05$) but most significantly with raised levels of malarial antibodies ($P < 0.001$).

<u>Caucasians with P.falciparum malaria</u> Malarial antibodies showed a wide variation and tended towards the lower range of positive values. Compared to the virtual absence of malaria antibodies in the Caucasian blood donors, the levels observed were significant. 17% of this group however, did not show detectable levels of malarial antibodies although they were parasitologically positive and showed raised levels of serum IgM. The malaria antibody levels were lower than those observed for healthy Ghanaian adults. On the other hand, Caucasians with malaria showed detectable levels of IgM antibodies(less than 1:320 by IFA and less than 0.50 by ELISA),malarial IgM antibodies were not seen in adults or Ghanaian children. The difference in malarial IgM antibody means, between these 2 groups (Caucasians with malaria and healthy adult Ghanaians) were statistically significant.

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Serum IgG, IgA and IgM were all correlated with malarial antibodies at least at the 1% level and an even higher correlation was shown with higher levels of malarial antibodies at the 0.005% level for IgG and IgM.

DISCUSSION

Group 1

The present data are consistent with previous reports of raised IgG, IgM, IgE, comparable levels of IgA, and low albumin in Africans compared with Europeans (Edozien, 1957; Stiehm and Fudenburg, 1966; Turner and Voller, 1966; Sturiko, 1968; Johansson and Berg, 1968; Rowe, 1972; Mohammed et al, 1973; Nantulya and Lindqvist, 1973; Cook and Lewis, 1974).

Several factors are known to influence immunoglobulin levels in health, race, sex and age have all been reported (Maddison et al, 1975). Foremost though is the precision of the assay system used, its reproducibility and individual variations (Hobbs, 1970). In fact, Hobbs reiterates that in order for immunoglobulins to be used as absolute standards, they should represent the variation in their class expected in the sera to be tested. IgG, for example, should be from a normal serum pool, representing all 4 subclasses and normal allotypic variation and also measured using antisera raised against themselves or adequately representative antigen. Hobbs (1970) on the question of assay reproducibility asserts that a single estimate by most current Mancini methods show a 2 Standard Deviation (2SD) variability of less than 10% and stresses the importance of quality control. In this study a high

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standard of quality control and reproducibility of assay system was achieved and maintained. Hobbs (1970) further reports that individual variation in any given normal subject remains within ±20% over 20 years.

The sex difference in the levels of serum IgM and albumin observed in both Ghanaians and Caucasians in this study is in agreement with widely reported studies (Butterworth et al, 1967; Grundbacher, 1972; Rowe et al, 1968; Rhodes et al, 1969; Maddison et al, 1975): females have significantly higher serum IgM and lower albumin than males. The serum IgG and IgA, on the other hand, do not differ significantly between the 2 sexes. On the other hand, Nantulya and Lindqvist (1973) studied serum protein concentrations of normal Tanzanian subjects and reported significantly higher immunoglobulin levels for females than males. Grundbacher (1972) suggested that in humans X-chromosomes carry genes with an effect on IgM concentration, but their observations do not exclude the effect of hormones (Washburn et al, 1965) or that of bacterial flora associated with the sexes. In some studies a sex difference in IgM has not been observed and it has been suggested that intense antigenic stimulations resulting in very high IgM levels may obscure the sex difference (Yadav and Shah, 1977).

It is suggested that genetic and environmental differences may explain differences in albumin observed between Ghanaians and normal British adult sera because a diet deficient in protein and hepatic dysfunction do not entirely explain the low albumin levels seen in Africans (Edozien, 1958). Buckley and Dorsey (1971) suggested that socio-economic instead of genetic differences could account for the differences attributed to race in the different immunoglobulin levels

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observed between blacks and whites in Africa. However, in a study of high and low income groups in Tanzania, no significant differences in albumin to globulin ratios could be observed (Isichei, 1975). The healthy urban Ghanaian adults used in the present study show significantly lower albumin levels from Europeans despite an adequate diet, so this must be due to a social or the environmental differences.

Hobbs (1970) reported that genetic factors potentially affect immunoglobulin levels and that apart from the obvious deficiencies and familial hypergammaglobulinaemia, studies in healthy twins indicate that actual normal levels of IgG and IgA have a small genetic contribution. He further observed that germ-free animals have very low levels of immunoglobulins and that it is environmental challenge which largely maintains even normal serum immunoglobulin levels. It is suggested here that the higher values of serum IgG and IgM when compared to Europeans would appear to result from a combination of environmental and genetic factors. The role of environment on serum immunoglobulin levels is confirmed by the observations of Schofield (1957), Cohen et al (1961) and Hobbs (1966). These workers observed that subjects from tropical regions run higher IgG and IgM levels than populations living in temperate zones, but after long term residence in temperate regions, their IgG and IgM levels decrease to low levels comparable to those for normal Caucasians. There is always a residual deviation which may be due to racial differences and may reflect genetic survival value in these subjects' country of origin.

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IgA has generally been found to be similar in Africans and Europeans (Hobbs, 1970) and the findings from the present study confirm this observation. This lack of difference may be due to little difference in leak-back into the serum from the sites of increased antigenic challenge , e.g. the gut.

Very high serum IgE levels were observed in Ghanaian sera and these were significantly higher than those observed in the British controls. Jarrett and Bazin (1977) reported that parasitic infestation can act as an adjuvant for IgG, IgA as well as IgE. This observation may be relevant here. In which case the raised levels of IgE may simply be a reflection of environmental difference and increased incidence of parasitic infestation. The raised levels may be antiparasitic antibodies and responsible for expulsion of gut nematodes and helminthes (Stanworth, 1973b). Houba and Rowe (1973) observed similar raised levels of IgE in African sera compared to Europeans. They further observed that the raised levels were not associated with malaria, vesical schistosomiasis or onchocerciasis. In the same study Ghanaian asthmatic children gave levels of IgE similar to levels in matched normal controls.

The marked increases of IgE observed in healthy Ghanaian adults clinically free of any helminthic infestation add to the growing unexplained immunological phenomena in African sera. Indeed the biological significance of high IgE in African sera remains obscure. It is suggested here that the levels observed in the present study may have a protective role, and furthermore on teleological grounds it is difficult to accept that the sole role of IgE antibody is an immunopathological one. The suggestion that 2 types of genes are involved in the control of IgE formation (Stanworth, 1972; 1973a) may be relevant here, with one gene controlling the production of the reaginic type of IgE and the other (Ir) gene may be concerned with immunoglobulin production in general.

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The mode of production of IgE is not fully understood, but it is thought that other immunoglobulins may influence their production and function (Stanworth, 1973b). Stanworth, in a recent review on the role of IgE, reported an observed intimacy of association between IgE and IgA production. In certain adults with IgA deficiency, IgE is known to act as a reserve line of defence where the supply of IgA proves inadequate. It is suggested here that the inapparent stimulatory effect of malaria infection on African serum IgA levels and raised levels of IgE in these populations may be related. It is possible that malaria immunosuppression, associated with defects in T cell regulatory mechanisms (Greenwood et al, 1977) may cause associated defects in the normal differentiation and maturation of IgA plasma cells (Butler and Oskvig, 1974). The biological role of secretory IgA is one of defence at the level of the mucous membranes (Bienenstock, 1970) and prevents entry of toxic and virulent agents into the bloodstream. Defects in secretory IgA or reduction in its functional integrity will enhance entry of parasites both gut and respiratory agents into the circulation and thus account for the increases in IgE levels. This requires investigation. However, the observed wide range of serum IgE makes the estimation of IgE in Ghanaian population of little diagnostic value.

The role of malaria in hypergammaglobulinaemia of African populations living in endemic malarious regions has been variously reported (McGregor et al, 1956; Gilles and McGregor, 1959; McGregor and Gilles, 1960; Turner and Voller, 1966; Rowe et al, 1968). In studies carried out in non-immune volunteers (Tobie et al, 1966) serum concentrations of IgG, IgA and IgM rose simultaneously shortly after parasitaemia became patent. The

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increases were greater in IgG and IgM than in IgA. Population studies made in the Gambia (McGregor et al, 1970) showed malaria parasitaemia to be associated with elevated levels of IgG over the first 20 years of life but with elevated IgM levels only over the first 2 years. Cohen and McGregor (1963) demonstrated conclusively that prolonged exposure to malarial infection leads to greatly enhanced daily rate of synthesis and to high serum levels of IgG. Cornille Brogger et al (1978) provide a further confirmation of the important role of malaria in elevated levels of immunoglobulin. They observed that IgM was significantly lowered in serum of a Nigerian population after 1½ years of malarial control measures. The role of malaria is implicit here, but it may be that the aetiology of hypergammaglobulinaemia in the tropics is complex resulting from antigenic stimulation from a wide range of viral, bacterial, protozoal and helminthic organisms. The study by Voller et al (1971) is relevant here as it further confirms the prominent role of malaria in the hypergammaglobulinaemia associated with African sera. These workers showed that populations resident in high altitude regions of Tanzania with a lowered malarial endemicity showed lower serum IgG and IgM compared to significantly raised levels observed in a corresponding population living at a lower altitude area with high malaria endemicity.

This further suggests the singular role of malaria in raised serum immunoglobulin levels. The fact that malaria is endemic in Ghana is also consistent with the findings of this present study.

The above study has enabled the establishment of local reference standards. It is therefore suggested that the ranges at the 95% confidence limits shown in Table 54 can be used as reference ranges for IgG, IgA, IgM and albumin for interpretation of normal values in urban Ghanaians.

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TABLE 54 Normal ranges (g/L) and(iu/ml) at the 95% confidence limits for IgG, IgA, IgM and albumin in normal serum in urban Ghanaians

	Protein		Range g/L
A)	IgG		4.7 - 27.0
	IgA		0.4 - 2.9
	IgM	male	0.4 - 3.1
		female	0.3 - 4.6
	Albumin	male	18 - 46
		female	19 - 36

	Protein		Range iu/ML
B)	IgG	male and female	58.46 - 335.82
	IgA	male and female	28.17 - 274.66
	IgM	male	47.23 - 366.00
		female	35.42 - 543.01

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Group 2

In order that valid comparisons with published data be made, all values obtained are expressed in iu/ml and also in percent of mean levels of the normal Ghanaian adults. Such comparisons are of value as they aid in the diagnosis of hyper- and hypogammaglobulinaemia states.

Cord sera The notable findings in this group are the high concentrations of IgM and IgG. The high frequency of IgM (27%) greater than 24 iu/ml levels in excess of those found in temperate regions (Stiehm and Fudenberg, 1966; Alford et al, 1969; Hobbs, 1970; Logie et al, 1973) are significant findings and confirm the reported increases in neonatal IgG and IgM in Gambia (Logie et al, 1973) and in Guatemalan neonates (Mata and Villatoro, 1977).

The level of IgG in cord blood approaches normal adult levels because of placental passage of this immunoglobulin (Stiehm and Fudenberg, 1966). Mattensen and Fudenberg (1965) further confirm the maternal origin of cord IgG and the present results would confirm this observation. Ghanaian urban cord IgG concentrations were similar to those observed for healthy Ghanaian controls and averaging a 100% of MNA. Cord IgG were also similar to levels in pregnant sera but cord levels showed less variation. Differences in IgG concentration as a function of foetal growth have been reported and reviewed (WHO, 1972; Caceres and Mata, 1974; Faulk et al, 1974; Mata and Villatoro, 1977), preterm infants, having a lower level of maternal antibody whereas the highest values were in term newborns. Cord samples used in this study were from full-term pregnancies and the placentae showed no evidence of malaria parasitaemia. The adult levels of IgG observed for cord sera in the present study are therefore in keeping with published data.

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Low levels of IgM less than 20 mg/100 ml (23.6 iu/ml) have been reported in normal Caucasian neonate serum (Stiehm and Fudenberg, 1966; Logie et al, 1973) but elevated levels have classically indicated congenital infection (Koch et al, 1956; Hobbs et al, 1968; Mata, 1975; Mata and Villatoro, 1977). IgM represents the chief immunoglobulin synthesized by the neonate, and it is also well known that it is the most primitive of the antibody classes in terms of evolutionary appearance (Marchalonis and Edelman, 1965) and therefore in vivo immunisation would lead to IgM antibodies first before IgG appear considerably later (Uhr, 1964). In populations where elvated neonatal IgM is found, there is always an associated high prevalence of antenatal infection. Mata et al (1972) and Mata (1975) made similar observations in 4 Guatemalan villages. Also elevated neonatal IgM and high prevalence of antenatal infection has been shown among infants of low social class in the United States (Alford et al, 1969). Logie et al (1973) studied immunoglobulin levels in sera from mothers and newborn infants in Gambian, Nigerian and Swiss populations and reported neonatal IgM levels above 23.6 iu/ml in 31% of Gambian neonates compared to 7% and 3% in the other 2 populations. They suggested that the higher levels in the Gambian neonates may have been due to frequent antigenic stimulation in utero. The high frequency 27% of cord IgM shown in this study is in keeping with the above observation. Several explanations could be advanced for such elevated increases, but it is most likely that in the Ghanaian urban context the foetus responds to microbial antigens or soluble parasite antigenic components (primarily malarial) which provoke foetal antibody production primarily IgM.

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Detectable levels of cord IgA were shown in 11% of Ghanaian cord sera. This frequency is lower than frequencies reported for normal Caucasian cord and neonate sera. Stiehm and Fudenberg (1966) reported cord IgA in 33% of subjects tested. They showed a range of 0-11 mg/100 ml and mean value of 2 ± 3 mg/100 ml. Logie et al (1973) found detectable levels of IgA in neonates were present in 25% of Swiss, 3% Nigerian and 46% of Gambian serum samples. However, only 3% of Gambian samples showed levels greater than 2 iu/ml. In the present study a level of 6 iu/ml was detectable in only 11% of the cord sera. It is difficult to interpret the relevance of the cord IgA levels observed in this study since levels compatible with health have not been properly defined. However, reported evidence would seem that the Ghanaian cord levels were much lower than frequencies seen in normal Caucasian sera. If raised cord IgM is attributable to intra-uterine infections, then the lowered frequency of IgA seen in Ghanaian cord sera may be related to a possible immuno-suppressive mechanism, of IgA differentiation in foetus, provoked by parasites or their products.

Cord immunoglobulins and malaria antibodies There was a high correlation between cord IgM and elevated levels of malaria antibodies observed in cord sera, but no association was shown between cord IgG and IgA and malarial antibody levels. It is suggested here that malaria infection may be a contributing factor to the elevated levels of cord IgM. Evidence of this is shown by the lowered levels of malarial antibodies in pregnant sera compared to non-pregnant women observed in this study. This provides an indirect evidence of loss of protective immunity to malaria during pregnancy and enhanced susceptibility to malarial infection, a well documented phenomena (McGregor and Smith, 1952; McGregor et al, 1965; Gilles et al, 1969). Susceptibility of pregnant women to malaria will enhance

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intra-uterine infections to malaria, products of malaria antigen at subclinical doses may cross the placenta and stimulate IgM synthesis in the foetus. It is further suggested that levels of cord IgM and IgA may be directly related to the extent of malarial infection in utero, and that enhancement or suppression of foetal synthesis of IgM and IgA may be associated with, and even dependent on the foetal growth stage at which stimulation with malaria parasite antigen or their products occur. The elevated levels of cord IgM and lowered frequency of detectable IgA may be interrelated. It is postulated that early in vivo malaria infection may cause defects in T cell responses as shown by Greenwood et al (1977). The role of the thymus in the differentiation and maturation of IgA-producing cells has been reported (Butler and Oskvig, 1974) and it is possible that T cell defect in malaria will affect IgA-producing cells maturation, and IgA synthesis.

Pregnant sera In general immunoglobulin levels were lower during pregnancy compared to healthy adult controls and non-pregnant sera. Differences in levels were more marked with IgM and IgA but not so much with IgG and the mean differences observed were not statistically significant. The differences observed here may primarily be due to the effects of haemodilution of pregnancy. The plasma volume in normal single pregnancy was found to be 34% above non-gravid control levels at 21-24 weeks of gestation (Rovinsky and Jaffin, 1966; Rovinsky , 1977) and rose to a peak increment of 49% at 33-36 weeks of gestation and remained essentially at this level to term. Kortmann (1972) in a study of malaria and pregnancy in Tanzania observed significant decreases in immunoglobulins IgG, IgA and IgM as much as 20% and confirmed the possible role of haemodilution of

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pregnancy in these decreases. Other workers, however, have associated the marked decreases in immunoglobulin levels in pregnant women in endemic malarious areas (Cohen et al, 1961; Rowe et al, 1968; McGregor et al, 1970) to immunosuppression of pregnancy with subsequent decrease in the synthesis of serum immunoglobulin.

Pregnant immunoglobulin levels and malarial antibodies There was a complete lack of correlation between serum IgG, IgA and IgM levels with malarial antibodies in this group. Pregnancy leads to immunosuppression with subsequent enhancement and susceptibility to infections and this immunosuppression is attributed to hormone changes of gestation (Vennig, 1946; Robinson et al, 1955). Lowered resistance to malaria during pregnancy has been well documented but the mechanisms are not yet fully understood. In the present study malarial antibodies in pregnancy were decreased compared to levels observed in non-pregnant females. It is possible that the lowered immunoglobulin levels may be correlated with lowered malaria antibody, a possible indication of loss of immunity to malaria. But it is possible that the level of haemodilution that occurs in pregnancy may obscure any likely association that may exist between serum immunoglobulin levels and malarial antibodies.

Children sera Hobbs (1970) observed that normal ranges of serum IgG, IgA and IgM are available throughout childhood. The results obtained for Ghanaian urban schoolchildren with no detectable malaria parasitaemia nor evidence of clinical illness showed levels of IgG 25% of MNA, an IgA 90% of MNA and IgM 51% of MNA. These results are consistent with observations made in Tanzania (Voller et al, 1971). They showed that the age

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at which adult levels of serum immunoglobulins are reached can be influenced by the level of malarial endemicity in a given population. In an area of high malaria transmission, a steep rise in the mean IgG occurred from early childhood and the plateau reached by about 5 years was maintained into adult life. In an area of sporadic transmission a similar early rise in IgG was observed but the plateau was not reached until 10-16 years. On the other hand, in a non-exposed group, IgG levels were much lower, but an increase occurred with increasing age, with a plateau achieved between 5-9 years after which little change occurred. The IgM levels in both intense and sporadic transmission areas showed an age-related increase, but the non-exposed population showed no clear cut pattern and mean IgM levels in children were higher than in adults.

Cornille Brogger et al (1978) compared immunoglobulin profiles of different age groups in a Nigerian population with those obtained for a Gambian population (Rowe et al, 1968). Both areas are endemic for malaria. These workers showed that the average level of IgG reached its adult plateau by age 5 years in both areas. In the Gambian population there was little or no systematic change in the average level of IgM between 6 months and 6-7 years and from then on levels increased with age. In the Nigerian population however, the increase occurred early and continued throughout life.

Serum IgA levels do not seem to be affected by malarial endemicity in a given population, and levels in Africans are consistently similar to those observed in Europeans (Hobbs, 1970). Stiehm and Fudenberg (1966) noted that IgA levels are detected in small quantities in a third of cord sera, the levels increased slowly during infancy and childhood,and the rate

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of increase though much lower was generally parallel to that of IgG. IgA does not gain full maturity until adolescence about 16 years of age. The levels observed in the present study, i.e. 90% of MNA, would suggest that adult level of serum IgA is not reached till after 11 years in Ghana.

Children serum immunoglobulin and malarial antibodies The causal relationship between malarial incidence and serum IgG, IgM has been numerously reported (McGregor et al, 1956; McGregor, 1964; McGregor and Rowe, 1967; Rowe et al, 1968; Voller et al, 1971; Molineaux et al, 1978) and protection of children living in malarious regions from malarial is significantly correlated with increase in serum IgG and IgM, and malarial antibodies. In this study strong correlations were observed between serum IgG and IgM levels with high levels of malaria antibodies, and thus reflects the role of malaria in the levels of IgG and IgM in Ghanaian children.

The levels of serum IgA attained in malarious regions, however, seem to play very little role in the hypergammaglobulinaemic state observed in Africans. In fact IgA levels in Africans do not show marked difference compared to levels observed in normal Caucasians (McGregor et al, 1970), and yet non-immunes exposed to malaria do show simultaneous increases in IgG, IgA and IgM (Tobie et al, 1966) and also confirmed in the present study. The low levels of serum IgA as compared to IgG and IgM increases in children and adults in malarious endemic regions may be due to partial suppression of IgA differentiation and maturation as a result of T cell regulatory defects associated with malaria (Greenwood et al, 1977), or due to tolerisation of pre-B cells with IgA antigenic receptors, during early intra-uterine malarial infection. In man it is thought that the major sites of IgA synthesis are the laminal propria underlying mucous

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membranes (Crabbe and Heremans, 1955) and 60% of IgA is synthesised in such sites (Hobbs, 1971) and the bone marrow and other sites synthesise the rest. Hobbs (1971) further suggests that bone marrow may be largely populated by IgA precursors which largely arose in the laminae propriae and then migrated. Furthermore the differentiation and maturation of IgA producing cells is controlled by the thymus (Butler and Oskvig, Thus intra-uterine infection with malaria causing defects in T cell responses may affect the migratory pattern of precursor IgA cells, their differentiation and maturation in bone marrow and with possible low dose tolerance to secondary malarial infections. It is possible that Caucasian non-immunes, who, not being previously tolerised in utero, show normal elevated IgA responses to malarial infection.

Nephrotic sera Serum IgG levels were significantly lowered compared to adult Ghanaian controls, and measured nephrotic levels, gave 82% of MNA level. Similar lowered levels of serum were reported by Kibukamusoke and Voller (1970) in quartan malaria associated nephrotic syndrome in Uganda. Powell et al (1977) observed similar levels in nephrotic syndrome in Papua New Guinea. In the present study serum IgM showed variable results, 12% gave lowered values and 82% showed raised levels when compared to Ghanaian adult control levels. The IgM levels in Ugandan and Papua New Guinea nephrotic syndrome sera (Kibukamusoke and Voller, 1970; Powell et al, 1977) were raised. Proteinuria is a known feature of nephrotic syndrome reported in Africa (Kibukamusoke et al, 1967; Gilles and Hendrickse, 1963; de Paillerets et al, 1972; Hendrickse, 1978). It is suggested that the low IgG may be due to loss in urine and the low IgM observed in 12% of cases may also be associated with poorly selective proteinuria seen in some Ghanaian nephrotics with chronic renal lesions (Adu et al, in preparation).

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Nephrotic syndrome serum immunoglobulins and malarial antibodies Specific malarial antibodies detected were predominantly P.falciparum specific. When P.malariae and P.vivax antigens were used very low levels of antibodies were detected. Serum IgG correlated significantly with raised levels of malarial antibodies but serum IgM did not show similar association. Malarial antibodies were lower though compared to levels observed in adult controls. The lowered levels may be due to protein loss through the glomerular basement membrane and coupled with a depressed response to malaria due to T cell suppression (Logme, 1975) as a result of lymphokine release as seen in nephrotic syndrome of other aetiologies other than malaria. T cell suppression could also primarily result from malaria infection (Greenwood et al, 1977).

Tropical splenomegaly syndrome Serum IgG levels in Ghanaian tropical splenomegaly syndrome (TSS) showed similar wide variations to those observed in Ghanaian adult controls. The measurable levels of IgM averaged 108% of the MNA level. The mean IgG in TSS although slightly raised above that for adult controls was not significantly different. Other reports and reviews (Wells, 1968; Marsden and Crane, 1976; Crane, 1977) show that the total circulating IgG in TSS is significantly greater than in controls. However, this difference can be masked by the associated expansion of plasma volume due to overproduction of immunoglobulin and albumin (Crane et al, 1974; Crane, 1977). In the present study both IgG and IgA concentrations were comparable to adult controls and thus agree with other reported studies (Wells, 1968; Stuiver et al, 1974; Marsden and Crane, 1976).

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Serum IgM in Ghanaian TSS were markedly high with a higher mean level of 214% of MNA level. Similar massive rise in serum IgM in TSS has been reported in Nigeria (Sagoe, 1970; David-West, 1974), in Uganda (Ziegler et al, 1969; 1974), in New Guinea (Wells, 1968; Marsden and Crane, 1976; Crane, 1977). In fact raised serum IgM is one of the diagnostic features of TSS associated with malaria and the results of the present survey are consistent with the above.

TSS serum immunoglobulins and malarial antibodies Tropical splenomegaly syndrome is closely linked to malaria on clinical and epidemiological grounds (Pitney, 1968; Hamilton et al, 1969; Ziegler et al, 1969; Sagoe, 1970; Stuiver et al, 1971). The initial evidence implicating malaria in the pathogenesis of the syndrome was the demonstration or raised malaria antibody titres by indirect fluorescence (Brown and Brown, 1965) and further work has confirmed this finding (Marsden et al, 1965; Marsden and Hamilton, 1969; Butler et al, 1973). Voller et al (1971b) further reported the association in immune Tanzanians of high IgG anti-malarial antibody titres with high serum IgM levels, and the association in TSS show even higher correlation (Crane, 1977). Recent refinements in immunochemical techniques and malaria serology have allowed measurement of class specificity of antibodies to malaria. A remarkable finding in this study was that while adult Ghanaians showed a virtual absence of IgM specific malarial antibodies, sera from TSS patients showed detectable levels, often at low titres when compared to IgG specific malarial anti-bodies which show significantly higher titres. Similar low levels of IgM malarial antibodies were reported by Stuiver et al (1973) in a study of malarial antibodies in TSS in Uganda. Crane et al (1977) in a

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recent study of TSS in New Guinea also reported significant levels of malarial IgM antibodies. The further significant correlation between the low levels of IgM malarial antibody and high levels of serum IgM in the present study is a relevant observation and may be diagnostic for potential TSS cases. The role of P.falciparum infection in TSS is suggested by the higher titres obtained for P.falciparum and very low titres with P.malariae and P.vivax antigens.

Group 3

Caucasians with P.falciparum malaria Serum immunoglobulin IgG, IgA and IgM were markedly raised, and compared to Caucasian reference standards, showed levels which were 178% of MNA IgG, 114% of MNA and 216% of MNA IgM level. On the contrary these raised levels showed remarkable similarity with IgG, and IgM concentrations observed in healthy Ghanaian adult controls. Interestingly the exaggerated responses seen in non-immune adults may even exceed those in African sera as observed for IgA responses which was significantly raised compared to levels in adult Ghanaian sera. Tobie et al (1966) observed similar increases in serum immunoglobulin IgG, IgA and IgM in non-immune Caucasians exposed to malaria for the first time. Tobie and his co-workers in carefully controlled experiments infected 12 human volunteers with Plasmodium vivax and 5 volunteers with Plasmodium cynomolgi, and serial estimations of the serum immunoglobulins were made during and after the primary malarial attack and in one case after relapse. Results showed that large amounts of IgM globulin were produced in all subjects. Twenty-six days after infection with P.vivax and P.cyanomolgi the serum IgM concentrations reached its maximum of 560 mg/100 ml from a pre-infection value of 113 mg/100 ml. Both serum IgG and IgA increased by 42% and 55% respectively after malarial infection. The present findings further substantiate the role of malaria in hypergammaglobulinaemia observed in populations living in malarious endemic regions.

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Caucasians with malaria serum immunoglobulins and malaria antibodies

Malaria antibodies were present in 86% of patients tested and the levels correlated with the age of infection. Malarial IgM antibodies were detected in the majority of cases, but in very low titres $<$1:320 by IFA and less than 0.50 by ELISA. There was a highly significant correlation between serum IgG and IgM with malarial antibodies ($P < 0.002$) and a significant correlation ($P < 0.05$) was also observed between serum IgA and malarial antibodies. A significant correlation at the 1% level was observed between serum IgA and high malarial antibody levels. The study by Tobie et al (1966) also showed a direct correlation between rise of malarial fluorescent antibody production and the increased serum immunoglobulin levels. Also malarial antibody activity has been shown to be present in all 3 major immunoglobulin classes (Voller, 1974; McGregor, 1975). Abele et al (1965) followed the course of malaria antibody production in 10 hospitalised human volunteers infected with P.vivax and P.cyanomolgi and observed an impressive rise in IgM malarial antibodies later followed by an increase in the IgG after repeated malarial stimulation. These observations indicate that the high initial IgM was due to the primary response whereas the subsequent rise of the IgG globulin was due to the secondary immune response.

The above observation infers that populations living in an endemic malarial area are subjected to repeated malarial infection and therefore exhibit an immune response characteristic of the secondary immune response, thus the fact that IgM malarial antibodies were not detectable in serum from Ghanaian children might indicate a primary intra-uterine infection with malaria and therefore neonates are born already primed to malaria stimulation, and therefore produce predominantly IgG malaria antibody during the course of repeated infections.

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The overall implications of the present findings Although the present findings implicate malaria in the high serum immunoglobulin levels in Ghana, given the polyparasitic African environment, the possibility exists that other parasitic infections may have contributed to the raised levels observed. Viruses (Rubin et al, 1971), bacteria (McKelvey and Fahey, 1965; Malomo et al, 1970), protozoa (Fahey, 1965; McKelvey and Fahey, 1965; Michaux et al, 1966; Dunn, 1972; Bolton, 1972), helminthes (Michaux, 1966; Crandall et al, 1967; Atunes et al, 1971; Bassily et al, 1972) have all been implicated in raised levels of immunoglobulins in the tropics. Indeed malnutrition in African children, which is often a side effect of some of the above listed infectious diseases, is known to produce direct immunologic effects which affect immunoglobulin synthesis. Also the singular effects of malnutrition on humoral and cellular immune responses (Douglas and Faulk, 1977; McFarlane et al, 1977) have been documented. It is therefore obvious that a complex nature of factors work together in the tropics and contribute to the raised immunoglobulin levels. However the epidemiological evidence suggests that the role of malaria on raised IgM and IgG in Ghanaian sera is more conclusive.

Hypergammaglobulinaemia as a result of malaria is only one of the many serological abnormalities associated with malaria infections. The succeeding chapters examine the role of other immunological parameters; complement; immune complexes and autoantibodies in the exaggerated disturbed immunological responses seen in malaria.