PD-AAW-222

AGENCY FOR INTERNATIONAL DEVELOPMENT

PROJECT DATA SHEET

1. TRANSACTION CODE: A = Add, C = Change, D = Delete — Amendment Number: 51295

DOCUMENT CODE: 3

2. COUNTRY/ENTITY: S&T/Interregional

3. PROJECT NUMBER: 931-0453.35

4. BUREAU/OFFICE: S&T/Health — 10

5. PROJECT TITLE (maximum 40 characters): Malaria Immunology - (BRI-Zolg)

6. PROJECT ASSISTANCE COMPLETION DATE (PACD): MM 03 DD 31 YY 87

7. ESTIMATED DATE OF OBLIGATION (Under 'B.' below, enter 1, 2, 3, or 4)

A. Initial FY 84 B. Quarter 2 C. Final FY 86

8. COSTS ($000 OR EQUIVALENT $1 =)

A. FUNDING SOURCE	FIRST FY 84 B. FX	C. L/C	D. Total	LIFE OF PROJECT E. FX	F. L/C	G. Total
AID Appropriated Total	1,500		1,500	2,250		2,250
(Grant)	( )	( )	( )	(2,250)	( )	(2,250)
(Loan)	( )	( )	( )	( )	( )	( )
Other U.S. 1.						
Other U.S. 2.						
Host Country						
Other Donor(s)						
TOTALS	1,500		1,500	2,250		2,250

9. SCHEDULE OF AID FUNDING ($000)

A. APPROPRIATION	B. PRIMARY PURPOSE CODE	C. PRIMARY TECH. CODE 1. Grant	2. Loan	D. OBLIGATIONS TO DATE 1. Grant	2. Loan	E. AMOUNT APPROVED THIS ACTION 1. Grant	2. Loan	F. LIFE OF PROJECT 1. Grant	2. Loan
(1) S&T/H	540	542				2,250		2,250	
(2)									
(3)									
(4)									
TOTALS						2,250		2,250	

10. SECONDARY TECHNICAL CODES (maximum 6 codes of 3 positions each)

11. SECONDARY PURPOSE CODE

12. SPECIAL CONCERNS CODES (maximum 7 codes of 4 positions each)

A. Code

B. Amount

13. PROJECT PURPOSE (maximum 480 characters)

The purpose of this project is to conduct research on red blood cell antigens in malaria using recombinant DNA techniques.

14. SCHEDULED EVALUATIONS

Interim MM YY MM YY Final MM YY

15. SOURCE/ORIGIN OF GOODS AND SERVICES

☐ 000 ☐ 941 ☐ Local ☐ Other (Specify)

16. AMENDMENTS/NATURE OF CHANGE PROPOSED (This is page 1 of a ____ page PP Amendment.)

17. APPROVED BY

Signature: George Curlin, M.D.

Title: Director, Office of Health

Date Signed: MM 01 DD 12 YY 84

18. DATE DOCUMENT RECEIVED IN AID/W, OR FOR AID/W DOCUMENTS, DATE OF DISTRIBUTION

MM DD YY

AID 1330-4 (8-79)

PROJECT AUTHORIZATION

Name of Entity: Interregional

Project Title: Malaria Immunity and Vaccination Research

Project No.: 931-0453.35

Grantee: Biomedical Research Institute

1. Pursuant to Section 104 of the Foreign Assistance Act of 1961, as amended, I hereby authorize the research project entitled "Malaria Immunity and Vaccination Research" involving not to exceed $2,250,000 of S&T Bureau grant funds over a three year period from the date of authorization subject to the availability of funds in accordance with the A.I.D. OYB/Allotment process to help in financing the costs of the project.

2. The project will conduct research on the intraerythrocytic protozoan agent, Plasmodium falciparum, to develop a vaccine based upon red blood cell antigens released by the parasite during infection and to screen the sequencing genes of P. falciparum.

Clearances:

A. ST/H: G. Curlin Date 1/3/84
B. ST/PO: G. Eaton Date 1/4/84

S&T/HP, J. Sarn, M.D.
Agency Director
for Health and Population

1-9-84
(Date)

ACTION MEMORANDUM FOR THE AGENCY DIRECTOR
FOR HEALTH AND POPULATION

FROM: S&T/HP, George Curlin, M.D. George Curlin M.D.

Action: Your approval is requested for a grant of $2,250,000 from Section 104 of the Foreign assistance Act of 1961 as amended for project 931-0453.35, Malaria Immunity and Vaccination Research, Biomedical Research Institute.

Discussion: This unsolicited research project proposes a carefully planned 10-step approach to the development of an effective red blood cell (merozoite) specific malaria vaccine using recombinant DNA techniques. By optimization of an automated mass-culture system based on hemodialysis, sufficient parasite material will be available for this project as well as other network laboratories to isolate the total RNA, enrich the m-RNA, synthesize the cDNA and clone the cDNA into various suitable vectors. This project will be highly integrated into the red blood cell component of the A.I.D. collaborative network, especially the University of Missouri, the University of Hawaii and Scripps Clinic to quickly accelerate RBC stage antigen isolation and recombinant genetics work.

Justification to the Congress: Project Funding is included in the FY 1984 Congressional Presentation, Annex V, Centrally funded Programs, page 53.

Clearances Obtained: This project was reviewed and approved by the AID external expert panel on the basis of scientific methodology, qualifications and experience of the proposed investigators, and the institutional capability in accordance to FPR1-4.909.

Recommendations:
1. That you sign the attached authorization.
2. That you sign the attached justification for non-competitive procurement.

Attachments:
1. Project Authorization
2. Project Paper

Clearance:
S&T/PO, G. Eaton [illegible] Date 1/4/84

Research Proposal

entitled

Molecular Cloning of the Dihydrofolate Reductase Gene of *Plasmodium Falciparum:* A Model Gene to Study Expression and Regulation of Malaria Genes in Procaryotic Cells

submitted to

Agency for International Development
Washington, D. C.

submitted by

J. Werner Zolg, Ph.D.
Biomedical Research Institute
12111 Parklawn Drive
Rockville, Maryland 20852
(301) 881-3300

Proposed Starting Date: 1 January 1984
Proposed Duration: Three Years
Date of Submission: 28 April 1983

J. Werner Zolg, Ph.D.
Principal Investigator

James L. Leef, Ph.D.
Director, Biomedical Research Institute

CONTENTS

1. Background 4

2. Rationale

2.1 Problems arising using recombinant DNA techniques 6

2.2 The dihydrofolate reductase (DHFR)-model system 9

2.3 The integration of the DHFR-model system into the search for malarial antigens 13

3. Specific Objectives 14

4. Experimental Design

4.1 Mass culture system for the *in vitro* growth of *P. falciparum* 16

4.2 Selection of overproducing strains 18

4.3 Isolation of RNA, *in vitro* translation of RNA 18

4.4 Synthesis of cDNA, cloning 20

4.5 Raising of anti-DHFR-monoclonals 21

4.6 Isolation of DHFR 24

4.7 Screening the libraries for the presence of *non-expressed* genes 26

4.8 Screening the cDNA libraries for gene expression 27

4.9 Sequencing genes of *P. falciparum* 28

5. Computerized Data Analysis 30

6. Collaborative Arrangements 35

7. Facilities 36

8. References 37

9. Personnel 42

10. Budget

10.1 Budget for Year 01 46

10.2 Budget for Year 02 51

10.3 Budget for Year 03 53

10.4 Summary Budget 55

10.5 Budget Justification

10.5.1. Justification: Personnel 56

10.5.2. Justification: Basic Equipment 58

10.5.3. Justification: Specific Research Equipment, Synthesis and Journals 59

10.5.4. Justification: Travel and Consultant Fees 63

ADDENDA

1. Publications of the Principal Investigator relative to this program (see copies within the folder).

2. Fold-out flow chart summarizing the strategy.

expression of the parasite-specific genes and thereby to identification of those clones containing the wanted gene coding for the protective antigen.

2. RATIONALE

2.1 PROBLEMS ENCOUNTERED USING RECOMBINANT DNA TECHNIQUES

Our understanding of the molecular biology of the human malaria parasite *P. falciparum* is still in its infancy, and most conclusions are drawn from the rodent and avian models (2-3).

Our initial studies in the group of Dr. J.G. Scaife in Edinburgh, Scotland, using recombinant DNA techniques, revealed that one has to overcome initially unexpected difficulties on the way to obtaining a malaria vaccine by means of genetic engineering and that the early optimism was not wholly justified. The results obtained - which have to be taken into account when planning an approach where recombinant DNA is used - can be summarized as follows:

a) The genome of *P. falciparum* is 3 to 7 times the size of the genome of *E. coli*, and, assuming an average insert size of 0.5-16 kb, one needs ~10,000 clones in order to have the whole genome represented.

b) The DNA of *P. falciparum* has the most unusual base composition yet identified in any organism (81% AT). The implications are two-fold: Such DNA is notoriously difficult to cleave with restriction enzymes, and cloned fragments of this DNA are far more unstable compared with DNA composed of equal amounts of AT and GC base pairs.

c) Complete *genomic* DNA libraries were constructed and the genes for ribosomal RNA, repetitive sequences, and actin genes were identified using heterologous and homologous probes. No *gene expression*, however, could be detected by techniques such as complementation and monoclonal antibodies. These negative results strongly suggest that the genes of *P. falciparum* are interrupted by non-coding sequences (introns), a structural feature that the malaria parasites seem to share with the overwhelming majority of other

eucaryotic organisms. This, in sum, means that genomic DNA will probably not be expressed in procaryotic hosts such as E. coli, which lack the appropriate splicing mechanisms. Consequently, the cloning of genomic Plasmodium DNA is highly unlikely to be helpful as far as antigen expression and a malaria vaccine are concerned. Therefore, one has to synthesize complementary DNA (cDNA) and insert the genes in the right reading frame into suitable vectors in order to overcome this problem.

d) The necessity to work with cDNA implies the isolation of RNA and the purification of mRNA. Although we were able to isolate 0.01-0.02 pg DNA/parasite (4) and comparable amounts of total RNA (0.04 pg RNA/parasite 5), more than 90% of this RNA consists of rRNA, tRNA and degraded mRNA, and is by definition not suitable for the synthesis of the cDNA wanted. Therefore, a number of parasites at least 10 times higher is needed for cDNA libraries than for genomic libraries. A frequent supply of 5×10^{10} parasites or multiples thereof is needed to extract 1-1.5 mg of total RNA, a fraction of which in turn is used for cDNA synthesis. Those amounts of parasite material are very difficult to obtain by established tissue culture methods, and one has to overcome these problems by establishing more productive continuous culture systems. An even greater supply of parasites will be needed to purify antigens used in an in vivo assay to test whether or not they are protective.

e) The main difficulty one is faced with, however, centers on the problem of gene expression when using recombinant DNA techniques as a tool to produce a malaria vaccine.

The obvious approach for detection of an antigen expressed by a gene is the use of protective Mabs. In order to get a positive signal, not only must the epitope be expressed, but it is very likely that in the recognition of the antigen-antibody complex the folding of the antigen protein chain is essential

for the right epitope configuration. This correct folding demands the synthesis of great parts of the protein and might be impaired by fusion of the antigen to procaryotic genes, regulated under the direction of strong promoters (e.g., β-galactose gene). These fusions are often used to get maximal gene expression. Because nothing is known about the regulation of Plasmodial genes, the promotor structure, or gene expression in general, any approach to working with relatively unknown complex proteins such as surface antigens in order to study gene expression will be risky.

We therefore propose the use of a multi-testable model system in order to gain fundamental knowledge about the processes involved in plasmodial gene expression. This model system will be described in detail below (2.2.), and this system in respect to a malaria vaccine will be discussed in 2.3.

2.2. THE DHFR-MODEL SYSTEM

The enzyme dihydrofolate reductase (EC 1.5.1.3.) (DHFR) catalyzes the reduction of dihydrofolate to tetrahydrofolate, an essential co-factor in the biosynthesis of various amino acids and nucleotides. The inhibition of the DHFR invariably leads to a depletion of tetrahydrofolate and consequently to an impaired synthesis of proteins, RNA and DNA, and therefore to cell death (6). Much attention has been given to this enzyme isolated from a variety of eucaryotic cells such as hamster, mouse, and human cells (7-8), procaryotic cells (9), and plasmids (10). Extensive studies have revealed the detailed molecular structure of the DHFR from these sources, including the expression of the DHFR gene in E. coli (11), and after insertion in viral vectors, murine DHFR genes could be expressed even in eucaryotic cells. In addition, the DHFR of Plasmodia is important in relation to antimalarial therapy, being the target enzyme for extensively used antifolates such as pyrimethamine, or antibiotics such as methotrexate and trimethoprim (6). Antifolate resistance of Plasmodia is currently a significant problem in malaria prophylaxis and has been shown in the P. berghei model to be due to either an altered DHFR or to overproduction of the sensitive enzyme (13), although the possibility of altered transport was not taken into account. The overproduction of the DHFR gene in resistant strains renders this gene a most promising one for cloning. This view is based on the following observations and assumptions: If certain P. falciparum isolates are up to 50,000 times more resistant to pyrimethamine compared to sensitive strains (14) and this resistance is due to an overproduction of the sensitive enzyme, the DHFR-specific-mRNA is probably also increased accordingly. Because one has to use cDNA derived from mRNA (see 2.1), the chances of synthesizing a full-length gene are consequently

increased by a similar factor compared to a single gene represented by few mRNA molecules such as the much-sought protective antigen.

The DHFR from P. falciparum is not yet characterized in detail. A previous report concerning the partial purification of this enzyme from parasites derived from monkeys (15) presented data which could not be verified by the Principal Investigator in spite of exhaustive attempts to repeat the reported measurements of stability, kinetic properties, and activity of the enzyme. The results of this study are therefore not taken into consideration in the following outline.

The DHFR from all known sources has a unique feature: the enzyme binds not only to its co-factor NADPH, forming a stable complex, but also to the substrate analogue methotrexate (MTX). MTX is available in radioactive form and, because of its binding characteristics, is an ideal tag to identify the enzyme. Preliminary titration of crude extracts with ^{3}H-MTX suggests that the resistance of P. falciparum isolates to pyrimethamine is due to an altered DHFR or to gene amplification. In addition, the MTX-enzyme complex can be used to immunize mice in order to raise anti-DHFR-monoclonal antibodies, which in turn can be used to purify the enzyme itself and thereby acquire a probe by obtaining the amino acid sequence and the synthesis of the corresponding polynucleotides. These probes are invaluable in detecting cloned gene fragments which are not expressed. These clones can then be used to screen the libraries for more complete genes and, equally important, these clones can also be used to look for sequences upstream of the coding region likely to contain regulatory elements. This strategy has been used successfully earlier by the Principal Investigator in characterizing the DHFR gene and the promoter region of a trimethoprim-resistant R-factor (10).

Another distinct advantage of the DHFR model is based on the following observations: When fragments of DNA carrying the DHFR gene derived from resistant cells are inserted into plasmids which are then used to transform E. coli, these bacteria become resistant to trimethoprim (12). One has reason to believe that a DNA fragment derived from a pyrimethamine-resistant P. falciparum strain carrying the DHFR gene, when inserted into a carrier, will render the trimethoprim-sensitive E. coli host into trimethoprim-resistant cells. Therefore the complex problem of detecting gene expression of plasmodial genes in E. coli is reduced to the simplest of all possible screening principles when a marker such as the DHFR model is used: the detection of antibiotic-resistant transformants. This extremely powerful selection principle allows the screening of any number of hybrid clones in a single-step procedure.

SUMMARY OF THE ADVANTAGES OF THE DHFR-MODEL SYSTEM

1. Some of the pyrimethamine-resistant P. falciparum strains overproduce the enzyme dihydrofolate reductase.

2. This overproduction is due to a gene amplification, and these cells contain an increased level of DHFR-mRNA.

3. The probability of synthesizing full-length complementary DNA, necessary for gene expression, is increased by the same factor the mRNA is enriched in the resistant cells.

4. The expression of the DHFR gene in E. coli can be detected in several ways:

a) use of anti-DHFR monoclonal antibodies

b) binding of radioactive methotrexate to the plasmodial enzyme having different size and binding characteristics than the E. coli host enzyme.

c) *E. coli* cells carrying the plasmodial DHFR gene become resistant to trimethoprim once the gene is expressed.

These combined features make the DHFR an ideal marker gene to clone into *E. coli* in order to study gene expression of malaria genes in procaryotic organisms.

2.3 INTEGRATION OF THE DHFR-MODEL GENE INTO THE SEARCH FOR MALARIAL ANTIGENS

As seen in the flow chart at the end of this proposal, the genetic engineering part of the proposal is placed in the center of the research program, symbolizing that it is common to both the DHFR program (left side of flow chart) and the search for an antigen (right side). All the steps aiming towards a cDNA library, such as mass culturing of P. falciparum, extraction of RNA, in vitro translation of RNA, synthesis of cDNA, and cloning of this cDNA into vectors, are identical for both programs. That means that every cDNA library will be tested in parallel for the presence of the DHFR and the antigen genes and eventually for the expression of these genes. As outlined in 2.2, the multi-testable DHFR gene system will be used to establish and modify a great variety of techniques used in creating a cDNA library. Because the target is identifiable (the DHFR enzyme), this system can be used as a positive control for i) the in vitro translation system, ii) the use of polynucleotides derived from the amino acid sequence of the purified enzyme in hybridization screenings of the libraries, iii) the use of monoclonal antibodies to detect minute quantities of expressed gene products, and iiii) most important: to answer the question if P. falciparum promotors can be used effectively to express P. falciparum genes in E. coli or if it is necessary to fuse genes onto E. coli promotors.

3. SPECIFIC OBJECTIVES

A ten-step approach to the development of an effective merozoite-specific malaria vaccine using recombinant-DNA techniques is proposed. The imperative aim remains the expression of P. falciparum genes in procaryotic cells. In order to gain fundamental knowledge about the structure, expression, and regulation of malaria genes in E. coli, we propose the molecular cloning of the malaria-specific dihydrofolate reductase gene (DHFR). The major advantage of this model system (see 2.2) is its feasibility in tracing the expression of the gene by two unique characteristics of the gene product: i) the malarial DHFR enzyme from resistant strains, when expressed in procaryotic cells, renders these cells resistant to trimethoprim, and (ii) the malarial DHFR enzyme binds methotrexate with different kinetics than does the well-known E. coli enzyme. Thus, the results obtained using this model gene can be directly applied to other genes, such as genes coding for immunogenically relevant merozoite-specific surface antigens. We propose to carry out investigations which meet the following specific objectives:

1. Optimization of an automated mass-culture system based on hemodialysis, capable of providing a constant supply of synchronized erythrocytic forms of P. falciparum in sufficient quantities to meet objectives 2, 3 and 4.
2. Isolation of total RNA, enrichment of messenger RNA and selection of mRNA coding for the DHFR using in vitro translation systems
3. Synthesis of full-length complementary, double stranded DNA (cDNA)
4. Cloning of the cDNA into different suitable vectors
5. Identification of P. falciparum strains which overproduce DHFR
6. Raising anti-DHFR monoclonal antibodies used for steps 7 and 8
7. Purification of the DHFR in order to synthesize polynucleotides

corresponding to the amino acid sequence of the enzyme as a probe for screening libraries in step 4 for non-expressed genes.

8. Screening libraries for expressed DHFR genes using the Mabs (step 6) as a probe
9. Characterization of the DNA regions upstream of the coding regions and comparison of these regions from overproducing and non-overproducing strains (study of promoter structure)
10. In addition to the characterization of the DHFR model gene, the same cDNA libraries (step 4) will be screened, once protective monclonal antibodies are identified and made available to us. The purified protective antigen(s) will be sequenced and the corresponding synthesized polynucleotides will be used for identifying non-expressed antigen-gene fragments (analog step 7). The Mabs from NMRI and Bogota will be further used for the detection of translation products in an in vitro system (analog step 2) and to screen the libraries for expression (analog step 8).

The order in which the specific objectives will be met within the 3-year period will be 1-5-2-3-4; step 6 will be carried out simultaneously with steps 2,3, and 4. Once the Mabs are available, steps 7-9 will be undertaken. Step 10 depends entirely on the progress of the research groups in Bogata and Bethesda. Step 10 is planned to be carried out in parallel to steps 7-9 (see flow chart, right-hand side).

4. EXPERIMENTAL DESIGN

4.1 MASS-CULTURE SYSTEM FOR THE *IN VITRO* GROWTH OF *P. FALCIPARUM*

Based upon data we obtained within the last two years from the group in Edinburgh, a supply of 5×10^{10} parasites is necessary per RNA isolation. For the purification of the DHFR, multiples of these numbers are required (Zolg, unpublished results). Such numbers are difficult to obtain by simply scaling up the established *in vitro* culture conditions established by Jensen and Trager (16). Studies concerning the media composition, serum requirements, storage of red blood cells, and the use of suspension cultures (a precondition to develop a mass-culture system) have resulted in a 2-4 fold increase in the parasitic yield (17). A major problem in growing adequate numbers of *P. falciparum in vitro* has been solved recently by using synchronized cultures and monitoring the accumulation of the metabolic product, lactic acid in the supernatants. It could be shown that this metabolic product inhibits parasitic growth when the threshold of 12 mM is exceeded. By knowing the amount of lactic acid produced per time unit by uninfected rbc and the amount produced by parasitized cells, and taking the developmental stage of the parasites into account, the rate of lactic acid production can now be predicted for any hematocrit value and any parasitemia. These calculations are essential for tailoring a dialysis schedule for the system described below. The mass-culture system developed within the last 2 1/2 years by the Principal Investigator is based on 3 separate circuits: i) A special blood pump is used to pump the blood-medium-serum mixture through a single flow parallel dialyzer (used for renal dialysis), through a Kolobow-membrane lung (used in extracorporal circuits), and back into a venous reservoir. The lung is gassed with 5% CO_2, 3% O_2, in N_2, so that the blood leaving the lung has the pCO_2 critical for parasite development. ii) The dialysis fluid is pumped

by a second pump through the outside of the dialyzer in the opposite direction to the blood flow, ensuring maximal solute transfer from the blood to the dialysis fluid according to the cut-off characteristics of the dialysis membrane chosen. iii) Because of the hemofiltration effect of the system, a third circuit replenishes the medium which ends up on the dialysis side after prolonged dialysis.

A blood-gas monitor is used to adjust the pCO_2 according to the pH value reached, thus stabilizing the system. Knowing the stage-specific lactic acid production outlined above, the pump in the dialysis circuit can be automatically activated so that the transfer of lactic acid from the blood to the dialysis compartment is complete during one dialysis period. The main advantage of the system is that it is an open system, i.e., there are no size-limiting surfaces which have to be considered in regard to gas diffusion as is the case in thin-layer systems. Because the kidney and the lung are designed to cope with the blood volume of a human body (ca. 6 L, which under tissue culture conditions equals 60 L of blood-medium suspension) the system could theoretically be scaled up to such volumes without alteration. The standard system planned contains 200 ml of whole parasitized blood (0.5-1% starting parasitemia) and 1800 ml medium-serum mixture, and is dialyzed against 10 L of dialysis fluid, changed according to the lactic acid content present after a series of dialysis periods. In previous trials (without automation), 1 ml of blood could be harvested (4×10^9 rbc/ml) with usually 20% parasitemia (segmented schizonts). Thus, in 84-90 hours after synchronization, $150 \times 4 \times 10^9 = 600 \times 10^9$ rbc were present containing 120×10^9 ($\hat{=}$ 20%) or 1.2×10^{11} schizonts. From this number of parasites > 4 mg total RNA can be isolated which is an amount sufficient to purify enough poly A mRNA to fulfill objective 2, to synthesize cDNA (objective 3), and to

clone the cDNA in different vectors (objective 4). For the isolation of the DHFR (objective 7) several of these preparations will be frozen in stabilizing agents and the proteins will be extracted after pooling at least 2×10^{11} parasites.

4.2 IDENTIFICATION OF *P. FALCIPARUM* STRAINS OVERPRODUCING DIHYDROFOLATE REDUCTASE

P. falciparum strains, especially those recently isolated in Thailand by G.H. Beale, Edinburgh, are up to 50,000 times more resistant to pyrimethamine than sensitive control strains from Africa. The molecular reasons have been described in 2.2. To select a strain overproducing the enzyme and hopefully having amplified DHFR genes, the methotrexate-titration of Flinthoff and Essani (18) will be applied. The procedure devised to measure ^{3}H-MTX binding is based upon the observation that DHFR in the presence of $NADPH_2$ forms a stable complex with MTX. Crude extracts of *P. falciparum* (300 µg protein) will be reacted with the labeled drug and the ^{3}H-MTX-DHFR complex will be separated from the unbound ^{3}H-MTX by column chromatography. Any drug bound to the enzyme is excluded from the column, whereas the unbound drug will enter the resin and will be eluted from the column at a later time. The free drug is determined by subtracting the bound material from the total. The specific amount of bound material (^{3}H cpm/µg protein) together with the Kd for the MTX-DHFR-$NADPH_2$ complex will identify those *P. falciparum* strains which have an amplified wild-type DHFR. Such a strain will be used for the isolation of RNA (4.3), for absorbing the enzyme to MTX-Sepharose to raise anti-DHFR-Mab's (4.5), and for purifying the enzyme (4.6).

4.3 ISOLATION OF RNA, *IN VITRO* TRANSLATION

Parasites will be freed by saponin lysis and the total RNA will be extracted in the presence of a mixture of RNase inhibitors by a method worked

out by Dr. J.E. Hyde, Edinburgh (unpublished results). The best results are obtained by repeated extractions with hot phenol, taking great care to use ultrapure glassware treated with diethylpyrocarbonate, followed by repeated autoclaving and rinsing with ultrapure reverse osmosis water. Maintaining sterility of all buffers and avoiding all sources of RNase contaminations allows isolation of 0.04 pg undegraded RNA/parasite. The RNA will be further purified using the common oligo dT-columns (19) in order to select for poly A containing RNA. This fraction and the non-binding RNA fraction (containing nRNA, tRNA and non-poly A mRNA) will be used in the rabbit-reticulocyte *in vitro* translation system. The ^{35}S-methionine labeled proteins synthesized will be separated on SDS-PAGE gels. The dihydrofolate reductase will be identified by one of the following methods. i) Use of anti-DHFR Mabs (4.5) to absorb the DHFR before loading on gels. One protein band should be missing compared to the untreated control sample in a parallel track. ii) The proteins synthesized in the *in vitro* system are titrated with unlabelled methotrexate bound to Sepharose and run as described in i). iii) The ^{35}S-labeled proteins are titrated with ^{3}H-methotrexate, separated on gels, the gels are cut into 1 mm pieces and the ratio of ^{35}S/^{3}H is determined. The slice containing the DHFR binding ^{3}H-MTX should be easily identifiable. iiii) The proteins are run on nondenaturing gels and stained for enzyme activity using a color reaction specific for NADPH-reduction. Once a positive signal is obtained with one of the detection methods, one knows if the message for the DHFR is polyadenylated or not, thereby identifying the desired fraction from the oligo dT-column. Based on the size of the protein and whether or not it is made up of subunits identifiable on SDS gels, an appropriate size range of mRNA will be selected from the RNA gels, thereby allowing significant enrichment for those particular mRNA classes to be used in the synthesizing of

the cDNA. The same selection principle will be applied to antigens once protective monoclonals from the Bogota group become available (objective 10).

4.4 cDNA-SYNTHESIS, CLONING

Potent RNase inhibitors such as RNasin and vanadyl-ribonucleoside complexes will be used to protect the mRNA from degradation. An excellent summary of the techniques used in the synthesis of double-stranded cDNA can be found in chapter 7 of reference 20. In brief, the first and second strand of the cDNA will be synthesized using AMV-reverse transcriptase. The protruding 3' single-stranded termini will be removed with the 3'→5' exonuclease activity of E. coli DNA polymerase I, resulting in blunt-ended, double-stranded cDNA. T4-ligase is used to add a synthetic linker to one end of the cDNA before the hairpin loop deriving from the synthesis of the second strand of the cDNA is cleaved with S1 nuclease. A second different synthetic linker is then attached to the second end of the cDNA, the linker-flanked molecule is cut with appropriate restriction enzymes and the resulting DNA fragments are cloned into vectors.

A second novel method for high-efficiency cloning of full-length cDNA will be followed with high priority. Published first by Okayama and Berg earlier this year (21), the method is very complex but brilliantly conceived. The basics are as follows: A hybrid pBR322-SV40 vector is cleaved and tailed with poly(dT) tails. Another hybrid vector is tailed with poly (dG), the tailed part serving as a linker. The first poly(dT) vector is linked with the poly A m-RNA (!) and used as a primer to synthesize the first cDNA strand. This first strand is then supplied with poly(dC) tails and the oligo (dG)-tailed cDNA-mRNA plasmid is ligated to the oligo (dG) linker derived from the second plasmid. RNase H is used to degrade the RNA in the RNA-DNA hybrid, E. coli polymerase carries out a nick-translation repair of the second cDNA

strand, and DNA ligase is used to covalently link the double-stranded cDNA to the vector DNA. 100,000 transformants per microgram of starting mRNA can be obtained with up to 6500 bp inserts capable of coding for 2100 amino-acids and therefore proteins with a molecular weight > 200,000 daltons. This size range of course is interesting in view of schizont-specific surface antigens reported recently by various investigators.

E. coli recipients (HB 101, DH1 and K802) will be transformed either by the common calcium chloride procedure (22) or by the calcium chloride/rubidium chloride procedure (D. Hanahan, personal communication).

4.5 PRODUCTION OF MONOCLONAL ANTIBODY SPECIFIC FOR DIHYDROFOLATE REDUCTASE ENZYME ISOLATED FROM CULTURED *PLASMODIUM FALCIPARUM* (in collaboration with Dr. Yupin Charonevit (NMRI).

(a) Myeloma cell lines:

- P3-X63-Ag8: A mouse myeloma cell line secreting IgGlk, derived from MOPC-21, developed by E. Milstein.
- X63-Ag8-653: A non-secreting mouse myeloma cell line, derived from P3-X63-Ag8, developed by John F. Kearney, Andreas and Klaus Rajewski.

(b) Antigen:

Dihydrofolate reductase coupled to MTX-Sepharose (DHFR-MTX-SE). Methotrexate will be coupled to aminohexyl-spacers linked to CNBr-activated Sepharose as described (23). After the first Sephadex G50 column (to eliminate the human DHFR, see 4.6 for details) will be absorbed onto the MTX-Sepharose beads, centrifuged, and after extensive washing the DHFR-MTX-Sepharose complex will be used to immunize mice.

(c) Immunization protocal:

Balb/c Jackson mice will be immunized intravenously at 7- or 14-day intervals with optimal doses of the DHFR-MTX-SE complex. Three days after the third immunization spleen cells will be taken from each of these mice for fusion with one of the myeloma cell lines specified in (a).

(d) P3-X63-Ag8 or X63-Ag8-653 cell line will be maintained in log phase growth in Dulbecco's modified Eagle's medium containing 20% heat-inactivated fetal calf serum (DMEM-FCS). These cells will be used for fusion with cells from a single spleen ($\sim 10^8$ cells) obtained from each of the immunized mice using a procedure slightly modified from a method described by Gefter et al (24). Briefly, 10^7 myeloma cells will be mixed with 10^8 immune spleen cells and the cell mixtures will be pelleted by centrifugation. The pellet of mixed cells will be fused using 30% polyethylene glycol (mw. 950-1050) in D-MEM. The cell pellets will be resuspended in 30 ml of D-MEM-20 containing hypoxanthine and thymidine (HT medium), distributed into wells of microtiter plates (50 µl/well), and incubated at 37°C in 10% CO_2. Fifty microliters of HT medium to which 8.6×10^7 m aminopterin has been added will be pipetted into each well 24 hours later. The resulting medium consists of 4.3×10^{-7} m aminopterin in HT, the so-called HAT medium. An additional fifty microliters of HAT medium will then be added on day 6 after fusion. When growth appears (usually within 7-14 days) supernate will be removed and replaced with HT medium; the medium will be changed twice the first day. Supernates will be tested for the presence of antibody specific against DHFR three days later.

The cells from wells containing DHFR-specific antibody will be divided into 2 portions. One portion will be expanded, frozen in 10% dimethysulfoxide (10% DMSO), and stored in liquid nitrogen. The other portion will be cloned

by limiting dilution with and without feeder cells (irradiated Balb/c mouse spleen cells). The positive clones will be expanded, frozen in 10% DMSO, and stored in liquid nitrogen for future use.

(e) Detection of the anti-DHFR-Mab's

Parent lines will be tested for the presence of anti-DHFR-Mab's as follows:

i) Crude extracts of P. falciparum (see 4.5b) will be loaded onto Sepharose columns where the antibodies present in the parent lines will be coupled to protein A. The bound proteins will be too numerous and complex to analyze so that the flow-through of the protein A-column will be assayed for the presence of DHFR by the ^{3}H-MTX titration procedure (see 2.2). The presence of anti-DHFR-Mab's in the parent lines will therefore be detected by the absence of proteins binding to MTX in the flow through compared to the untreated control extracts.

ii) Alternatively, the crude extracts can be titrated first with ^{3}H-MTX and then passed over a protein A-antibody column. Among the proteins bound when using the parent lines will be the ^{3}H-labeled-DHFR, which can be later detected by autoradiography after elution of the columns and PAGE-electrophoresis.

iii) Among the materials used to immunize the mice (see b) will be MTX-Sepharose without DHFR bound to it. These molecules might have immunogenic properties and therefore there is a slight chance that anti-MTX-Mab's will be among the antibodies produced by the parent lines. These particular antibodies would be extremely important because they could not only be used to purify P. falciparum DHFR, but would identify all DHFR irrespective of its origin, since they would bind any DHFR by binding the attached substrate MTX. Therefore, anti-MTX-antibodies would give easy access

to the DHFR from rodent, avian, and monkey Plasmodium species and would allow inclusion of these different enzymes in a comparative analytical study. The following screening procedure will be used to detect anti-MTX-Mab's.

The protein A-columns with all the IgG's of the parent lines bound will be loaded with crude extracts of P. falciparum as described above. Among the antibodies produced will be antibodies which can be classified into 3 broad specificities - antibodies directed against various parasite proteins other than DHFR; antibodies directed against different parts of the DHFR which may or may not include the substrate binding site ; and hopefully Mabs against MTX. The first two types of antibody will bind their corresponding proteins and can be recognized by a test for binding of DHFR: no binding to ^{3}H-MTX in the flow through. The same column is then loaded with a crude extract reacted before with an excess of ^{3}H-MTX (for separation of bound and unbound MTX, see 2.2). Because all the antibodies except the anti-MTX antibodies have already reacted in the first step described above, the disappearance of ^{3}H-MTX-protein complexes in the flow-through of the second cycle indicates the presence of anti-MTX antibodies in the parent lines tested. The parent lines showing activities directed against the DHFR and/or MTX will be selected for cloning and the individual clones will be tested as described above.

4.6 ISOLATION OF DIHYDROFOLATE REDUCTASE

One unit of DHFR of malaria origin is defined as the amount of enzyme necessary to reduce 1 nanomole of substrate per minute under standard conditions compared to one enzyme unit from E. coli, hamster or mouse cells, which reduces one micromole substrate under the same conditions. This factor of 1,000 indicates that the DHFR from malarial parasites is far less active or less abundant than DHFR's obtained from other sources. Another feature of the malarial DHFR is its extreme instability causing 50% loss of activity after

holding the preparations for 12 hours in ice (25, and unpublished observations). Earlier investigations of the Principal Investigator revealed conditions under which the enzyme retains 80% of its activity by stabilizing the preparations with Ficoll-BSA-EDTA-DTT mixtures. This improved stability together with the mass-culture system brings the P. falciparum DHFR within reach of purification.

Earlier purification procedures were based on the molecular weight difference between the P. falciparum DHFR (180,000 daltons) and the human DHFR (60,000 daltons). Separation on a Sephadex G200 column resulted in a near total inactivation of the enzyme due to the minimum 18-hour separation time necessary. In this approach enzyme activity was the measure using the common photometric assay based upon the reduction of the cofactor $NADPH_2$ (26). For this reason an alternative purfication scheme will be followed:

1) Crude extracts obtained by ultracentrifugation (100,000 g) are passed over Sephadex G50 columns in order to remove the hemoglobin and the human DHFR (running as shoulder of the Hb peak).

2) The flow-through is loaded in small quantities onto the FPLC and by using the programable fraction collector, the identical fractions of each run will be collected in the same tube. This method shortens the separation time from 18 hours to 10 min. The DHFR containing fractions will be identified not by the time-consuming photometric assay but by their ability to bind ^{3}H-labelled methotrexate (see 4.2). The combined fractions will then be tested for activity using the assay above.

3) The pooled fraction will be absorbed batchwise onto Red Sepharose CL-6B, specific for NAPPH-requiring enzymes.

4) Impurities detected on SDS-gels will be removed by folate affinity chromatography where the ligand is reduced to dihydrofolate while bound using ascorbic acid (27).

Alternatively, the anti-DHFR-Mab's (4.5) are bound to Sepharose - protein A-columns and used to absorb the DHFR. Once we obtain a single band on heavily overloaded SDS gels after staining with sensitive silver stains and a reproducible pattern after peptide mapping, the protein will be given on a contract basis to companies for amino acid sequencing. A polypeptide consisting of 5-7 amino acids, having little variations in their codons (Meth, Trp, Tyr, Phe, Lys) will be selected to synthesize all possible 15-21 bp long polynucleotides to be used as a probe in 4.7. In addition the purified DHFR per se allows proper kinetic studies relevant to understanding the antifolate resistance of P. falciparum strains (see 2.2 and 4.2).

4.7 SCREENING OF THE cDNA LIBRARIES FOR THE PRESENCE OF NON-EXPRESSED P. FALCIPARUM GENES

The first cloning will be done using the hybrid pBR322-SV40 vector system as described in 4.4. The labeled polynucleotides (4.6) will be used as a probe to search for complementary sequences. Positive clones after hybridization will be tested for the insert size using the rapid Birnboim lysis method (28). Inserts having the size capable of coding for the entire DHFR molecule (or the antigen, see objective 10) will be selected and tested if they indeed code for the wanted products by a hybrid-select screening (29). The inserts are denatured and immobilized on activated cellulose filters (30) and hybridized to total P. falciparum mRNA. The RNA in the RNA-DNA hybrid formed is eluted and used in the in vitro translation system. In principle one protein should be found in each individual assay. The identification for the DHFR in such translation assays has been described in detail (4.3). The antigen can be detected by common immunoprecipitation, e.g., passing the ^{35}S-labeled proteins over protein A-Mab-columns, followed by PAGE gels. From theoretical considerations, up to 20 different plasmids can be used on one

filter before the filter is cut and separately eluted. Since the efficiency of the hybridization is only between 10-30%, only 0.1-0.3 μg of every microgram of total mRNA can be functionally recovered. The conditions for the hybridization have to be worked out because of the extremely high AT content of the P. falciparum DNA (for discussion, see 2.1). One translation experiment will be run with about 0.2 μg of eluted mRNA.

4.8 SCREENING THE cDNA LIBRARIES FOR EXPRESSED P. FALCIPARUM GENES

The expression of complete functional P. falciparum genes remains the ultimate aim of this proposal. Out of the whole variety of possible cloning strategies, only those are considered suitable for gene size to be expressed (4.7). Because new, better cloning vectors are developed constantly, a definite choice of what vector will be used will be made when the program is more advanced.

The following general considerations will influence the strategy:

1) A strong promotor should be present to initiate the mRNA at high frequency with an intact Pribnow box in position - 10 upstream of the initiation triplet and a second conserved region at - 35 (for review, see ref. 31). Because eucaryotic promotors function extremely poorly, if at all in E. coli, the choice of the right promotor carrying plasmid is very important.

2) In order to increase stability of the insert carrying plasmid, a terminator of transcription has to be placed downstream of the promotor.

3) Hybrid promotors (tac) will be used to start the search for expressed P. falciparum genes. Such promotors use the tryptophan - 35 region fused to the lactose - 10 region and are regulated by the lac repressor (32).

4) To achieve high levels of gene expression in E. coli, it is necessary to use not only strong promotors to generate large quantities of mRNA, but also ribosome-binding sites to ensure that the mRNA is efficiently

translated. A complete Shine-Dalgarno-sequence (33) is therefore essential. Therefore the cloning into a hybrid-ribosome binding site will be anticipated (34-35). Details are given in ref. 20.

The assays used to detect the expression of the cloned DHFR gene can be summarized as follows (see also flow chart at the end of the proposal).

1) Plating the transformed E. coli recipients onto trimethoprim-containing plates (rationale, see 2.2). Resistant colonies are likely to have the P. falciparum DHFR gene expressed.

2) Replica-plating of transformed E. coli onto NC-filters, lysis of the colonies and titration of the filters with increasing amounts of ^{3}H-MTX. E. coli expressing the P. falciparum DHFR gene will bind more ^{3}H-MTX than cells containing only the "standard" amount of E. coli DHFR.

3) The NC filters will be reacted with an anti-DHFR Mab, and a second antibody will be used carrying a fluorescent label.

4) The procedure in 3 is identical to the screening procedure for the expression of antigen-genes. The protective Mab, tested before in vivo (see objective 10) will be used to screen the cDNA libraries.

4.9 SEQUENCING GENES OF P. FALCIPARUM

Once gene(s) of P. falciparum libraries are identified either by hybrid select procedures (4.7), by hybridization with contract-made polynucleotides (4.6) or by expression (4.8), the DNA will be analyzed on the molecular level as follows:

1) Detailed restriction maps will be established by multiple digestion with two or more enzymes simultaneously, sequencial digestion of isolated fragments obtained after cleavage with one enzyme and subsequent cleavage with a second enzyme, and/or partial digestion of fragments specifically labeled at only one terminus (Smith and Birnstiel technique (36).

2) Depending on the distribution of the restriction cleavage sites the DNA will be subcloned for sequencing according to the Maxam-Gilbert technique (37). This technique has been used by the Principal Investigator to establish the complete DNA sequence coding for the DHFR of the R-factor R388 (12.2).

3) Alternatively, the didetoxy-sequencing method of Sanger (38) will be used after subcloning of the P. falciparum DNA into the M13 system (39).

4) Special attention will be given to the sequences flanking the gene (promotor, terminator regions).

5) cDNA clones will be used to identify genomic DNA segments. Sequencing selected parts of this DNA will reveal the structure of the splicing points. The information obtained will be processed and analyzed using computer programs as outlined in section 5.

5. COMPUTERIZED DATA ANALYSIS

The complexity of the expected results demands their evaluation with the help of a suitable computer. Firstly, the data have to be stored in a way that cross-checks can be routinely carried out, and secondly, the data have to be processed as described below, which is virtually impossible without computerization.

Once cloned genes (or fragments thereof) of P. falciparum are identified, a vast amount of data will accumulate in a short period of time, such as the length of restriction fragments, followed by sequencing data (see 4.9). To establish a complicated restriction map or handle several thousand sequenced nucleotides which are partially overlapping without computer help is not only extremely time consuming, but has such a high margin of error that the data cannot be trusted. Simple computer programs were developed several years ago by the Principal Investigator and his colleagues, but these programs are wholly inadequate for the complexity of the data expected. Software dealing with recombinant DNA research has been developed to answer a tremendous variety of sophisticated specific questions that arise once several kb of DNA sequence are available (see entire supplement 9, Nucleic Acid Research, 1981). The following selection of programs may illustrate the development using a XY scanner linked to a desk top computer using the appropriate software: Once DNA is cleaved with restriction enzymes, the scanner simply measures the distances from the origin after agarose gel electrophoresis.

1) The computer will calculate the actual molecular weight of the fragments.

2) The program analyzes the size of partial fragments and can therefore plot a provisional restriction map. These maps are the basis for subcloning strategies (4.9).

3) Once the DNA sequence is known the following tasks will be performed using the computer:

a) The DNA is scanned for all known recognition sequences of all restriction enzymes characterized (see ref. 46 for complete list). A printout of all those cleavage sites will be given, starting with base 1 from the ATG codon, coding for the start methionine.

b) The length of the restriction fragments obtained with every individual restriction enzyme will be calculated.

c) The length of the restriction fragments obtained from every double digest, e.g., the mixture of any of two restriction enzymes, will be calculated.

d) The plotter will draw one linear map for any of the selected enzymes.

e) The DNA will be scanned for any ATG start codon, in any of the six possible reading frames.

f) All the proteins in each of the six reading frames will be printed and knowing the approximate size of the wanted gene products only those proteins will selected, which fulfill the preconditions regarding the size

g) A total amino acid composition will be given of every protein encoded, which meets the size criteria.

h) Possible secondary structures in one DNA strand can be revealed by a "homology" program, e.g., are inverted repeated sequences or direct repeates present which are separated by non-complementary

sequences? What type of theoretical possible loops (promotor terminator sequences) are encoded in the DNA and do they obey thermodynamic laws?

i) Is the sequence found unique to P. falciparum DNA or are there extensive homologies between the malaria genes and the DNA sequenced from other organisms?

It is evident from even this incomplete selection of questions, that DNA sequences can only be handled adequately with computer assistance. The same is true for analyzing enzyme kinetics and comparing different enzyme preparations, prepared at different times with one another. The computer evaluation of this type of data is mandatory and therefore it is proposed to purchase an Apple IIe computer which interfaces with the ultraspec and for which the critical software is available. The capacity of the Apple IIe is sufficient to handle the amount of data to be expected of the proposed program. The fact that the "Applegration" from Dynamic Solutions Corporation is now available makes this computer very efficient (see justification).

To optimize use of the computer, a part time (75% effort) systems analyst/programmer/operator will be hired. The qualified person should have an insight into the problems outlined above and should screen the various marketed research software packages to see if it can be used without alterations for the questions arising such as listed under 1-3 in this section. He should be able to modify the existing programs and tailor them to the specific needs outlined. In addition, new programs will have to be written based on continuous in-depth discussions between the Principal Investigator and the analyst/programmer to facilitate the use of the computer

and make it accessable to other group members. Frequent searches for homologies between the DNA found in our lab and those stored in the comprehensive data bank in Los Alamos and the EMBO data base in Heidelberg, FRC should be performed routinely by the operator via the MODEM. Entry of data and operation of the computer will be the responsibility of the computer professional. In view of the variety and extent of the tasks to be performed and the problems to be solved, a computer professional should devote his time to these essential aspects of the recombinant DNA research proposed, so that the Principal Investigator can concentrate his efforts on other critical parts of the program.

In addition, the "raw data" would be processed as follows:

1) All known restriction sites would be listed.

2) Restriction maps would be drawn.

3) The size of all restriction fragments obtained by single or double digestion with any one of the known restriction enzymes would be given.

4) The given DNA sequences would be tested for overlapping sequences in order to establish the correct overall DNA sequence. This is critically important after shotgun cloning in M13 (ref. 40) and sequencing random inserts.

5) The distribution of initiation and termination codons will be indicated.

6) The length of all possible proteins and their amino acid sequence would be determined in all six reading frames.

7) A search for possible promotor-like structures would be conducted.

8) Any possible inverted or parallel repeats exceeding 5 base

pairs would be localized.

9) Possible secondary base matches would be searched for detecting possible loop structures.

10) Any given DNA fragment (> 50 bp) would be tested individually for extensive homologies with other known DNA sequences stored in the central data banks.

6. Collaborative Arrangements

It is planned to collaborate with various groups within and as well as outside the AID network. Up until now, an agreement with two research groups has been reached: Dr. Yupin Charenovit (Naval Medical Research Institute, Bethesda, has agreed to raise anti-DHFR-Mab's (see 4.5) using purified DHFR. The AID group in Bogota is willing to supply relevant hyperimmune sera to test the expression of cloned antigens. Once the cDNA libraries are established it is planned to approach other AID groups for hyperimmune sera and/or Mabs. *Protective* Mabs serve as the basis for the program illustrated on the right-hand side of the flow chart at the end of this proposal (see also objective 10 in Chapter 3). The selected Mabs are not the only useful probe to detect the expression of relevant antigen-genes in the cDNA libraries (4.8) but are evidently a prerequisite to purifying the antigen itself. The identical approach used for both the anti-DHFR-Mab's and the protective Mabs derived from this collaboration is discussed in the sections 2.3, 4.7 and 4.8 and emphasized in the mirror-image arrangement of the objectives in the flow chart.

SUPPLY OF ISOLATED MEROZOITES

Once the dialysis system (4.1) has produced a sufficient quantity of parasites to start the immunization program to raise anti-DHFR-Mab's (4.5), to isolate the RNA (4.3) and to purify the DHFR (4.6), the parasites will be harvested and separated (see 6.3 below), and purified, isolated merozoites will be made available to other groups in exchange for Mabs. The system is expected to produce 1.2×10^{11} schizonts within 90 hours (for detailed calculations, see 4.1) and under the assumption that 8 merozoites per schizont are present at the time of harvest, 9.6×10^{11} merozoites could be obtained. A 60% yield after purification would still allow for harvesting $5\text{-}6 \times 10^{11}$ separated, highly purified merozoites.

7. Facilities

It is planned to establish a new self-contained AID laboratory at BRI. About 2,500 sq. ft. of space will be available so that a tissue culture room, a walk-in 37°C incubator, a walk-in refrigeration room 4°C, a small radioisotope lab, a darkroom, glass washing facilities, general lab space and office space can be established. It is planned to complete the structural alterations two months after the beginning of the project. This ensures the total independence of this AID project from the ongoing NMRI project at BRI.

8. REFERENCES

1. Cochrane, A.H., Santuro, F., Nussenzweig, V., Gwadz, R.W. and R.S. Nussenzweig. 1982. Monoclonal antibodies identify the protective antigens of sporozoites of Plasmodium knowlesi. Proc. Natl. Acad. Sci. 79: 5651.
2. Sherman, I.W. 1979. Biochemistry of Plasmodium (malarial parasites). Microbiol. Rev. 43: 453.
3. Homewood, C.A. and K.D. Neame. 1980. Biochemistry of malarial parasites. In: J.P. Kreier (ed.), Malaria, Vol 1, Academic Press, New York, p. 345.
4. Goman, M., Langsley, G., Hyde, J.E., Yankovsky, N.K., Zolg, J.W. and J.G. Scaife. 1982. The establishment of genomic DNA libraries for the human malaria parasite Plasmodium falciparum and identification of individual clones by hybridisation. Molec. Biochem. Parasitol. 5: 391.
5. Hyde, J.E., Zolg, J.W. and J.G. Scaife. 1981. Isolation and characterisation of ribosomal RNA from the human malaria parasite Plasmodium falciparum. Molec. Biochem. Parasitol. 4: 283.
6. Blakley, R.L. 1969. The biochemistry of folic acid and related pteridines. North Holland, Amsterdam, p. 139.
7. Nakamura, H. and J.W. Littlefield. 1972. Purification, properties, and synthesis of dihydrofolate reductase from wild type and methotrexate-resistant hamster cells. J. Biochem. 247: 179.
8. Bostock, C.J., Clark, E.M., Harding, N.G.L., Mount, P.M., Tyler-Smith, C., van Heyningen, V and P.M.B. Walker. 1979. The development of resistance to methotrexate in a mouse melanoma cell

line. Chromosoma 74: 153.

9. Stone, D., Phillips, A.W. and J.J. Burchall. 1977. The amino-acid sequence of the dihydrofolate reductase of a trimethoprim-resistant strain of Escherichia coli. Eur. J. Biochem. 72: 613.

10. Zolg, J.W. and U.J. Hanggi. 1981. Characterisation of a R plasmid-associated, trimethoprim-resistant dihydrofolate reductase and determination of the nucleotide sequence of the reductase gene. Nucl. Acids Res. 9: 697.

11. Lee, F., Mulligan, R., Berg, P. and G. Ringold. 1981. Glucocorticoids regulate expression of dihydrofolate reductase cDNA in mouse mammary tumor virus chimaeric plasmids. Nature 294: 228.

12. Chang, A.C.Y., Nunberg, J.H., Kaufman, R.J., Erlich, H.A., Schimke, R.T. and S.N. Cohen. 1978. Phenotypic expression in E. coli of a DNA sequence coding for mouse dihydrofolate reductase. Nature 275: 617.

13. Ferone, R., Burchall, J.J. and G.H. Hitchings. 1969. Plasmodium berghei dihydrofolate reductase, isolation, properties, and inhibition by antifolates. Mol. Pharmacol. 5: 49.

14. Thaithong, S. and G.H. Beale. 1981. Resistance of ten Thai isolates of Plasmodium falciparum to chloroquine and pyrimethamine by in vitro tests. Trans. R. Soc. Trop. Med. Hyg. 75: 271.

15. Kan, S.C. and W.A. Siddiqui. 1979. Comparative studies on dihydrofolate reductases from Plasmodium falciparum and Aotus trivirgatus. J. Protozool. 26: 660.

16. Jensen, J.B. and W. Trager. 1977. Plasmodium falciparum in culture: Use of outdated erythrocytes and description of the candle jar method. J. Parasitol. 63: 883.

17. Zolg, J.W., MacLeod, A.J., Dickson, I.H. and J.G. Scaife. 1982.

Plasmodium falciparum: Modifications of the in vitro culture conditions improving parasitic yields. J Parasitol. (in press)

18. Flintoff, W.F. and K. Essani. 1980. Methotrexate-resistant chinese hamster ovary cells contain a dihydrofolate reductase with an altered affinity for methotrexate. Biochem. 19: 4327.

19. Aviv, H. and p. Leder. 1972. Purification of biologically active globin messenger RNA by chromatography on oligothymidilic acid-cellulose. Proc. Nat'l. Acad. Sci. 69: 1408.

20. Molecular Cloning. 1982. T. Maniatis, E.F. Fritsch, J. Sambrook, eds.) Cold Spring Harbor Laboratory, New York. 545 pages.

21. Okayama, H. and P. Berg. 1982. High-efficiency cloning of full-length cDNA. Mol. Cell. Biol. 2: 161.

22. Mandel, M. and A. Higa. 1970. Calcium dependent bacteriophage DNA infection. J. Mol. Biol. 53: 154.

23. Kaufman, B.T. 1974. Methotrexate-agarose in the purification of dihydrofolate reductase. In: Methods in Enzymology (W.B. Jakoby and M. Wiecheck, eds.) Vol. 34, Academic Press, New York, p. 272.

24. Gefter, M.L., Marguliers, D.H. and M.D. Scharff. 1977. A simple method for polyethylene glycol-promoted hybridization of mouse myeloma cells. Somatic Cell Genetics 3: 237.

25. Ferone, R. and S. Roland. 1980. Dihydrofolate reductase: thymidylate synthetase, a bifunctional polypeptide from Chrithidia fasciculata. Nat'l. Acad. Sci. 77: 5802.

26. Osborn, M.J. and F.M. Huennekens. 1958. Enzymatic reduction of dihydrofolic acid. J. Biol. Chem. 233: 969.

27. Then, R.L. 1979. Purification of guanosine triphosphate cyclohydrolase I and dihydrofolate reductase on a

dihydrofolate-sepharose affinity column. Analyt. Biochem. 100: 122.

28. Birnboim, H.C. and J. Dolg. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7: 1513.

29. Parnes, J.R., Velan, B., Felsenfeld, A., Ramanathan, L., Ferrini, U., Appella, E. and J.G. Sidman. 1981. A screening procedure for cDNA clones corresponding to rare mRNAs. Proc. Natl. Acad. Sci. 78: 2253.

30. Seed, B. 1982. Diazotizable arylamine cellulose papers for the coupling and hybridization of nucleic acids. Nucleic Acids Res. 10: 1799.

31. Rosenberg, M. and D. Court. 1979. Regulatory sequences involved in the promotion and termination of RNA transcription. Ann. Rev. Genet. 13: 319.

32. de Boer, H.A., Comstock, L.Y., Yansura, D.G. and H.L. Heynecker. 1982. Construction of a tandem trp-lac promotor and a hybrid trp-lac promotor for efficient and controlled expression of the human growth hormone gene in Escherichia coli. In: Promotor Structure and Function (R.L. Rodriguez and M.Y. Chamberlain, eds.), Praegar Publishers, New York (in press).

33. Shine, Y. and L. Dalgarno. 1975. Determinant of cistron specificity in bacterial ribosomes. Nature 254: 34.

34. Goeddel, D.V., Yelverton, E., Ullrich, A., Heynecker, H.L., Miozzari, G., Holmes, W., Seeburg, P.H., Dull, T., May, L., Stebbing, N., Crea, R., Maeda, S., McCandliss, R., Sloma, A., Tabor, J.M., Gross, M., Familetti, P.C. and S. Pestka. 1980. Human leucocyte interferon produced by E. coli is biologically active.

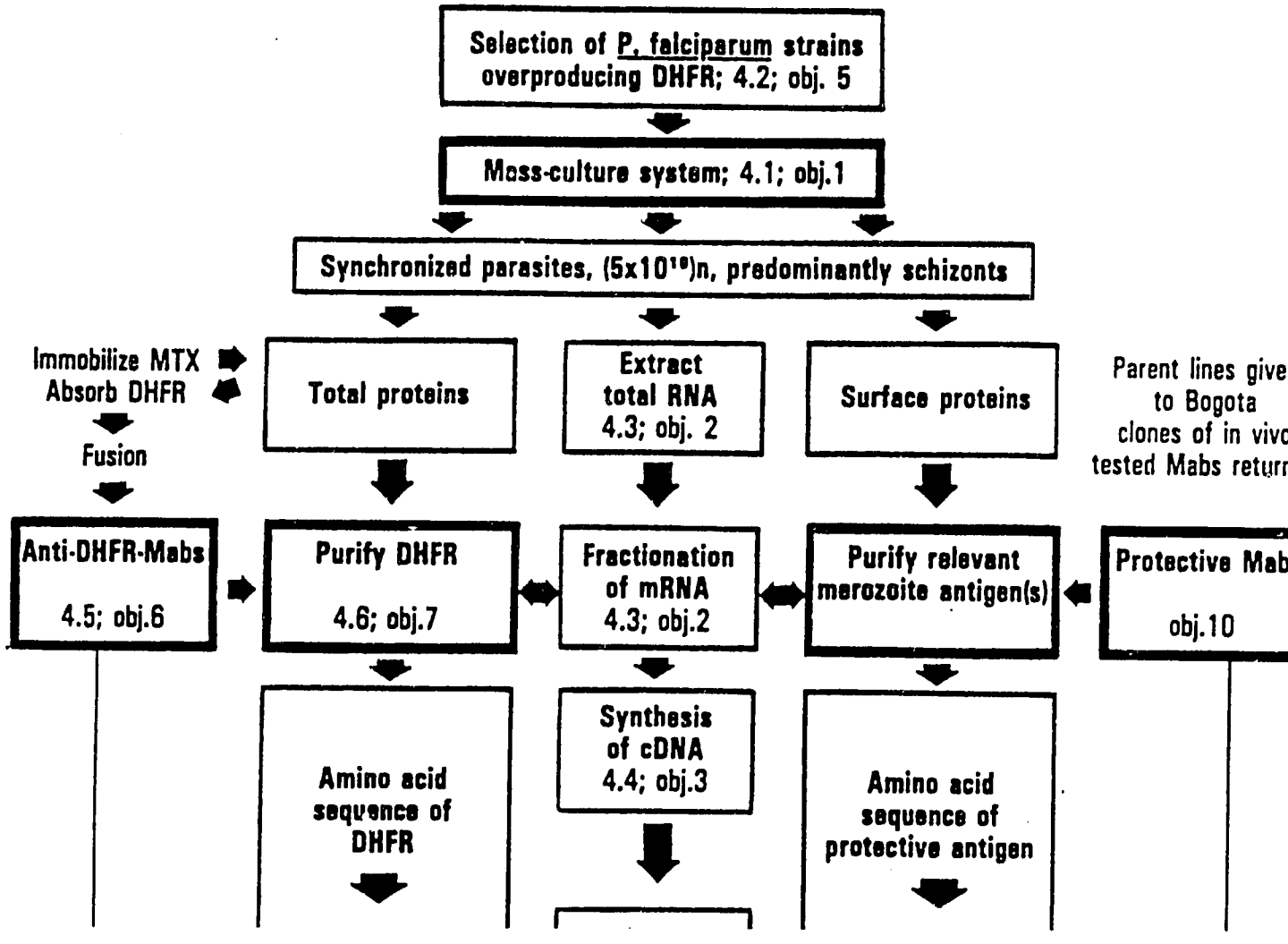
Nature 287: 411.

35. Edman, J.C., Hallewell, R.A., Valenzuela, P., Goodman, H.M. and W.J. Rutter. 1981. Synthesis of hepatitis B surface and core antigens in E. coli. Nature 291: 503.

36. Smith, H.O. and M.L. Birnstiel. 1976. A simple method for DNA restriction site mapping. Nucleic Acids Res. 3: 2387.

37. Maxam, A.M. and W. Gilbert. 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods in Enzymol. 65: 499.

38. Sanger, F., Nicklen, S. and A.R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Nat'l. Acad. Sci. 74: 5463.

40. Messing, J., Crea, R. and P.H. Seeburg. 1981. A system for shotgun DNA sequencing. Nucleic Acids Res. 9: 309.

SUMMARY OF THE RESEARCH STRATEGY FOR CLONING SPECIFIC GENES OF PLASMODIUM FALCIPARUM