IMMUNOSEROLOGY OF MALARIA

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SUMMARY

This literature review discusses the most frequently used serodiagnostic methods for the determination of the humoral immune response to malarial parasites. The importance of malaria as a global public health problem is stressed in the light of the new discoveries leading to the future development of an anti-malarial vaccine suitable for use in humans. Serological techniques are expected to play an important role in the assessment of the relative efficacy of these candidate vaccines. A discussion of the different antigen preparation techniques is also presented.

KEY WORDS: — Malaria — Serodiagnostic methods — Humoral immune response — Antigen preparation techniques.

Malaria, the parasites, and the disease. Human malaria is caused by infection with protozoans (Apicomplexa, Sporozoa) of the genus **Plasmodium.** Four species are known to infect man. **P. falciparum** is the most virulent species producing, in many cases, infections that lead to death. Frequently, the term "malignant" is used when clinicians refer to the malaria caused by this species. The other species, P. **vivax, P. ovale,** and **P. malariae,** have a more worldwide distribution and usually cause less severe infections. With the exception of **P. malariae,** human malarial parasites have a schizogonous cycle ranging between 46 and 50 hours. **P. malariae** has a longer cycle, usually 72 hours (CAMPBELL and CHIN, 1981).

The life cycle of these species includes both asexual and sexual phases. In the asexual phase, which occurs in the vertebrate host, slender, spindle-shaped sporozoites inoculated through the bite of an infected **Anopheles** mosquito (Diptera, Culicidae) develop into pre-erythrocytic stages in the parenchymal cells of the liver.

Upon completion of this stage, merozoites are released and penetrate red blood cells. There follows several rounds of erythrocytic stages through schizogony with subsequent increase of the number of infected cells in circulation. Some of the ring forms develop into gametocytes of either sex. When the susceptible mosquito takes its blood meal from the infected host, gametocytes are ingested, initiating the sexual cycle. Fertilization of the female gamete by the male takes place in the insect's stomach producing an oocyst, which upon development releases a large number of sporozoites. After maturation, these sporozoites migrate to the salivary glands and are eventually inoculated into another susceptible host, thus completing the cycle.

The pattern of cyclic fever coinciding with the termination of each schizogonic cycle is a major clinical sign which has been historically associated with the disease. Early signs and symptoms, however, may be nonspecific — with fever occurring in no particular pattern, chills

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ranging in intensity from mild-to-violent shuddering, headache, myalgia, and arthralgia — making up a set of signs and symptoms that resembles those caused by viral and some bacterial infections (CAMPBELL and CHIN, 1981).

Other malaria parasites occur in several orders of vertebrates. Approximately 100 different species have been described from reptiles, birds, bats, rodents, ungulates, and simians. Some simian **Plasmodium** species are infective to man, and studies have been carried out because of the possibility of simian zoonotic malaria acting as an additional reservoir for human infection (KALRA, 1980).

The global nature of malaria. Malaria is probably the number one parasitic disease affecting humankind. The morbidity and mortality associated with the disease represent a tremendous threat to public health programs in developing countries (WYLER, 1983).

Most of the significant advances in the biology, chemotherapy, and control of malaria have unfortunately emerged only as a consequence of the two major world wars. During World War II and immediately thereafter, the scientific literature was enriched by almost as many publications about malaria as had been accumulated in the preceeding century. Additional and more effective insecticides, and suppressive and curative drugs all emerged during intense basic and applied research programs worldwide (Most, 1974). Based on these technological advances the World Health Organization (WHO) in collaboration with the U.S. Agency for International Development (USAID) established in the 1950s an aggressive program to eradicate human malaria from the world. Considerable success had been attained in many of the developed countries earlier, where autochthonous malaria was completely eradicated. The joint program had initial sucess in many countries, but its major ambition, worldwide eradication, was not fulfilled due to a combination of problems, many of which plague most of the developing nations, especially in the tropics. Additionally, vectors and parasite strains genetically resistant to insecticides and drugs were gradually selected.

Operational factors played an important role in retarding the effectiveness of the program. Lack of adequate community health services, scarcity of trained professionals, and administrative and political obstacles have contributed to failure of malarial control measures in developing tropical countries.

In the past 15 years many countries have witnessed almost complete failure of malaria control programs. The resurgence of the disease has affected nations in southern Asia and Latin America, not taking into account the highly endemic areas of Africa where precise figures have always been difficult to obtain (Anonymous, 1978). It is estimated that the incidence of malaria on a worldwide basis now reaches 150 million cases annually. Malaria prevails in most of tropical Africa where approximately one million children die annually from this disease (SPEER and SILVERMAN, 1979). These figures are higher than for any other parasitic disease. Considering humanitarian, social, and economic values, malaria probably remains the most important public health problem in the world today.

One of the major reasons for resurgence of malaria has been negligence. During the mid 1960s several countries relaxed control measures based on the assumption that residual cases did not deserve sustained effort and eradication would eventually be attained by general health services. Financial allocations were sharply reduced, and malaria task forces were fused with other general health programs. The increase in morbidity due to malaria and the problems that ensued have led to a reawakening of interest in the disease as we see it today (WERNSDORFER, 1979).

Due to renewed interest in the disease, in the mid 1970s WHO activated a special program for research and training in tropical diseases. This organization established as a primary goal an aggressive attack on six of the major neglected diseases of mankind in which malaria has been included. Special working groups in malaria research were originally established for three different areas: chemotherapy (CHEMAL), immunology (IMMAL), and applied field research (FIELDMAL). The targets of the strategy utilized by these programs are defined as: (i) reduction of malaria mortality to negligible levels, (ii) alleviation of the effects of the disease on socio-economic development, and, ultimately, (iii) eradication of malaria whenever feasible (Anonymous, 1978).

The immune response to malarial parasites. There is currently a resurgence of interest in the immunology of malaria for several reasons. The development of an anti-malarial vaccine appears as a viable proposition. An increased understanding of the role of the host's response in acting synergistically with anti-malarial drugs has led scientists to new chemotherapeutic approaches (JAYAWARDENA, 1981). In addition, insights into the role of immunity in host-parasite relationships and even host-tumor interactions may be gained by studying the immune response against malaria (JAYAWARDENA, 1981).

The complexity of the immune response to malaria is not surprising if we consider the complex life cycle of the parasite and its mode of existence within the vertebrate host. The magnitude and effectiveness of the immune response depends on the extent to which the antigenic make-up of the parasite triggers individual responses to individual antigens. The final outcome of such a chain of events within the host is a net balance between various single manifestations of the immune response, all of which may not be fully effective. An effective immune response that leads to a self-limiting infection is frequently seen in natural infections. For instance, **P. knowlesi** infection in rhesus monkeys — an unnatural host — is frequently fatal; this, in effect, eliminates both host and parasite. In its natural host, the kra monkey, however, **P. knowlesi** induces a level of immunity that allows long-lasting coexistence of both host and parasite (JAYAWARDENA, 1981; RUITENBERG and BUYS, 1980).

Extensive research in the last decade has led to the current view that both antibody and cell mediated immunity occur in the response to malarial infection. In 1969 Cohen et al. showed that immune serum inhibited the development of malarial parasites by acting on free merozoites rather than on the intraerythrocytic forms. This experimental approach has been refined by other authors aiming at assaying specific immunity mediated by antibody (CAMPBELL et al., 1979; CHULAY et al., 1981; EPSTEIN et al., 1981; REESE and MOTYL, 1979). Protection against infection in vivo can also be obtained by passive transfer of anti-malarial immune serum (Speer and Silverman, 1979). Other experimental evidence for the role of B cells include the intensification of P.

yoelii infection in animals with B cell activity suppressed by anti-IgM, and the induction of fatal **P. gallinaceum** infections (an otherwise fairly innocuous parasite) in bursectomized chickens (PLAYFAIR, 1978). The inhibition of erythrocyte invasion by merozoites has been shown to be mediated by species specific IgG and/or IgM antibodies; this interaction seems not to be complement dependent, since $F(ab)_2$ fragments of specific IgG also effect inhibition (COHEN, 1979).

The level of protection conferred by anti-merozoite serum is, nonetheless, not well correlated with its in vitro titer (Cohen, 1979). This is not surprising since the net balance of anti-malarial immunity may also involve cell-mediated immunity. Development of protective immunity to malaria is clearly thymus dependent. The triad of evidences supporting this view has been provided by JAYAWARDENA (1981): (i) T cell-deficient animals lack effective responsiveness to malaria, (ii) T cell activation plays an important role in development of immunity, and (iii) transfer of immunity from immune to nonimmune animals can be accomplished by T lymphocytes in a syngeneic system. The firs direct evidence that the thymus played a role in controlling malarial infections came from the demonstration that P. berghei infections were significantly more severe in rats that were thymectomized at birth than in intact controls (BROWN et al., 1968; STECHSCHULTE, 1969). The same phenomenon is observed in congenitally athymic mice (COHEN, 1979). Additionally, it was later demonstrated that reconstitution with T cells restores the capacity of such animals to respond to infection (SPITALSNY et al., 1977). Whereas there is little doubt that T cells are essential for generating an effective anti-malarial immune response, very little is known of the manner by which these cells contribute to the development of the protective response. Close examination of the intensity of infections (**P. yoelii-mice**) in animals of different major histocompatibility complex (MHC) haplotypes has revealed no association between H-2 (mouse MHC) and the outcome of the malarial infection (JAYAWARDENA, 1981). This observation, however, does not rule on the possible role of MHC in the anti-**Plasmodium** immune response because the complexity of the response is always a product of both immunological and nonimmunological me-

chanisms which could ultimately mask some form of MHC-linked control.

Recently, a new mechanism has been proposed to explain the role of effector immune cells in anti-malarial immunity (ALLISON and EUGUI, 1982). The model proposed is based on the release of superoxide anion by macrophages and natural killer cells upon binding to parasitized red cells. This oxidant stress leads to degeneration of parasites within erythrocytes. Another macrophage-derived mediator acting under oxidized conditions is tumor-necrosis factor. This enzyme-like substance has been shown to inhibit multiplication of parasites in vitro (ALLISON and EUGUI, 1982).

The only stages of the parasite that are immediately within reach of circulating antibodies are the sporozoite and the merozoite. The other forms — rings, trophozoites, schizonts, and the intra-hepatocytic stages — are under various degrees of protection by cell membranes which are generally impermeable to antibodies. Gametocytes, on the other hand, are sexually specialized stages which may also be potential targets for humoral antibodies. It has been both empirically and experimentally shown that the extra-cellular stages are potential sources of immunogenic preparations for vaccine use.

As discussed above, the effect of anti-merozoite antibodies in serum is inhibitory to erythrocyte invasion. It, therefore, became apparent that merozoites may be susceptible targets in order to achieve protection of the host by either active or passive immunization (FALANGA et al., 1982). The use of such parasite stages in preparation of vaccines is expected to induce protective immunity that prevents erythrocyte infection. Despite difficulty in achieving effective long-lasting protection with malarial vaccines, some successful short-term immunization protocols have been presented (COHEN, 1979; COHEN et al., 1977; SPEER and SILVERMAN, 1979; SPEER et al., 1977).

A different immunization approach that has also been the subject of intensive research is the use of sporozoite-derived vaccines. Effective immunization against sporozoites is believed to confer protection by the elimination of the parasites at the very moment of entrance into the host's body (MURPHY and LEFFORD, 1979; SPEER and SILVERMAN, 1979; SPEER et al.,

1977). One major assumption of this approach is that the vaccinated host will be able to neutralize 100% of the invading sporozoites or else will become infected, since protection is not conferred to the other stages.

The third possible target in the parasite cycle is the gametocyte. The feasibility of immunizing against gametes for blocking mosquito transmission has been recently reviewed (SPEER et al., 1977). In the typical model (**P. gallinaceum**-chicken) specific IgG antibodies in the immunized animals block fertilization in the insect midgut by immobilizing microgametocytes. Although this approach of immunization does not protect the initial host against infection, since asexual multiplication can still occur (SPEER and SILVERMAN, 1979), this type of vaccine is gaining attention because of its possible use as a control measure in blocking transmission.

Information in the literature has been fragmentary regarding the most appropriate protocols for vaccine administration. Major objectives in experimentation with candidate vaccines have been compiled by SPEER et al. (1977). Ideally, a malaria vaccine should be: (i) capable of providing long-lasting protection against the species in question; (ii) free of host cell contamination to avoid possible autoimmune responses; (iii) storable for reasonably long periods without losing potency; (iv) free of microbial contaminants; (v) easily mass-produced and transported by conventional methods; and (vi) free of undesirable side effects that could potentially outweigh its protective benefits.

Recent studies have indicated the feasibility of vaccination with peptides from sporozoites of different species of **Plasmodium**. These peptides have been show to be the target antigens of protective immunity. Genetic expression of cDNA encoding these peptides has been successfully obtained in bacteria using suitable vectors. Libraries of genes encoding peptide fragments of both erythrocytic and sporozoite parasite stages have been established. Use of biosynthetic antigens from malarial parasites represent a great advantage over extracted antigens. Genetically engineered immunogens can be obtained in large quantities and are relatively free of contamination with host substances.

Cultivation. The bulk of the significant studies on vaccination against human and simian malarial parasites has been contributed since 1976. In that year Trager and Jensen published the first successful method for continuous cultivation of erythrocytic stages of **P. falciparum.** Since then, the Trager-Jensen technique has been used to cultivate strains of **P. falciparum** from different geographical areas around the world.

Parasites harvested from continuous culture provide a ready source of material for a variety of immunization protocols. Parasites from continuous culture can also be used as reagents for serological tests, thus abrogating the need for maintaining colonies of the valuable **Aotus** monkeys to be used as the source of infected blood.

Basically, cultivation of **P. falciparum** is achieved by the use of a settled layer of human red cells in RPMI 1640 culture medium that has been supplemented with HEPES buffer, a gas phase of 2 to 3% CO_2 and low oxygen tension, and provision for a periodical change (24 hr) or a slow flow of medium over the layer of erythrocytes (TRAGER, 1980). Despite the various minor technical variants that have been successfully used in different laboratories, continuous cultivation is normally performed through two major methods: the continuous flow (TRAGER, 1979) and the candle jar (JENSEN and TRAGER, 1977) techniques.

In addition to **P. falciparum** certain simian malarias have been adapted to continuous culture (CHIN et al., 1979; NGUYEN-DINH et al., 1980), but there has been little or no success in cultivating the other three species of human malarial parasites. The literature has two accounts of presumably successfull attempts in the cultivation of **P. malariae** in conjunction with **P. falciparum** (CHOWDHURI et al., 1980) and **P. vivax** alone (LARROUY et al., 1981). Unpublished data from different laboratories have shown that these two species can undergo maturation in vitro for only a few schizogonic cycles with decreasing numbers of viable parasites during the period in culture.

Antigen preparations. Various methods have been reported for the extraction and isolation of malarial antigens from parasites maintrained in culture or from infected blood of animals. KREIER (1977) has reviewed the existing methods for isolation and fractionation of malarial parasite-infected cells. He divided the most popular techniques into groups based on the principle of release of parasites from host cells. Kreier's classification categories include hypotonic lysis; freezing and thawing; lysis with saponin, ammonium chloride, antiserum and complement; sudden decreases in pressure; ultrasound; and natural release. Each of these methods has intrinsic advantages and disadvantages. Kreier found that continuous flow sonication combined with natural release of cultured schizonts provide intact free parasites of all the erythrocyte developmental stages. The major disadvantage of continuous flow sonication is cost of the ultrasound cell disruptor which limits its application to those laboratories most generously funded.

The techniques mentioned above were designed to be used with suspensions of infected cells of different stages of purification and enrichment. Depending on the concentration of infected cells and/or the relative abundance of a particular parasite stage, it may be necessary to treat the infected blood prior to release of parasites, to enrich the preparation and to isolate fractions containing, primarily, stages that are more relevant to the experiment. This is especially true of **P. falciparum** which in culture loses synchronicity within a few cycles even if the original inoculum contained parasites of only a single developmental stage. The Ficoll gradient centrifugation, PlasmagelTM, and gelatin methods are the most used to concentrate and isolate blood stage parasites. The former has been used to extract ring forms and to harvest merozoites from preparations but it is difficult to execute. The other two methods (JENSEN, 1979; REESE et al., 1979) are based on the principle of differential settling of parasitized red cells in a buffered solution of gelatin. Non-parasitized red cells and those infected with ring forms tend to aggregate and stack in rouleaux formations that sediment rapidly. Erythrocytes infected with more mature stages are usually deformed — losing their biconcave shape — and do not stack to form aggregates. Therefore, they remain in suspension due to the high viscosity of the gelatin solution. The advantage of the Plasmagel solution is its ready availability as a commercial plasma expander for clinical use (3% partially degraded gelatin in physiological saline). Another desirable characteristic of the Plasmagel preparation is its

resistance to gelation when stored at 4°C, as happens with gelatin solutions.

Another frequently used method for purifying ring forms and young trophozoites is the sorbitol technique (MREMA et al., 1979). This compound destroys schizonts and mature trophozoites, yet is innocuous to younger forms. Althrough the sorbitol technique is not an enrichment method, it can be used alone or in conjunction with one of the gelatin techniques to obtain inocula to start synchronized cultures of **P. falciparum.** By the use of such homogeneous cultures, it is possible to obtain relatively pure suspensions of a single stage by harvesting at appropriate intervals after the inoculation of the culture. Unlike the infection in human or simian hosts, however, the parasite population tends to gradually lose synchronicity in the in vitro system. Therefore, harvest of parasite stages must be done during the cycles immediately after inoculation with the ring forms.

BILLIAULT and AMBROISE THOMAS (1980) devised a technique for isolating **P. falciparum** merozoites from cultivated parasites bound to concanavalin A. This technique may prove to be very useful for immunological studies. It is based on the principle that infected cells (and normal red cells) bind and become attached to concanavalin A molecules that have been covalently linked to Sepharose 4B beads in a column-packed assembly. Because parasite (merozoite) membranes lack the carbohydrate residues that bind to the lectin, they are not retained in the column, and thus are collected in the effluent. The merozoites are released by natural rupture of the mature schizonts and exit the column with the void phase with minimal contamination.

A major shortcoming of almost all isolation techniques has been contamination with host material. Red cell stromata and other host substances — at best in trace amounts — are frequently harvested with the parasite antigen phase. Even when using a relatively mild isolation technique such as the saponin method, free parasite suspensions will invariably contain host cell derived material, while presenting apparently acceptable cleanliness by microscopical inspection. These host derived antigens may mask the immune response in vaccination studies and may even lead to autoimmune reactions. In serological reactions these host antigens may decrease sensitivity of the assay and may be a cause for nonspecific reactions.

With the advent of recombinant DNA techniques biosynthetically- produced plasmodial antigens have been successfully expressed in bacterial hosts. These genetically engineered peptides have been obtained by initial characterization of the amino acid sequence and reverse translation of $_m$RNA segments into $_c$DNA which is in turn transferred to bacterial hosts via a suitable vector (ANONYMOUS, 1982). Antigens produced in this manner represent an obvious advantage in terms of their cleanliness (with respect to host-derived substances) and availability in large quantities. It is expected that the techniques currently used to produce these peptides will be improved from the present state-of-the-art. This will allow the production of genomic libraries encoding larger antigenic proteins. As a derived benefit from the use of genetically translated antigens is the possibility of using nucleic acid probes for detection of circulating malarial antigens (FRANZEN et al., 1984). These probes can hybridize with the correspondent parasite peptides present in the plasma of infected persons, thus allowing detection of infection.

Serodiagnostic techniques. Serological techniques as applied to a variety of diseases serve three main purposes: (i) to detect the presence of current infection, (ii) to detect past experience with the parasite, and (iii) to assess levels of protective immunity. The contribution of serological methods for the study of malaria has been limited to the second of the above purposes in the form of seroepidemiological surveys. Although serological techniques such as the indirect immunofluorescence (IIF) test can aid in presumptive diagnosis of current malarial infection, definitive diagnosis can only be made by demonstrating the parasite in smears of peripheral blood. In addition, detection of protective immunity has only been achieved through the use of antibody-mediated bioassays of inhibition of in vitro growth of parasites (CAMPBELL et al., 1979); CHULAY et al., 1981; REESE and MOTYL, 1979).

Serodiagnostic methods have attained an importance second only to the more classical technique of examining stained blood slides in the diagnosis and epidemiology of malaria. In fact, serological testing has provided most of

our present knowledge on the humoral immune response to malaria.

Humoral response plays an important role in anti-malarial immunity. This role can be better exemplified by observing the course of infection in a nonimmune person traveling to a hyperendemic malarious area as compared to that in an immune native. The former will have high parasitemia and severe clinical symptoms which, especially in untreated **P. falciparum**, may prove lethal, whereas the native will pro- bably have low intermitent parasitemias with mild clinical symptoms. It is unfortunate, ho- wever, that despite this strong evidence for the role of protective antibodies, our state of the art in immunology of malaria can only empiri- cally provide clues about individual levels of immunity.

Ideally, serodiagnostic tests should provide the same information as that obtained in the diagnosis of viral infections such as rubella and measles, where antibody titers often indicate the presence of effective immunity.   In the case of malaria and many other diseases caused by eukaryotic organisms, antibody response is complex and, expectedly, represents a combina- tion of monoclonal responses against a large variety of antigens of differing degrees of re- levance to the welfare of the parasite. Ideally, for estimation of the immune state, only protec- tive antibodies should be detected. These are the antibodies that compromise the survival of the parasite, presumably, by combining with, and inactivating some essential antigen com- ponent of the cell.

The only available tests for evaluation of protective immunity are bioassays based on antibody mediated inhibition of parasite growth in vitro. Such tests were originally proposed (MITCHELL et al., 1976; WILSON and PHILLIPS, 1976) as empirical approaches for detection of anti-plasmodial antibody responses that could impair maturation of **P. faciparum** in vitro. With the advent of continuous culture techniques for this parasite species those tests were also used to detect antibody mediated inhibition of merozoite invasion of erythrocytes (BROWN et al., 1982; CHULAY et al., 1981; EPSTEIN et al., 1981; REESE and MOTYL, 1979; VERNES et al., 1984).

Although not correlating with protective immunity, the serological techniques that have been more or less successfully applied to the detection of antimalarial antibodies in human sera are the indirect hemaggluttination test (IHA), the indirect immunofluorescence (IIF) test (also designated as indirect fluorescent antibody test: IFA), and the enzyme linked immunosorbent assay (ELISA) (also as enzyme immunoassay: EIA). The latter two tests are considered to be immunoassays since they employ second antibodies (usually in labeled form) as an indicator system. The IHA test expresses antigen-antibody binding directly by increased adhesiveness of the coated cells which leads to visually detected agglutination.

The IHA test has been used extensively for seroepidemiological purposes (KAGAN et al., 1969; VOLLER and DE SAVIGNY, 1981). Ori- ginally, **P. berghei** antigen was used in the test for the detection of antibodies in homologous antisera (DESOWITZ and STEIN, 1962). In the initial system there was already an attempt to provide long lasting reagents by the use of formol and tannic acid-treated sheep erythro- cytes (STEIN and DESOWITZ, 1964). Results with this early system were reviewed by KAGAN et al. (1969). Various problems in executing the test were soon realized, such as lack of repro- ducibility due to variations in reagent batches, autoagglutination of cells, and nonspecific agglu- tination factors in sera. Introduction of techni- cal variants gradually improved the test. These included the use of antigens from simian pa- rasites (LUNDE and POWERS, 1976; WILSON et al., 1975; WILSON et al., 1971) or even better antigens from human malarial parasites (MEUWISSEN et al., 1974; VOLLER et al., 1974) that had been grown in AOTUS monkeys or extracted from infected human placentas. Other refinements included the use of lyophilized cells, double aldehyde fixation, and serum adsorption with red cells coated with extracts of cells from noninfected monkeys to reduce nonspe- cific reactions (DRAPER and AKOOD, 1983). In two comprehensive studies comparing the IHA and the IIF tests (1971), and later the complement fixation (CF) test to both the IIF and IHA tests (1975), WILSON et al. demons- trated that the latter tests were of superior sensitivity as compared to the CF test; these comparisons were made using sera from U.S veterans from Vietnam.

MEUWISSEN et al. (1974), testing sera from an endemic area in Africa, showed that

the IHA yielded twice as many nonreactors as the IIF. A major pitfall of the IHA test was pointed out by VOLLER et al. (1974). The test was defective in revealing antibody patterns in children living in endemic areas. The Authors expressed fear that surveys based solely on this test could yield misleading results by underestimating the number of infected young children. One possible explanation for this lack of sensitivity is that the antibodies produced in the child's response to the infection would be of lower affinity, although this has not been proven experimentally.

The IHA test has always been plagued by a number of seemingly insurmountable problems. Despite its simplicity of execution, the test is primitive in both interpretation and performance. As does the CF test, which was purposely omitted in this discussion, the IHA test uses reagents of animal origin (erythrocytes and diluent serum) and, thus, is difficult to standardize. In addition, this test, as well as others of earlier generations in serology, is interpreted subjectively and is not easily automated. Possibly the IHA test will eventually be replaced by the ELISA test which as the former, uses soluble antigens and is rapidly executed but, unlike the IHA, can be interpreted objectively.

The IIF test was used originally for direct diagnosis of malaria by detecting parasites in blood slides (BROOKE et al., 1959). The test as it is known today was first used by KUVIN et al. (1962). The early history of the IIF test has been presented in a review by SULZER and WILSON (1971). An initial difficulty was presented by antibodies in the antigen donor's plasma that could react autologously with the antigen. To avoid autologous reaction between the donor's antibodies in the serum and the antigen preparation, parasitized blood had to be drawn from donors before appearance of antibodies (SODEMAN and JEFFERY, 1966). This reduced the number of blood smear donors available. Gamma globulin in the donor's serum often coated the slide producing background staining with the anti-species conjugate and consuming a fraction of its activity. This secondary staining due to the conjugate reaction with the plasma globulin in the preparation was prevented by a technical variant proposed by COLLINS and SKINNER (1972). They used dilute hydrochloric acid to lyse erythrocytes,

fix the antigen, and remove the donor's plasma from the slides prior to reacting with serum. Although this procedure seems to provide good results in eliminating the background reaction, it may also remove or inactivate some acid-sensitive antigenic determinants from the preparation which reduces test sensitivity. It seems, however, that the acid-lysis technique has been largely accepted.

A method to circumvent the problem of background nonspecific reactions was provided by SULZER and WILSON (1967) and SULZER et al. (1969). Their approach was strikingly simple. Since only the parasitized red cells are required in the test, plasma proteins can be removed by washing the cells in buffered saline in a few centrifugation cycles. Any antibody in the antigen donor's serum is removed before it can react with the antigen. Since the presence of specific antibody in the donor's serum is no longer of any concern, this procedure increases the number of potential donors of parasitized blood for the test. Because nonspecific staining was no longer a problem, thick smears could be made, which effectively concentrated the parasites and thus reduced time required to read tests. Another derived benefit was the possibility of using blood samples with low parasitemia which would otherwise be unsuitable for the thin smear technique.

Another phenomenon that was soon noticed by serologists was the variation in results obtained when using different antigen species. Reactions were more intense and to greater titer in homologous systems, i.e., when using antigens derived from the same malarial species that caused the infection in the donor of the serum being tested (GLEASON et al., 1971; SALATA et al., 1981; SULZER and WILSON, 1971; SULZER et al., 1973; VOLLER, 1977). This led to a number of variants according to the antigen species used in the test. For many laboratories, availability of malarial parasites from either simian or human hosts is an acute problem and, hence, many resorted to the use of rodent malarias such as **P. berghei** which can be easily maintained through passages in mice or rats (MAIER and PIEKARSKI, 1979; SALATA et al., 1981), or even avian malaria parasites such as **P. gallinaceum** (GIACOMETTI and SUTER-KOPP, 1972; KIELMAN and WEISS, 1968). Simian-host malarial parasites seem to provide the most satisfactory reagent since

cross reactivity between simian and human parasites is usually much higher than that of human compared to rodent or avian malarial parasites. **P. knowlesi** has been the preferred simian parasite when none of the human malarial antigen preparations is available (CHANDANARI et al., 1981; COLLINS et al., 1966). With the increasing scarcity of non-human primates for biomedical purposes, very few laboratories in the world can use entirely homologous antigen sets in their tests. This problem led one laboratory to propose the creation of a malarial antigen distribution center to supply IIF slides of all four human malarias on a regular basis to serology laboratories devoted to the diagnosis of malaria (SULZER, 1975).

A more suitble source of variability in the IIF test (and also other tests) has been represented by the relative abundance of the different erythrocytic stages of the parasite that are present in the antigen. Earlier studies had mentioned the greater reactivity of more mature stages of the parasite with most human sera (SODEMAN and JEFFERY, 1966; SULZER et al., 1969). In a more detailed study, TARGETT (1970) corroborated these observations by using an elegant protocol that compared **P. falciparum** antigen "in natura" as obtained from the infected blood against a portion of the same antigen that had been matured in vitro to the point of greater abundance of schizonts. Titers were dramatically higher when using the matured antigen. Targett's choice of **P. falciparum** as antigen in the study highlighted the problem of the scarcity of schizonts in the peripheral blood of infected hosts. It has been known for many years that schizonts of **P. falciparum** sequester in deep tissues prior to schizogony, therefore early trophozoites are the stage most commonly seen in the blood. The importance of using a matured homologous antigen system for diagnosis of malaria, although dramatic, has been taken into consideration by only a few investigators (AMBROISE-THOMAS et al., 1974). Paradoxical observations in the early literature — such as the fact that heterologous reactions may be greater than homologous reactions in simian malarias (COLLINS et al., 1965) — may have been due to lack of equal abundance of more mature stages in the antigens utilized. As a result of their concern with the problem, SULZER and LATORRE (1977) developed a method for the maturation of parasites in blood

which is applicable to all four human malarial species. This method, further evaluated by FRANCO and SULZER (1983), enables serologists to prepare optimally matured antigens for evaluating infections at the species level on the assumption that, once differences in stage reactivity among the four antigens are abolished, only species-specific antibodies would play a role in causing differences among titers. More recently, **P. falciparum** antigens obtained from in vitro cultures have been used in the IIF or other tests (GUPTA et al., 1981; HALL et al., 1978). It is unfortunate, however, that continuous cultivation in vitro is not possible for the other three species of human malarial parasites which would obviate the need to maintain parasites in nonhuman primates or to depend on infected human subjects as a source of antigen preparations.

The ELISA technique was originally introduced as an alternative to radioimmunoassay (ENGVALL and PERLMANN, 1971) and was later adapted to the serology of malaria in 1974 by VOLLER et al. Since its introduction this test has acquired importance in the seroepidemiology and immunology of malaria (TANDOM et al., 1982). The great advantage of the ELISA technique is its short execution time which makes it a convenient test in assaying large numbers of specimens simultaneously. Of the various tests described for malaria serology the ELISA is the one most likely to supplant the IIF test due to the comparable sensitivity and specificity that this test exhibits to the latter (DRAPER and AKOOD, 1983). The principle governing the degree of response in the ELISA is analogous in design to the IIF test since both are immunoassays (serological tests that use a second antibody). Nevertheless, a great deal of commonality also exists between the ELISA and the IHA since both tests are based on selective binding of antibodies to soluble antigens of the parasite.

A crude extract of **P. knowlesi**-infected blood from monkeys was used in the first studies on ELISA in malaria diagnosis (VOLLER et al., 1975). Since then, a variety of antigens from several host systems has been used. The more successful combinations include sonicated **P. falciparum** extracts from matured (VOLLER et al., 1974) **Aotus** blood (BIDWELL and VOLLER, 1981; QUAKYI, 1980; VOLLER et al., 1980), sonicated **P. falciparum** extracts from

continuous cultures (SPENCER et al., 1979; SPENCER et al., 1979) and from infected pla-centas (QUAKYI, 1980), and the originally pro-posed system of sonicated P. knowlesi antigen from infected blood of rhesus monkeys (TAN-DOM et al., 1982; VOLLER et al., 1975).

At present it is not wise to draw conclu-sions about any particular behavior of the ELISA as compared to the IIF test regarding sensitivity and specificity. Because enzyme im-munoassays in general represent a relatively novel approach in serology, several problems that are intrinsic to the ELISA test must be solved, such as purification of antigen, and standardization of solid phases and antibody-enzyme conjugation. Problems that have arisen through the use of the ELISA are lack of sen-sitivity and/or specificity (particularly in test-ing specimens from younger age groups), and poor reproducibility (DRAPER and AKOOD, 1983). Despite these shortcomings, ELISA is the only diagnostic system besides radioimmu-noassay that can objectively measure antibody activity in specimens, since there is a conti-nuum o results provided by colorimetric read-ing. This represents a great analytical advantage as compared to tests based on titers which are geometrically spaced, e.g., IIF and IHA. This characteristic of enzyme immunoassays makes them potentially useful tools for all areas of malaria immunology, particularly in the recen-tly expanded field of hybridoma technoloy.

Use of hybridoma-derived monoclonal anti-bodies is likely to bring new perspectives in serological testing. These monospecific antibo-dies bind to a single antigenic moiety and, hence, through their use one is able to distin-guish between background reactivity in a given assay and genuine antigen sharing among para-sites (MITCHELL et al., 1979).

PROCEDURES

In vitro cultivation of P. falciparum. For the cultivation of **P. falciparum** in human ery-throcytes the Jensen-Trager candler jar method is used. Parasites are cultivated as 10% infec-ted-cell suspensions in RPMI 1640 medium (GIBCO Laboratories, Grand Island, NY) sup-plemented with 10% group O, Rh+ human se-rum, 30 mM HEPES buffer, and 0.6 M NaHCO$_3$. Cultures are usually started as 0.5 ml suspen-sions in 24-well flat bottom plastic plates. The

medium is changed daily and thin smears are prepared and stained with Giemsa for the eva-luation of the percentages of infected cells and determination of parasite stages. When appro-ximately 4-5% of the erythrocytes are parasitiz-ed, cultures are diluted 4-5 fold by the addition of washed noninfected human erythrocytes as a 10% suspension in the above medium. For larger culture suspension volumes either 5 or 10 ml capacity Petri dishes are used instead of the multi-well plates. Cultures are incubated at 37°C in gas-tight jars under reduced O$_2$ ten-sion and elevated CO$_2$ concentration as in the original candle flame extinction procedure.

Enrichment of infected-cell suspensions. Suspensions of infected erythrocytes obtained from in vitro culture are aseptically made in either gelatin or Plasmagel to allow separation of red cells infected with older trophozoites and schizonts from noninfected and ring-con-taining cells. Basically, culture-derived cells are washed once in culture medium by centri-fugation to remove cell debris and resuspended again in fresh medium as a 25% suspension. An equal volume of prewarmed (37° C) gelatin (Sigma Chemical Co., St. Louis, MO) or Plas-magel (HTI Corporation, Buffalo, NY) solu-tion is added. The suspension is gently, but throughly mixed and incubated at 37°C in a water bath for 30 min. Tubes used for this in-cubation step are never filled with more than the volume of suspension necessary to provide a fluid height of 10 cm. Because the efficacy of the procedure is based on differences between settling speeds of noninfected (and ring-infect-ed) and older stage-infected cells height, and not volume, of fluid is a critical variable in the procedure. Following incubation, tubes are gen-tly removed from the water bath and the upper phases of the suspensions are transferred with a Pasteur pipette to a separate tube (taking care not to disturb the sedimented erythrocy-tes). Cells from this top layer are sedimented by centrifugation at 1,000 x G for 10 min and resuspended in an equal volume of fresh cold medium. For the evaluation of the percentage of infected red cells, smears are made and stained for microscopical examination. The bottom layers remaining in the original incu-bation tubes can be either discarded or frozen in liquid N$_2$ following the addition of 10% di-methyl sulfoxide (DMSO) (Baker Chemical Co., Phillipsburg, NJ). These are retained as para type stock cultures for further reference.

Preparation of antigen slides of P. falciparum. **P. falciparum** infected-cell thick smears are made in multi-well glass slides for use with the IIF test as follows: Plasmagel-enriched suspensions are washed once in PBS (pH 7.6, 0,01 M) and resuspended at 10% in the same buffer. The density of parasites per 1,000 X microscopic field is then recorded following staining of trial thick smears made in one multi-well slide. If the density is outside the range of 5-50 parasites per field the suspension can be concentrated or diluted properly to allow uniform parasite density. Slides are prepared (12-well slides) by allowing thick films of the suspensions to air dry at room temperature. Slides are coded, wrapped in paper and stored in vapor phase over liquid N_2 until used. Giemsa-stained slides of all isolates are kept for each of the three steps of the procedure: (i) culture, (ii) Plasmagel-enriched suspension, and (iii) thick smear on 12-well slide.

Extraction of soluble P. falciparum antigen. For the enzyme immunoassay (EIA) reactions enough antigen material is obtained from large culture volumes (10 ml Petri dishes). The untreated culture suspension to be used in the preparation of EIA antigen should range in volume between 80 and 100 ml. Following enrichment with Plasmagel, infected cells are washed once by centrifugation in could incomplete culture medium (without serum and $NaHCO_3$) at 1,500 x G for 15 min. The supernatant is discarded and 5 ml of a solution containing 0.04% saponin in incomplete medium is added to the brownish pellet of cells. Following brief homogenization by vortex-mixing the suspension is incubated at room temperature for 20 min to allow lysis of red cells. The suspension is then centrifuged at 1,500 x G for 15 min to allow sedimentation of parasites. The hemoglobin-tinted supernatant is discarded and the pellet resuspended in 12 ml of cold incomplete medium. After another identical centrifugation cycle the supernatant is discarded and the pellet frozen at -20°C for periods not exceeding four days.

Pellets are resuspended in 2 ml of cold extracting buffer (PBS pH 7.2, 0.01 M or 8 M urea in the same buffer) and exposed to 300 Watt ultrasonic pulsed oscillations (Sonifier Cell Disrupter, Heat Systems Ultrasonics, Inc., Plainview, NY) with a microtip transmitter for times ranging between three and four minutes with 30 second idle intervals for every 30 seconds of sonication. The tube containing the suspension is immersed in an ice water bath during the entire sonication procedure. The resulting dark solution is then centrifuged for 15 min at 12,100 x G and 4°C. After centrifugation the yellowish supernatant is carefully removed and transferred to a gas-thight plastic vial and stored in vapor phase over liquid N_2 until assayed. The black pellet in the bottom of the tube is composed mostly of malarial pigment and cell debris and, therefore, can be discarded.

To determine the protein concentration Lowry or Bradford techniques can be used. Because these procedures differ in principle a standard curve should be prepared to allow derivation of results using the linear regression estimates by the least-squares method.

Indirect immunofluorescence (IIF) test. The basic method is that of SULZER et al. (1969) with some modifications: Specimens to be tested are used either undiluted (for screening purposes) or serially diluted in PBS, pH 7.6. Antigen slides are brought to room temperature immediately prior to use. To avoid water condensation on smears, slides are exposed to an air current provided by a fan for about 15 min. Drops of approximately 0.02 ml of each specimen are dispensed into antigen wells using a Pasteur pipette. Slides are then placed on a tray containing a moist piece of tissue paper, covered, and incubated for 30 min at 37°C. Slides then are flooded with PBS, washed once by dipping for about 30 sec in the same solution, and air dried using a fan. To reveal antibody binding, fluorescein (FITC) — labelled anti species antibody conjugate (Tago, Inc., Burlingame, CA) (diluted in PBS containing 0.5% Evans blue, as a counterstain) is dispensed on the slide wells (approximately 0.02 ml drops). Slides are placed in the humid tray and incubated again as described above. Following another washing cycle, slides are air dried and covered with mounting fluid (a 1:9 mixture of 0.2 M Na_2HPO_4, pH 9.0 and glycerol) and cover slips. To evaluate reactivity, slides are examined with a microscope (American Optical) equipped with a mercury vapor (HBO 50) epiilluminator, an 2077 fluorocluster, and oil immersion objectives. Intensity of fluorescing organisms per field (1,000 x) are recorded for each preparation.

Enzyme immunoassay (EIA). EIA reactions are performed in flat-bottomed, 96-well polystyrene plates (Immulon 2, Dynatech Laboratories, Inc., Alexandria, VA). Plates are sensitized with **P. falciparum** soluble antigen extract (prepared as described above) diluted in PBS 7.2 (adjusted to 1-10 mg/L of protein concentration). Fifty microliters of freshly diluted antigen extract are dispensed into all wells. Plates are vibrated using a plate vibrator (Thomas Shaking Apparatus, Arthur H. Thomas Co., Philadelphia, PA) to allow formation of uniform coating areas for all wells — and sealed with adhesive tape. Incubation follows in two phases: first, for 2 hr at 37°C in a water bath (with the water level preadjusted to prevent flotation of plates, yet allowing all well bottoms to be in contact with water) and, second, overnight at4°C.

Prior to assay, specimens are brought to room temperature and vortex-mixed. Plates are removed from the refrigerator, unsealed, and the antigen solution is removed by inverting and shaking. Plates are then washed for three cycles of 3 min each by filling all wells with PBS pH 7.2 containing 0.05% Tween 20 (PBS-Tw) supplied by an 8 channel manifold fitted to a Cornwall syring that is connected to a solution reservoir. The use of the manifold allows all wells to be flooded with equal stream pressure. Removal of PBS-Tw is done by inverting and shaking. To remove the last possible traces of moisture from the wells each plate is inverted and struck sharply 10 15 times against a paper towel spread on a laboratory bench. Fifty ul of undiluted or serially-diluted specimens (in PBS-Tw supplemented with 1% BSA (PBS-Tw-BSA) are added to each well by means of an automatic 8-channel pipette (Flow Laboratories, Inc., Mc Lean, VA). Plates are incubated for 30 min at 37°C in a water bath as described above. Specimens are removed and plates washed as above and shaken dry. Previously diluted (in PBS-Tw-BSA) peroxidase (Donor: H_2O_2 oxidoreductase, EC 1.11.1.7, horseradish) labelled anti-antibody conjugate (specific for the antibody donor and heavy chain type) (Tago, Inc., Burlingame, CA) is added (0.05 ml) to each well. Following another incubation and washing cycle, solid-phase bound enzyme is revealed by adding 0.05 ml of a freshly prepared solution of substrate-chromogen (0.1 mg/ml o-phenylenediamine and 0.003% H_2O_2 in acetate buffer,

pH 4.5). Plates are then incubated at room temperature for 30 min in the dark and substrate catalysis is stopped by addition of 0.025 ml/well of 4 M H_2SO_4. The well contents are mixed by vibration, the bottom of the plates are wiped with soft absorbent paper and colorimetric readings (490 nm) are taken using a computer — interfaced plate reader (MR-580, Dynatech Laboratories, Inc., Alexandria, VA).

RESUMO

Imuno-sorologia da malária

Neste trabalho são revistos os métodos sorodiagnósticos mais frequentemente utilizados para a determinação da resposta imune humoral aos parasitas da malária. A importância desta doença como um problema mundial de saúde pública é enfatizada à luz das novas descobertas no campo imunológico que poderão culminar no desenvolvimento de uma vacina adequada para uso humano. A aplicação de técnicas sorológicas certamente será de extrema importância na avaliação da eficácia relativa destas potenciais vacinas. Discute-se também, no presente trabalho, as diferentes técnicas de preparação de antígenos.

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