6.1 CONCLUDING DISCUSSION

Parasitism involves the close relationship between two different kinds of organisms. One, namely the host, provides food and shelter for the other, the parasite (1). Although some parasites are beneficial to the host, the malaria parasite is not, causing much morbidity and mortality to the host (2). Malaria should be considered in any febrile patient who has travelled to an endemic area, as there is no specific clinical features for malaria infection (3). As we enter the last decade of this century, malaria is on the increase (See Fig. 1.2). The upsurge of the disease world-wide, especially Africa and Southern Africa can be attributed to the following: (a) The increasing population, especially in the high risk areas where malaria is endemic. (b) Increasing poverty and consequent lack of/or inefficient eradication programs. (c) Immigrants spreading the disease to areas where it was under control, or spreading of drug resistant strains to new areas (3). Furthermore, transmission is also expediated by the creation of agricultural communities in malaria areas, since it attracts more and more workers (4). (d) Insecticide resistance leading to an increasing mosquito population. (e) Drug resistance, which is one of the main problems in parasite control, as new drugs are not common and increasingly costly to develop.

By using our knowledge of the current status and occurrence of drug resistant isolates much of the morbidity and mortality can be avoided by correct treatment of the disease (5). With the development of techniques for the in vitro cultivation of malaria parasites a new era was entered.
with respect to research on and diagnosis of malaria. Using the principles of the candle jar/petri dish method for the continuous cultivation of *P. falciparum*, a short term micro-assay was developed for the *in vitro* testing of the drug resistant status of malaria parasites (6). Although the system developed by Rieckman has some disadvantages as mentioned in section 2.1 the test can be used to determine the degree of susceptibility of malaria parasites to most of the antimalarial drugs, except to combinations of sulfa-drugs and pyrimethamine. A reduction in the concentration of 4-aminobenzoic acid (PABA) and folic acid in the RPMI 1640 medium to those found in human blood, made it possible to also employ this assay for the above drugs (7).

The field evaluation in 1988 revealed that 11 % of the cases in the North-Eastern Transvaal had parasites that were resistant to chloroquine (Fig 2.2 and Fig 2.3 a). Thus, one out of every ten patients infected with malaria, might respond only to drugs other than chloroquine. A bigger concern is the evidence that in 22 % of the cases, the malaria parasites were resistant to mefloquine. Mefloquine is a relatively new drug and not yet available in South Africa and already it appears to be ineffective. Perhaps in this case it is a matter of insensitivity to the drug rather than resistance (8). Padua crossed a cloned line of a highly drug-resistant isolate with a drug-sensitive isolate of malaria parasites and found that many clones of the progeny exhibited a level of resistance midway between that of the parents (9). During meiosis of hybrid zygotes, the various resistance genes apparently had undergone recombination, thus producing the intermediate forms of resistance (9). This may explain why certain isolates from North-Eastern Transvaal were more resistant to chloroquine and mefloquine than others. The *in vitro* method
also does not take cognisance of the immune system of the host or the possibility that metabolites formed *in vivo*, may be more effective than the drug itself (8). It is thus possible that *in vitro* assays could be biased and not a true reflection of the *in vivo* situation.

Interpretation of results of the micro-test is dependent on the ability to cultivate parasites in the control wells. It is thus important to ascertain that no prophylactics were taken (urine test) prior to the assay and to be aware that a high parasitemia needs more chloroquine than a lower parasitemia to inhibit schizont formation (6). The micro-test can be easily accomplished if the hematocrit is kept below 3% and if fresh erythrocytes are added to reduce the parasitemia. The candle-jar can also be replaced by a dessicator filled with a special gas mixture (7). The short-term *in vitro* drug test is also more effective if the parasitised erythrocytes are washed prior to cultivation to remove any adsorbed inhibitory antibodies and if serum from a naive donor is used (7).

The *in vitro* testing of anti-malarial drugs thus broadens our knowledge of the isolates found in specific areas and will aid in the selection of the appropriate chemotherapeutic treatment as well as prophylactics. It is recommended that *in vitro* testing of the commonly used drugs such as chloroquine, quinine, sulphad Drugs and drug combinations should be continued on a regular basis to assist in the treatment of patients and to monitor changes in the antimalarial drug susceptibility profile, as well as to evaluate new antimalarial drugs (5).

The relatively late development of a long term *in vitro* culture method was mainly due to an inadequate knowledge of the biochemistry of the parasite (12). The extent of parasite growth depends on a number of
factors: strain, time of culture, suitability of the erythrocytes as well as the serum (7). Several modifications to the method developed by Trager and Jensen have now led to the development of semi-automatic apparatuses with various degrees of complexity, in order to maintain the physiological pH and remove toxic waste products (7).

The initial inability to establish a continuous in vitro culture in our laboratory appears to have been due to a difference in the composition of dissolved gases between Pretoria and Durban. Parasite growth at Pretoria was only observed in flasks which contained gas-equilibrated medium and were flushed with the special gas mixture (Fig 3.4). The lack of growth in flasks that were only flushed, was most likely due to the toxic effect of a too high oxygen concentration which could not be prevented by the prevailing concentration of dissolved carbon dioxide. Equilibration of the medium with the gas mixture, prior to culturing of parasites, apparently compensated for the lower partial pressure of gases at Pretoria compared to Durban. The consequent higher concentration of dissolved carbon dioxide protected the parasites against oxygen toxicity. It appears that only wild isolates are extremely sensitive to the composition of dissolved gases since after a few weeks in culture, parasites could be maintained in medium that was only flushed but not equilibrated with the gas mixture. It is recommended that gas mixtures with lower concentrations of oxygen be used in future studies, especially if wild isolates need to be established.

Serum is one of the most important supplements of the culture medium (7). From our results in Table 3.3 it is evident that bovine serum can be used as a substitute for human serum. However, growth in human
serum-supplemented medium was superior to medium which contained bovine serum. Culture mediums also required the addition of hypoxanthine. It is possible that further additions of hypoxanthine to bovine serum-supplemented medium may further improve the growth rate due to the substantial lower content of hypoxanthine in bovine serum compared to human serum (Table 3.3).

One of the fundamental goals of studies on the biochemistry of *Plasmodium* is to search for metabolic differences between the host and parasite which can be exploited for the design of new drugs. Particularly striking is the molecular differences between the dehydrofolate reductases of host and malarial parasites, which contribute to the exquisite sensitivity of malaria parasites to pyrimethamine (12). There is no doubt that other as yet undiscovered differences exist between the host and *Plasmodium* which could be used in the development of specific antimalarial drugs.

The successful invasion of the host erythrocyte by a merozoite involves specific binding to a surface receptor followed by endocytosis (2). The intra-erythrocyte parasite derives its energy via conversion of glucose into lactate (both L and D isomers) (13). The avian parasite oxidizes a portion of the pyruvate into CO₂ and H₂O by means of a citric acid cycle, whereas rodent and primate parasites lack a complete citric acid cycle and have only lactate as end product (13). The only evidence for an energy-yielding electron transport chain is at best circumstantial. In all the malaria parasites studied, the only enzyme which could be associated with this system is cytochrome oxidase (13).

Malaria parasites can synthesise pyrimidines *de novo* but not purines (12). Purines are obtained via a parasite-specific salvage pathway in
which hypoxanthines, derived from the extracellular medium and the host, is converted mostly to ATP and small quantities of GTP (14).

One of the conclusions drawn from our results was that lactate concentrations above 12 mM are inhibitory to parasite growth, in agreement with other researchers in the field (14). The rate of medium replacement is therefore largely determined by the parasitemia of the culture which should be kept below 10%. The amount of lactate produced by the infected cultures in comparison to the non-infected cultures cannot be accounted for by glycolysis alone. For the first 48h of culturing, the lactate/glucose ratio exceeded 2 whereas during the last 48h it was below 2 (Fig 3.8 and Table 3.4). Confirmation of these results requires further investigation. As only a fraction of the available glucose is used this is clearly not a limiting factor in parasite cultures. The concentration of ATP inside the host erythrocyte is considered to be critical for parasite development (16). Its concentration is influenced by various factors which include the pH and lactate concentration of the medium as well as utilization by the erythrocyte and parasite (16). Over the first 48 hours of culture the ATP concentration in the infected cultures were relatively stable. However, over the last 48 hour period the ATP concentration decreased by 44%, from 1690 to 950 nmol/ml PCV (Fig 3.8). Most importantly, this large decrease was obtained in a culture where only 14% of the erythrocytes was infected. Similar results were also found for some in vivo infections (12). From our results (Fig 3.8), it seems that the purine salvage pathway is inhibited since, even though high concentrations of IMP were available, the ATP concentration was lower than in the non-infected controls. The total adenylate nucleotide concentration was as suspected lower, due to the excessive demand (Table 3.4). The
decrease in the multiplication index (Table 3.5), could thus be due to a limiting adenylate nucleotide pool as well as the lesser susceptibility of non-infected erythrocytes to invasion, caused by ATP depletion (see Introduction). At present the cause for the block in the purine salvage pathway is unknown and requires further investigation.

Haematological complications are the most common of all in malaria infections (16). According to Kelton, thrombocytopenia in malaria infection was first observed by Hill in 1964 and is prevalent in *P. falciparum* and *P. vivax* infections (16). Two major systems induce thrombocytopenia in malaria, namely the increased destruction of platelets either by hyperactivity of the reticulo-endothelium cells and/or immune destruction and disseminated intravascular coagulation (DIC) (16,17). Strong evidence exists for the immunoglobulin destruction of thrombocytes as increased concentrations of thrombocyte-associated immunoglobulins (TAIg) have been measured both by us (18) and Kelton *et al.* (16). The modified micro-enzyme-immunoassay for the determination of thrombocyte-associated immunoglobulins which we developed, simplified the assay considerably. The method is faster and more economical and accurate than any other method employed at this stage. It also uses less blood and can be employed in antibody displacement studies to determine the specificity of TAIg. This method also lends itself to quantification of autoimmune antibodies present on cells or antibodies specifically bound to membrane surface antigens or receptors. It could be shown with this method that the inverse correlation between thrombocytes and thrombocyte-associated immunoglobulins decreased while patients received treatment, which helped to monitor their recovery (Table 4.1).
The reason for the increased association of antibodies with thrombocytes is not yet understood but the following models need to be considered: (a) It is an autoimmune disease in which antibodies produced against the parasite cross-reacts with epitopes on the thrombocyte surface. (b) The antibodies are immune complexes consisting of parasite antigens and their antibodies which adhere to the thrombocyte surface. (c) Parasite antigens first adhere to the surface of thrombocytes before being bound by antibodies. Future studies should be aimed at the identification of the idiotypic specificity of the antibodies to discover whether or not cross-reacting epitopes are responsible for the platelet destruction.

The parasite morphology and the host species infected by the parasite are primary attributes considered in the taxonomy of *Plasmodia* (19). The light microscope is the principle tool used in assigning morphological descriptors to the parasites. However, over the last three decades the electron microscope with its capacity for detecting fine structures has become increasingly important to the researcher (25). Erythrocytes infected by *Plasmodium* exhibit various changes in its morphology. However, it is still not known why one strain of malarial parasites produce changes which is only specific for that strain (19).

Cytoplasmic organelles which are considered to be involved in parasite trafficking from and to the host cell membrane and the parasite, were described by Aikawa (19). Similar structures were seen in our studies. We found that the fast fixation method is the preferred method for electron microscopy. The slow fixation method, was only good enough for scanning electron microscopical studies. It is now possible to conduct
immunocytochemical studies and to investigate the fine structures of isolated parasites (Fig 5.7).

Thus, new techniques for studying the biology of the malaria parasite and their interactions with the host, opened new doors for research on malaria. Culturing techniques are currently used to screen parasites for drug susceptibility and effectivity of vaccine candidates (7). The drugs developed against the parasites are in a sense only an aid to nature (20). Without an active immune response from the host it is unlikely that any antimalarial drug used today will completely cure a patient of his malaria infection (20). It is furthermore misleading to compare the eradication of a disease such as smallpox, which is easily diagnosed and prevented by vaccination, to malaria (21). The inadequacy and high cost of the currently available control programmes coupled to the resistance of vectors and parasites to insecticides and drugs, respectively, makes this disease much more complex and highlights the need for a cheap, effective vaccine (22). However, we must guard against the impression that once an effective vaccine is developed the problems of malaria will disappear (23). As in the case with other vaccines, its distribution to all the areas where it is needed, is near impossible (23).
6.2 REFERENCES.


SUMMARY

Malaria parasites are responsible for an increase in the morbidity and mortality in several tropical regions in Southern Africa. In this thesis, research was undertaken on *Plasmodium falciparum*, which is responsible for more than 95% of these cases.

Although pharmacological prophylaxis is available, a worldwide resistance against existing drugs have been encountered. A study on chloroquine-resistance in North-Eastern Transvaal in 1988, indicated that 11% of the strains in this area were resistant against chloroquine. Only 78% of these parasites were sensitive to the new drug, Mefloquine, which could serve as a substitute in chloroquine-resistant infections. Furthermore, no strain was found to be resistant or insensitive to both anti-malarial drugs.

Malaria parasites can be obtained for research purposes from long term in vitro cultures. The initiation of cultures of some wild isolates of *P. falciparum* appears to be problematic at certain levels above sea-level. The gas composition of the medium was identified as a probable cause due to its dependence on the partial pressures of gasses at different heights above sealevel. The toxic effect of a too high oxygen concentration on the parasite, caused by the lower atmospheric pressure of the Highveld area, was prevented after the concentration of dissolved carbon dioxide in the medium was increased through equilibration with the special gas mixture.

Possible shortcomings in the long-term culture method for malaria parasites that could retard optimum growth, were also investigated. Local parasite isolates could be supported on medium enriched with bovine serum although the growth rate was lower than when human serum was
used. By increasing the frequency of medium replacement with progressing parasitemia, less stress were placed on the system since parasites was exposed for shorter periods and to lower concentrations of byproducts such as lactic acid. In addition, the ATP-concentration in infected cultures decreased by nearly 50% over a growth period of 4 days. The stress in the host cell was reflected by the decrease in the total adenyl-nucleotide pool and the increase in AMP-concentration. An increase in the intracellular IMP-concentration indicated that the purine salvage pathway was inhibited which may explain the decrease in the ATP-concentration. It therefore appears that the regular replacement of medium and replenishment of erythrocytes only partially contribute to the successful establishment of malaria cultures. More research is necessary to identify the factors that are responsible for the inhibition of the purine salvage pathway.

The stress placed on the human body by the parasite is complicated by the thrombocytopenia observed in some infections. Increases in the concentrations of thrombocyte-associated immunoglobulin G and M which are a characteristic for this condition, can be determined by a modified micro-method developed in our laboratory. By monitoring the parasite-infected patient over a period of time with the micro-method, the increase in thrombocytes and decrease in thrombocyte-associated antibodies were correlated with the recuperation of the patient. This method does not destroy the thrombocytes, thereby allowing displacement studies to be undertaken with purified parasite antigens of synthetic peptides.

An investigation of parasite-infected erythrocytes by means of light microscopy, transmission and scanning electronmicroscopy, indicated that
the local isolate could be composed of a mixture of strains, of which some have the ability to induce knobs on the erythrocyte. Furthermore, the investigation illustrated that fast fixation with gluteraldehyde is superior to slow fixation when transmission electronmicroscopy is performed. However, no difference could be observed when scanning electron microscopy was performed on infected erythrocytes that had been fixed by either of these methods.
OPSOMMING

Malaria parasiete is verantwoordelik vir 'n toenemende morbiditeit sowel as mortaliteit in verskeie tropiese streke in Suider-Afrika waarvan meer as 95 % van die gevalle deur Plasmodium falciparum veroorsaak word. Navorsing in die tesis is gevolglik op hierdie parasiet toegespits.

Ten spyte daarvan dat far'makologiese profilakse toegepas word, kan malaria nog steeds opgedoen word as gevolg van 'n wêreldwyse weerstandigheid teen bestaande geneesmiddels. 'n Ondersoek van die klorokien-weerstandigheidstatus in die Noord-Oostelike Transvaal het in 1988 getoon dat 11 % van die stamme in die area weerstandig is teenoor klorokien. Slegs 78 % van die parasiete was sensitief vir die nuwe middel, Meflokien, wat as moontlike plaasvervanger in klorokien-weerstandige parasietinfecties gebruik kan word. Geen parasietstam was egter weerstandig of onsensitief teen beide middels nie.

Langtermyn kultuurkweking van malaria parasiete is essensieel vir die verkryging van uitgangsmateriaal vir navorsing op die parasiet. Dit blyk egter dat inisiasie van kulture van wilde stamme van P. falciparum problematies by sekere hoogtes bo seespieël is. Die gassamstelling van die medium is as 'n moontlike oorsaak geïdentifiseer aangesien dit afhanklik is van die parsiële drukke van gasse by verskillende hoogtes bo seespieël. Die toksiese effek van te hoë suurstofkonsentrasies op die parasiet a.g.v. die laer atmosferiese druk op die Hoëveldstreek, is oorkom deur die konsentrasie van opgeloste koolsuurgas in die medium te verhoog deur vooraf 'n spesiale gasmengsel daardeur te borrel.
Moontlike tekortkominge in die langtermyn kultuur metode vir malaria parasiete wat optimale groei kan belemmer, is ook ondersoek. Plaaslike parasietstamme kon op medium wat verryk is met beesserum onderhou word, alhoewel die groeitempo laer is as wanneer mensserum gebruik word. 'n Toenemende tempo van mediumvervanging soos die parasitemia in kulture toeneem, het getoon dat minder stres op die sisteem geplaas word deurdat die parasiete vir 'n korter periode en aan laer konsentrasies van byprodukte soos melksuur blootgestel word. Die ATP-konsentrasies in geïnfekteerde kulture daal egter met ongeveer die helfte oor 'n groeiperiode van 4 dae. Tesame hiermee, is daar ook 'n daling in die totale adeniul-nukleotied poel van die geïnfekteerde rooibloedsel en 'n styging in die AMP-konsentrasie wat die stres in die gasheersel weerspieël. 'n Verhoging in die intrasellulêre IMP-konsentrasie dui op 'n moontlike inhibisie van die purienherwinningspadweg wat die verlaagde ATP-konsentrasie mag verklaar. Uit die studie blyk dit dus of meer gereelde vervanging van medium en toevoeging van rooibloedselle slegs 'n gedeeltelike bydrae maak tot die suksesvolle vestiging van malariakulture. Die identifikasie van die faktore wat lei tot die inhibisie van die purienherwinningspadweg, verg nog verdere ondersoek.

Die stres wat die parasiet in die menslike liggaam veroorsaak, word ook weerspieël deur onderandere die trombositopenie wat in sommige infeksies waargeneem word. Verhogings in die konsentrasie van trombosiet-geassosieerde immunoglobuliene G en M wat kenmerkend is van die toestand, kan gemeet word deur 'n gemonifiseerde mikrometode wat in ons laboratoriums ontwikkel is. Deur die parasiet-geïnfekteerde pasiënt oor 'n paar dae met die mikrometode te monitor, kon die verhoging in trombosiete en afname in trombosiet-geassosieërde antiliggame met herstel
van die pasiënt gekorreleer word. Die metode veroorsaak geen skade aan die trombosiete nie sodat antigeenverplasingstudies ook na die tyd gedoen kan word.

In 'n ondersoek van die parasiet met behulp van ligmikroskopie, deurstraal- sowel as skandeer-elektronmikroskopie, is gevind dat die plaaslike isolaat moontlik uit 'n mengsel van stamme bestaan, waarvan sommige die vermoe het om knoppe op die rooibloeedsel te induseer. Verder het die ondersoek getoon dat indien deurstraal-elektronmikroskopie gebruik word, 'n vinnige fikseringsmetode met gluteraldehied beter is as 'n stadige fikseringsmetode. In vergelyking hiermee kon geen verskil waargeneem word met skandeer-elektronmikroskopie van geparasiteerde rooibloeedselle wat met enige van hierdie metodes gefikseer is nie.