Memoranda
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Memoranda are statements concerning the conclusions or recommendations of certain WHO scientific meetings; they are signed by the participants in the meeting.

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The epidemiology of drug resistance of malaria parasites: Memorandum from a WHO Meeting*

This Memorandum presents current knowledge concerning the epidemiology of drug resistance of malaria parasites and outlines 33 research proposals which could lead to a better understanding of that epidemiology and to a better management of the problem.

The epidemiology of drug resistance of malaria parasites is important for the planning, implementation and evaluation of malaria control programmes. Several factors have contributed to the deterioration of the malaria situation in the past decade; prominent among these is drug resistance. Antimalarial drugs are now widely available so that the regulation of their distribution and use presents problems. In addition, the appearance of *Plasmodium falciparum* with varying degrees of insusceptibility to chloroquine and related compounds in many parts of Asia and the Western Pacific, South America, and eastern and central Africa is seriously jeopardizing the value of this drug which is commonly used by the primary health care systems in developing countries. Elucidation of many aspects of this complex problem is urgently required through carefully designed and executed research work, which should examine general epidemiological issues as well as specialized parasitological and chemotherapeutic problems.

* This Memorandum is based on a report (document TDR/FIELDMAL/SWG (4)/86.3) which was drafted by the participants at a meeting organized by the Scientific Working Group on Applied Field Research in Malaria of the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR). The meeting was held in Geneva, Switzerland, on 10–14 November 1986 and the names of the participants are listed on pages 815–816. Requests for reprints of this Memorandum or for copies of the full report should be addressed to the Office of the Director, TDR, World Health Organization, 1211 Geneva 27, Switzerland. A French translation of this Memorandum will appear in a later issue of the *Bulletin*.

REVIEW OF CURRENT KNOWLEDGE

Current knowledge about the epidemiology of drug resistance of malaria parasites was reviewed in 19 working papers, which were discussed at the meeting.[a] While some differences of opinion persisted, it is hoped that they will be resolved by further research. The main points in all the areas discussed are presented below.

Historical and geographical review of malaria parasite resistance to drugs

South-east, central and western Asia.[b] Resistance of *P. falciparum* to inhibitory compounds of dihydrofolate reductase (DHFR) was observed shortly after their introduction in several independent foci. This resistance was closely associated with, and subsided upon the removal of, the drug pressure.

Resistance of *P. falciparum* to chloroquine is believed to have arisen on the Thai–Kampuchean border about 1957 and spread in all epidemiologically-feasible directions—eastwards across the Indochina peninsula and west across Thailand—within 3 years. One of the leading epidemiological

[a] A limited number of copies of individual working papers are available, on request, from the Secretary, Scientific Working Group on Applied Field Research in Malaria, MAP/EME, World Health Organization, 1211 Geneva 27, Switzerland.

[b] Working paper presented by D. Clyde.

factors that favoured the spread of chloroquine-resistant *P. falciparum* appears to be migration of non-immune and semi-immune persons, carrying resistant gametocytes to the receptive vectors. It seems the resistant parasite may possess a biological advantage over the sensitive parasite, but this has to be confirmed. The variety of efficient vectors in these parts of Asia shows no constraints in carrying the resistant parasite. Control operations have had little success in containing resistance, and indeed the widespread use of chloroquine for presumptive treatment has probably facilitated the dispersal of resistance.

In the early 1960s, chloroquine-resistant *P. falciparum* spread across Thailand and entered Western Malaysia. Under continuing chloroquine pressure in the core area, the parasite not only consolidated to higher grades of resistance but subsequently showed a diminishing susceptibility to amodiaquine, quinine, sulfadoxine–pyrimethamine and mefloquine when these drugs were used during the ensuing 20 years.

The resistant parasite was carried south to Indonesia around 1970, and reached all the Indonesian islands and Irian Jaya by 1974. From the north-west of Thailand it was carried across Burma to Bangladesh in the late 1960s, and was first confirmed in north-east India in 1973. After consolidating there, the parasite was carried to Orissa (1978) and then rapidly moved westwards across central India, with extensions to the north and south. It was identified as being transmitted in Pakistan in 1983, in Sri Lanka and Nepal the following year, and at about the same time was reported from eastern Iran. The possibility that the parasite was carried to East Africa around 1977 from one or other of the many Asian foci cannot be refuted.

Africa south of the Sahara.[c] In the review of *P. falciparum* resistance in Africa to the standard antimalarial drugs, particular attention was paid to the rapid changes in the situation with respect to chloroquine resistance and the epidemiological factors related to its apparent geographical spread from east to west across the continent. Resistance of *P. falciparum* to DHFR inhibitors was found in at least 13 countries. Cross-resistance between proguanil, chlorproguanil, and pyrimethamine has been reported from different areas, although the pattern of cross-resistance is not uniform.

The first well-documented case of chloroquine resistance was found in a tourist who had contracted the disease in Kenya at the end of 1977. *P. falciparum* chloroquine resistance rapidly spread over East, Central and southern Africa and more recently in West Africa. *P. falciparum* resistance to the combination pyrimethamine/sulfadoxine (Fansidar) was reported from Kenya and the United Republic of Tanzania in 1982, and to the combination pyrimethamine/dapsone (Maloprim) from Kenya in 1982. A reduction of *P. falciparum* sensitivity to quinine has been recorded in recent years in Tanzania, Burundi, Kenya and Zaire, and a case of falciparum malaria resistant to mefloquine was reported from Tanzania in 1983.

The mobility of the human population and the consequent spread of resistant parasites, associated with heavy malaria transmission, might have contributed to the emergence and spread of *P. falciparum* chloroquine resistance in Africa. It is suggested that other factors which might have played a role are an association between pyrimethamine and chloroquine resistance; heavy drug pressure and the utilization of subcurative doses of the drug; and a possible biological advantage of chloroquine-resistant parasites over sensitive ones.

East Asia and Western Pacific.[d] Resistance to chloroquine of *P. falciparum* in the Western Pacific Region has been observed since 1961, spreading south-eastwards across the malarious countries of the Region in a way that suggests geographical diffusion during a period of 20 years from the Indochina peninsula to Vanuatu. The speed of this diffusion and its intensity appeared to be greater under conditions of heavy transmission and drug pressure. Chloroquine resistance appeared more frequently in young children than in adults in endemic areas, suggesting the involvement of immunological factors.

Since 1980, *in vitro* testing for chloroquine-resistant *P. falciparum* indicated a stabilization in intensity and distribution of resistance, compared with the previous 5 years, in the countries that have long harboured resistant parasites. Sensitive strains still occur focally. Differences in *P. falciparum* sensitivity to mefloquine occur, but below the level of resistance, and a similar situation is apparent in respect of sulfadoxine–pyrimethamine. Consequently chloroquine or amodiaquine in full courses are still widely in use, local policy being guided by the results of *in vivo* and *in vitro* monitoring.

Experience in the region suggests that there are no practical ways to contain chloroquine-resistant *P. falciparum* once it is well established in an endemic area.

The genetics of drug resistance in malaria parasites; genetic markers and their uses in the epidemiology of drug resistance

Genetics of the response of malaria parasites to drugs.[e] The genetics of resistance to some antimalarial drugs has been studied in rodent models.

[c] Working paper presented by E. Onori.

[d] Working paper presented by T. Matsushima.

[e] Working paper presented by D. Walliker.

Resistance to both pyrimethamine and chloroquine is found to be due to spontaneous gene mutation. For pyrimethamine, a high level of resistance may be due to a single mutation in the gene for the enzyme DHFR, causing a marked decrease in its ability to bind to the drug. There appears to be no increased quantities of the enzyme, which implies that gene amplification is not a factor.

For chloroquine, high resistance appears to be due to the simultaneous presence in a parasite of several mutations, each conferring a low degree of resistance. The mutants are difficult to produce in the laboratory because prolonged exposure is required, and, when produced, they may be stable or unstable. They have not developed following single-step treatment. Crosses of chloroquine-resistant and sensitive clones with pyrimethamine-resistant and sensitive clones, which confirmed that each type of resistance is discrete and controlled by genes at different loci, have resulted in high proportions of chloroquine-resistant progeny and low proportions of pyrimethamine-resistant progeny, suggesting differential selection.

Implications of the evidence from rodent models have been applied to *P. falciparum*. Since many patients having falciparum malaria harbour mixed infections of more than one genetically distinct parasite of this species, recombinant-type parasites are produced in the mosquito at high frequencies, and these result in varied characteristics of the sporozoites inoculated into fresh hosts, including genetic patterns differing from the parasites which gave rise to the mosquito infections.

The genetic basis of resistance to a given drug, and the ability of drug-resistant genes to recombine, have important implications. For a drug such as chloroquine, widespread use of a low dose of the drug is likely to select resistant forms, which may be due to mutations of different genes in different parasites. Mosquito transmission of mixed parasites could bring more than one gene together in a single parasite, thus producing a higher level of resistance. Conversely, if drug pressure is released, it can be predicted that hybridization between highly resistant and sensitive parasites may occur, resulting in the segregation of different resistant genes from one another, thus breaking down the level of resistance.

Distribution of genotypes within P. falciparum *isolates and within parasite populations.*[f] Characterization of *P. falciparum* parasites is carried out using various biological and biochemical markers, especially enzyme types and protein-variants. Cloning studies have shown the presence of different genotypes within isolates. Clones vary in their drug response which has important implications for (*a*) the selection of resistant parasites by subcurative treatment and (*b*) the definition of clinical resistance. However, reports of the emergence of chloroquine-resistant lines of *P. falciparum* from within the chloroquine-sensitive clones need verification, since contamination by resistant clones has not been ruled out.

P. falciparum resistance to mefloquine has been correlated with markers, in contrast to the situation in respect of chloroquine. Recent studies with a clone (W2) submitted to continuous mefloquine drug pressure *in vitro* have shown stable DNA changes that may be associated with the acquisition of resistance to this drug. At the same time, resistance to chloroquine diminished in intensity.

Methods for the characterization of malaria parasites.[g] The rapid changes in drug sensitivity patterns in several continents require better means of monitoring, e.g., by biological markers of drug resistance and of geographic origin of isolates. Immunologists and molecular biologists have developed for other purposes a wide range of new tools which are applicable.

Among the characterization methods available, most (e.g., 2d-gel maps of proteins, iso-enzymes, chromosome-sized DNA patterns) require a large amount of parasites (10^6–10^7) and need to be performed from cloned parasite populations (which require culture, and whose relevance to the clonal diversity of the original isolate has to be ascertained). A few methods allow identification of individual parasites in freshly collected isolates (e.g., monoclonal antibody labelling).

The techniques available can also be classified according to whether they concern the protein content (e.g., iso-enzymes, polymorphic proteins) or the DNA content. In the latter case, identification methods may extend to genes coding for other stage proteins and non-coding fragments. They may therefore appear more appropriate, but at the present time are frequently more time-consuming.

Since the mechanisms of resistance to quinolines have not yet been identified, two types of approaches to define specific markers can be envisaged: (1) the empirical approach in which some correlation between the markers of a given assay and the drug-resistant character is looked for; (2) a targetted approach aimed at identifying the DNA fragment specific to the resistance mutation.

Biological and biochemical mechanisms of drug resistance

Mechanisms and characteristics of drug resistance in leishmania: relevance to studies of drug resistance

[f] Working paper presented by V. E. do Rosario & S. Thaithong.

[g] Working paper presented by P. Druilhe.

in malaria.[h] Studies of drug resistance in simpler model systems are relevant to studies of drug resistance in malaria. Resistance to the antifolate methotrexate (MTX) may be readily obtained in promastigotes of *Leishmania*. Three mechanisms are involved: overproduction of DHFR–TS (thymidylate synthetase) mediated by amplification of the gene encoding the DHFR-TS gene, decreased MTX transport, and an unknown mechanism mediated by an amplification of the H region of DNA. The three mutations may occur singly or in combination. The mutations may also occur in unstable and stable forms, the unstable types tending to evolve into stable types.

The properties of certain drug-resistant malaria parasites resemble those of *Leishmania* bearing amplified DNA. As amplified DNA may be readily visualized, it is appropriate to survey drug-resistant field and laboratory isolates for its presence.

The biochemical and biological mechanisms of drug resistance in malaria parasites[i] are summarized below:

(*a*) Related to the mode of action of the drug:

(i) decrease in affinity of the target (simple mutation);

(ii) increase in quantity of the target (gene amplification or reduction in turnover of target);

(iii) target no longer important (metabolic bypass);

(iv) increase in the quantity or affinity of a natural competitor for the drug (it could be an increase in quantity of a natural substrate for an enzyme).

(*b*) Unrelated to the mode of action of the drug:

(i) permeability change, which may be related to change in the affinity or quantity of a transport protein mediating influx or efflux of the drug, or to change in the lipid composition of a membrane;

(ii) increase in the quantity or affinity of an unimportant alternative target for the drug;

(iii) organism degrades the drug to an inactive product (e.g., penicillinase in bacteria).

Some of these mechanisms are known to occur in malaria. For example, resistance to drugs such as pyrimethamine which affect dihydrofolate reductase depends on a decrease in the affinity of the mutant enzyme for the drug (see (*a*) (i) above). Resistance to sulphonamides may in some cases be related to synthesis of *p*–amino benzoic acid (PABA) by the parasite (see (*a*) (iv) above). Resistance to blood schizontocides like chloroquine and quinine apparently relates to less effective total uptake of drug (see (*b*) (i) above), although the details of how this is achieved and the mechanism of action of such drugs in sensitive parasites are not yet clear.

A scheme for the mode of action of blood schizontocides and for the mechanisms of resistance was proposed, which could explain the facilitation of chloroquine-resistant development in pyrimethamine-resistant organisms, and the loss of natural insensitivity by *Plasmodium yoelii* under PABA depletion.

Further studies on the modes of action of antimalarials and on the mechanisms of resistance are desirable, in order to understand ways of designing more effective drugs and to develop strategies for overcoming resistance to existing drugs.

Experimental models and their relationship to the epidemiology of parasite resistance in the field

Drug pressure and drug response of malaria parasites: the experimental evidence.[j] Laboratory experiments using rodent malaria models have proved highly predictive of the risk for human malaria parasites of developing resistance to a variety of drugs. To what degree these models can predict the potential value of drug combinations in preventing such resistance has still to be confirmed since there are obvious deficiencies in the models employed up to the present time.

Nevertheless, certain parallels have been demonstrated in this respect and there is good reason to pursue the policy of avoiding monotherapy in the treatment of malaria in man, especially infections with *P. falciparum*. Some parallels may be drawn also from another protozoal infection with certain affinities with malaria, namely, avian coccidiosis.

Suggestions were made for a number of field projects to test the value of selected drug combinations in limiting transmission and impeding the emergence of resistance. At the same time a warning was given about possible enhanced toxicity from *ad hoc* drug mixtures. It was strongly recommended that evidence should first be obtained from laboratory studies before a new drug combination is field tested in man. The failure of chloroquine–pyrimethamine against falciparum malaria could have been predicted from the rodent malaria model.

Relative fitness of sensitive and resistant parasites

Biological fitness of drug-resistant and drug-sensitive malaria parasites in relation to different vectors.[k] Natural susceptibility or resistance of *Anopheles* to the malaria parasite has not been fully explained. Selective transmission of either drug-resistant or drug-sensitive parasites could affect the spread of resistant malaria. Chloroquine is unusual in

[h] Working paper presented by S. M. Beverley et al.

[i] Working paper presented by D. C. Warhurst.

[j] Working paper presented by W. Peters.

[k] Working paper presented by R. G. Andre.

that it appears to be beneficial to the survival of resistant parasites. Once parasites have become resistant to chloroquine, some results indicate that further exposure to this drug may lead to higher infection in mosquitos.

Studies were reviewed that have dealt with the relationship between the malaria parasites and antimalarial drug action in the vector. Results from studies on chloroquine, quinine, mefloquine, primaquine, pyrimethamine, sulfadoxine/pyrimethamine and proguanil against *P. falciparum* parasites were discussed. Also examined were reported data on chloroquine, mefloquine, pyrimethamine, sulfadoxine/pyrimethamine and proguanil against *P. vivax* parasites. Many laboratory studies have looked at the gametocytocidal and sporontocidal effects of various antimalarials in mosquitos using *P. gallinaceum*. These results were summarized in tabular form. Drug action against *P. cynomolgi* also was mentioned.

Secondly, the importance of certain anopheline species in the transmission of drug-resistant malaria was reviewed. The intensity of malaria transmission may directly affect the spread of drug resistance. Complicating this picture is the existence of sibling-species complexes whose individual member species may play different roles in the transmission of drug-resistant malaria.

Lastly, possible experimental steps were listed that may delineate some of the mechanisms behind the evolution of resistant malaria strains. These steps incorporate the use of mosquito transmission to allow for recombination of sensitive and resistant parasites.

Insecticide resistance in vectors: lessons to be learned

Factors involved in the epidemiology of resistance of vectors to insecticides.[l] The 1986 report of the WHO Expert Committee on Vector Biology and Control on resistance of vectors and reservoirs of disease to pesticides lists 139 species of vectors as displaying resistance. However, resistance does not evolve at the same rate in all vector populations that come under selection pressure. Numerous cases of divergent rates of evolution of resistance are known, and elucidation of the causes is useful for understanding the epidemiology of resistance and designing countermeasures.

(1) behavioural increased sensitivity to insecticide (DDT) and avoidance of treated habitats (many insecticides); (2) increased detoxication (DDT, carbamates, pyrethroids, organophosphates); (3) decreased sensitivity of target site (carbamates, organophosphates, pyrethroids, DDT, dieldrin, BHC (HCH)); (4) decreased cuticular penetration (most insecticides). The selection of resistance in the field is influenced by numerous factors—genetic, biotic, behavioural and ecological, and operational involving the nature and function of the insecticide and its application (timing, dose, formulation, extent of coverage), alone or alternating with other compounds.

Factors involved and their interplay; alternative drug utilization policies for field evaluation

The epidemiology of drug resistance in malaria: an unfinished conceptual model.[m] The measures advocated for the control of the drug resistance problem depend on conceptual models, explicit or implicit, of the epidemiology of the phenomenon. Although it is not yet possible to construct a comprehensive explanatory model, a review was made of the components required and of what we know about them.

The *basic genetics* is very imperfectly known. In the case of chloroquine, the most probable genetic mechanism consists of multiple additive mutations; that model fits Padua's crossing experiments very well indeed if one assumes variable contributions of gametocytes by the parent clones and a steep decrease in survival with increasing resistance. The *relative fitness* of sensitive and resistant parasites is also very imperfectly known. It is claimed that chloroquine-resistant *P. falciparum* has a "biological advantage" over the sensitive parasite, even in the absence of drugs; if this is so, then at least one additional hypothesis is required to explain that the resistance waited for drug pressure before emerging. Furthermore, the claims in favour of a biological advantage are questionable: in the vector they commonly involve the erroneous assumption of statistical independence between mosquitos fed on the same host or between oocysts found on the same gut; in the erythrocytic cycle, the advantage demonstrated by resistant *P. chabaudi* in mice or by resistant *P. falciparum* in culture may not apply to *P. falciparum* in man. In the presence of drugs, a mixture of clones of different sensitivities will, in theory, be shifted towards increased resistance by an increase in the drug concentration, as long as not all parasites are killed; there is also some experimental evidence that higher concentrations select resistance faster.

Basic genetics and relative fitness are influenced by the *parasite's environment*: drug pressure (depending on patterns of utilization and pharmacokinetics), intensity of transmission, innate and acquired immunity, diet, and population movements. Finally, the conceptual model was compared with the *epidemiological behaviour of drug resistance*, in particular with the "diffusionist" theory and with the incrimination of low ("subcurative") doses. Historical maps

[l] Working paper presented by G. P. Georghiou.

[m] Working paper presented by L. Molineaux.

of chloroquine resistance suggest actual diffusion, but do not prove it; it is difficult to reconcile diffusion from a single point of emergence with the hypothesis of multiple additive mutations and with expected mutation rates: furthermore, the alternative hypothesis of geographically uniform mutation rates and drug pressure, combined with baseline geographic variation, would produce rather similar historical maps of resistance, even in the absence of diffusion. The incrimination of low doses lacks satisfactory controls. In conclusion, we do not really know even some of the things we thought we knew, and further research is needed. Simulations may assist, at least in clarifying the discussion.

The control and prevention of malaria parasite resistance to antimalarial drugs in man: some thoughts and suggestions for research.[n] A distinction should be made between the absolute and relative frequencies of parasitic resistance. For instance, the suggested systematic addition of primaquine to the first-line drug, chloroquine, might well decrease the absolute frequency of resistant parasites in the human population by interfering with the sporogonic cycle. However, the relative frequency of the resistant genotype in the parasite population may well be increased as a result of its effect on the existing drug-sensitive gametocytes.

The major need in practical terms is to preserve the effectiveness of our existing drugs for as long as possible. This means drug policies and drug usage directed towards preventing, delaying, stabilizing and/or reversing drug resistance. Where feasible, vector control measures and individual and community action to reduce contact between man and mosquito are important for managing the problem of drug resistance. Concerning drug usage, four aspects in particular need to be studied. These are the value of drug associations providing an additive and/or potentiating schizontocidal effect or a schizontocidal and gametocytocidal/sporontocidal effect; the impact of alternating drugs, for example at different levels and seasons of transmission, or of using different drugs at different levels of the primary health care referral system; the activity of various dosage regimens, particularly the impact of low dosage regimens (e.g., 10 mg/kg of chloroquine base vs 25 mg/kg), on persistence of drug-resistant infections and on gametocytogenesis; and the effects of restricting the use of alternative drugs (alone or in combination) when resistance to one or more of these is apparent.

Geographical containment of spreading chloroquine and sulfadoxine/pyrimethamine resistance has been attempted, sometimes on a large scale, but has not been very successful. A natural barrier may exist in barring South American chloroquine-resistant *P. falciparum* from Panama, Middle America and Hispaniola, and this needs elucidation.

[n] Working paper presented by P. F. Beales.

Methodological issues

The validity of methods of assessing the response of malaria parasites to drugs for epidemiological studies.[o] The *in vivo* tests include the "WHO standard 7-day test", the "extended 28-day test", and the "single-dose test". The extended test is required to differentiate early recrudescence (i.e., between 7 and 28 days) and full sensitivity. In areas of on-going malaria transmission, the possibility of reinfection made the interpretation of reappearance of parasitaemia uncertain. The 7- and 28-day tests provided excellent data on the response to standard therapeutic regimens. The single-dose test was widely used in semi-immune populations, particularly in Africa, to establish minimum therapeutic dosages. There have been attempts at simplification of *in vivo* tests in order to save money, manpower, and supervision. This usually entails the reduction in the number of days of observation, the loss of the ability to determine the clearance time, and the reduction of close clinical supervision.

The *in vitro* tests for drug resistance have basically taken two forms: a macro test using serial dilutions to obtain the minimum inhibitory concentration (MIC) of the drug required to obtain inhibition of schizont maturation, and a micro test using a standard 96-well microtitration plate predosed with serial dilutions of the test drug. Test plates for chloroquine, mefloquine, quinine and amodiaquine have been produced. Development is in progress of *in vitro* micro tests for sulphonamides and pyrimethamine. A number of alternative *in vitro*-type tests have been used. These include a modified 48-hour test for chloroquine, mefloquine, quinine and sulphonamides, a similar test for pyrimethamine, modified 24- and 48-hour tests for pyrimethamine and sulphadiazine, a visual test, a radioassay test, a semi-micro test, an isotopic micro test, and a fluoroassay test. Evaluation of the reliability of these tests under field conditions is in progress. Of the *in vitro* test systems currently available, the WHO standard *in vitro* micro test appears to offer the best facilities for the global monitoring of *P. falciparum* susceptibility to chloroquine, amodiaquine, quinine and mefloquine.

Methods for the detection and measurement of antimalarial drugs in tissues and body fluids.[p] The choice of a proper analytical method for drug analysis depends on its application. Pharmacokinetic studies require blood sampling and use of sophisticated

[o] Working paper presented by D. Payne.

[p] Working paper presented by Y. Bergqvist.

sensitive and specific methodologies such as liquid chromatography. Compliance studies may only require a simple qualitative test of urine samples.

Analytical conditions are crucial. If biological specimens are taken without sufficient care in handling, the results may be invalidated even when the most sophisticated analytical techniques are used. As an example, chloroquine and amodiaquine are degraded by sunlight. Absorptive losses of drugs must be considered especially when the drug is in contact with glass surfaces. Analysis of chloroquine and sulfadoxine will give different results depending on whether whole blood, plasma or serum is used.

Validation is essential. Methods for determination of antimalarials must be repeatedly validated by analysing control samples of known concentration with the patients' samples. Standardization of analytical methods is essential as results must be comparable between and independent of the laboratories that performed the analyses. A reference laboratory and reference methods for antimalarial drug assays should preferably be established.

Immunoassays for antimalarial drugs.[q] Recent laboratory-based research has confirmed the potential of immunoassays for measuring antimalarial drugs in body fluids. Specificity and sensitivity of the most accurate methods depend on chromatographic separation of the drug and/or its metabolites from other compounds; sophisticated equipment and highly specialized staff are needed, which limits their use in many malaria endemic areas. Simple colorimetric tests such as the Dill-Glazko, lignin, and Bratton-Marshall are relatively insensitive and non-specific, and there is a need for relatively simple, specific assay tests usable in the field.

As a result of the production of high titre antibodies, ELISA or fluoroimmunoassay (FIA) techniques have been developed which are capable of measuring drug levels as low as 5 ng/ml for chloroquine, quinine and primaquine and as low as 20 ng/ml for pyrimethamine and dapsone. Several ELISA inhibition tests have been developed for this purpose, and are based on the antimalarial in solution competing with (*a*) solid-phase-bound drug for the binding of enzyme-labelled specific antibodies, or (*b*) enzyme-labelled drug for binding to solid-phase-bound specific antibody. The tests may be read visually (semi-quantitative) or spectrophotometrically (quantitative). Fluoroimmunoassay tests for drugs are becoming an alternative to radioimmunoassays and enzyme-immunoassays. The system is simple, stable, sensitive and rapid, but the reaction cannot be read visually. The tests are valid for chloroquine, quinine and primaquine.

[q] Working paper presented by P. I. Trigg et al.

Variations of the ELISA technique are being introduced for malaria diagnosis in many laboratories in endemic areas, and the equipment and trained personnel are widely available for using the same technology for determining drug levels.

Methods of assessing patterns of utilization of antimalarials through questionnaires and/or interviews of distributors and/or consumers.[r] Drug supply measured by import statistics and availability in pharmacies gives some indication of utilization but does not account for supplies being smuggled, sold through the informal sector, etc. Specific methodologies were suggested for investigating (i) what the health workers prescribe, (ii) what the people are actually taking for an episode of malaria, and (iii) the perceived side-effects that affect their choices.

The populations that should be sampled may be summarized as follows:

(i) *Health worker survey*. Sample as many ranks of health workers as possible. Keep these distinct as a stratified sample, and in analysis, compare the strata.

(ii) *Community survey*. Sample three groups: urban, peri-urban poor, and rural people. Keep these groups distinct as a stratified sample, and in analysis compare the strata. Include males and females, adults and children.

Statistical treatment of the in vitro *test data.*[s] Pooling the results from *in vitro* tests to obtain estimates of the EC_{50}, EC_{90} and EC_{99} provides no proper indication of the precision of these estimates. Two alternative approaches were described:

(i) *The use of a single concentration*. The degree of inhibition of schizont maturation at a single concentration is evaluated for each subject. The distribution of this value over subjects is characterized by the median and the inter-quartile range. It is shown by ordering countries in terms of resistance to chloroquine, that the use of this technique is very similar to the use of information from all wells.

(ii) *The use of individual EC_{50} values*. A simple interpolation method for determining individual EC_{50} values (for each subject) is proposed and compared to a statistically efficient method. The interpolation method provides a simple basis for comparing groups of subjects, using standard statistical methods.

Methodological issues in the epidemiological studies of drug resistance in P. falciparum *malaria.*[t] The issues involved belong to three main categories. The first involves *assessment of drug response* (the dependent variable). Problems of sampling arise

[r] Working paper presented by C. P. MacCormack.

[s] Working paper presented by M. Hills et al.

[t] Working paper presented by S. Goriup & L. Molineaux.

owing to lack of representativity and inadequate size. Measurement of drug response of parasites *in vivo* requires simpler standard tests, having fewer days of follow-up and larger samples; inclusion of information on clinical response, immune status, and detection and follow-up of treatment failures is necessary. *In vitro* testing is constrained by competition between different test methodologies without standard conversion factors, lack of tests for some of the drugs, excessive numbers of concentrations in the test, difficulties in preserving specimens for delayed testing, and inability to serve as a guide to individual treatment. Analysis and interpretation of results are hampered by use of different indices and lack of a standardized statistical approach. A simple method for measuring parasitaemia and drug level simultaneously would be useful.

The second category involves *assessment of the independent variables*. A number of issues arise in connection with the basic genetics of resistance and the differential fitness by genotype. Environmental variables that raise problems of assessment include geographic mobility of parasites and people, and patterns of drug pressure and utilization.

The third category involves *assessment of association between dependent and independent variables*. Serious problems are caused by lack of baseline data, for example, on the initial degree of drug susceptibility of *P. falciparum* in many areas; by lack of adequate controls, e.g., in evaluating the association of a developing resistance with population movement or use of subcurative drug dosage; and by the multiplicity of factors and variables involved, and the extent of their interdependence and unpredictability.

OUTLINES OF RESEARCH PROPOSALS

The meeting drafted 33 research proposals which are necessarily sketchy. While they go far beyond recommendations, they are obviously not blueprints. They express the suggestions of a selected group of experts, who quite understandably could not agree on all the proposals. Also, the inclusion of a proposal does not necessarily imply WHO or TDR approval or funding. The outlines, as given below, have been further condensed.

Basic mechanisms (and directly related methodological research)

1. *The genetic basis of resistance to antimalarial drugs* (see also No. 3 and 33 below). The objective is to determine the genetic basis of resistance to antimalarial drugs, especially to newly developed drugs, because the emergence and spread of drug resistance in microorganisms can be explained only by an understanding of the genetics, about which little is known. Genetic studies will identify the genes involved in drug resistance and studies on their inheritance will help the interpretations and predictions on their spread in the parasite population. It is important to establish the number of genes involved in resistance to a given drug, the number of alleles at a given drug resistance locus, and whether high-level resistance is due to a major structural gene, to an additive effect of mutations at different loci, or to gene amplification, etc. Such information has important bearings on our understanding of how resistance to a given drug builds up and on the possible lowering of resistance levels if the drug pressure is removed. This knowledge can lead to the development of specific gene probes to determine whether resistance to a drug which has emerged in different regions of the world is caused by mutations at identical or different loci.

With respect to methodology, in addition to studies on *P. falciparum*, it is important to develop experimental laboratory models. The following methods are examples of approaches that may be pursued.

(*a*) *Identification of drug resistance loci by genetic mapping.* Genetic analysis may demonstrate linkage of the drug-resistance phenotype with known loci. This requires: (i) a large number of marker loci, preferably previously mapped, scattered around the genetic map; these variable loci may consist of isoenzymes, antigens, or restriction enzyme cleavage sites; (ii) the ability to perform genetic crosses; this most likely will require rodent malaria models. The method involves crossing between drug-sensitive and resistant lines, examination of progeny for association of R phenotype with markers, and verification of the association by subsequent crossing.

(*b*) *Identification of drug-resistant loci by biochemical methodology.* Directed methods may be used for certain drugs for which current data suggest the nature of at least some mechanisms of resistance. The proteins responsible for the resistance should be identified and characterized biochemically. These data may then be employed in the isolation and development of specific reagents for the detection of the resistant protein, possibly using monoclonal antibody technology or alternatively by isolating the gene encoding the protein and developing specific oligonucleotide probes. Suggested examples include investigations of a possible chloroquine carrier, of DHFR, and of the enzymes responsible for the synthesis of folates and their precursors. Nondirected methods may be used for many drugs for which the biochemical lesions are unknown. As alterations in protein structure have been shown to lead to alterations in migration in two-dimensional

gel electrophoresis, this method may be employed to identify specific proteins altered in drug-resistant lines. Identification of a protein will lead to its isolation and use in the development of probes, as described previously.

(*c*) *Survey of drug-resistant lines for gene amplification.* In many organisms selected for resistance to a variety of drugs the resistance is mediated by amplification of specific loci. These loci may correspond to the drug target, or a locus responsible for detoxication. Amplified DNA may be visualized by the presence of amplified DNA fragments after cleavage by a battery of restriction enzymes. Both laboratory and field isolates should be examined; examination of clones may increase the sensitivity of the amplification assay.

2. *Drug resistance and uptake/efflux of drugs.* The objective is to determine whether the mechanisms of uptake and/or efflux of drugs and other substances differ in drug-sensitive and resistant forms. There are indications that resistant parasites have reduced accumulation of drugs. This may also be related to alteration in the functioning of carrier systems. Research in this area is likely to lead to a rational development of new drugs, as well as to strategies for overcoming resistance—by analogy with the use of β-clavulinic acid in overcoming β-lactamase production in penicillin-resistant bacteria.

The ability of resistant parasites to accumulate antimalarial drugs should be examined. Where decreased accumulation is found, studies should be carried out to determine whether the changes are due to decreased uptake or increased efflux. Given the multiplicity of membranes present, isolated parasites should be used in addition to parasitized cells. Cloned drug-sensitive and resistant parasites of *P. falciparum* and of rodent malarias should be used. The mechanism of uptake, whether carrier-related or otherwise, should be determined, and the effects of inhibitors which alter membrane potential and ion gradients examined. The role of drug carriers in normal cellular metabolism should be investigated.

3. *Identification of DNA probes specific for drug-resistant* P. falciparum (see also No. 1 above and 33 below). The objective is to develop one or several probes which will enable identification of various types of drug resistance in *P. falciparum* by means other than *in vitro* sensitivity assays. Such probes would be essential tools to monitor and analyse the spread of drug resistance.

As *P. falciparum* parasites differ from one another by numerous factors, the search for a specific DNA marker of drug resistance must be undertaken in artificially pure parasite populations, as alike as possible except for their drug response. It will be necessary to: (i) induce a stable resistance by *in vitro* drug pressure in a drug-sensitive *P. falciparum* clone; (ii) hybridize the DNA extracted from the original sensitive and the resistant clone, and isolate the non-hybridizing fragment(s). The next steps depend on the size and the number of the recovered DNA fragments. Basically they would be used to screen several other sensitive and resistant cloned lines, and isolates. The above steps may have to be repeated (eventually by inducing resistance in the presence of mutagenic substances) in order to identify the several distinct mutations involved in resistance mechanisms.

Experimental production/selection of drug resistance

4. *The effect of mosquito transmission on the development of resistance.* Most previous experimental studies on the selection of drug-resistant forms of malaria have been carried out on parasites maintained only by blood passage. As genetic recombination occurs readily during mosquito passage it is necessary to determine whether cyclical transmission is important for the emergence of resistance to a given drug or drug combination.

The most appropriate models for these studies are certain species of rodent malaria. The use of both cloned and uncloned mosquito-transmissible parasites will be necessary depending on the questions to be addressed. The procedures involve exposing drug-sensitive parasites to appropriate doses of the drugs under investigation, with cyclical passages carried out as frequently as possible. The response of the selected parasites should be monitored by established techniques such as the "2% test".

5. *The effect of drug combinations on the emergence of resistance* (see also No. 20 below). The objective is to investigate whether appropriate drug combinations can impede the emergence of resistance in experimental models which incorporate cyclical transmission. Data from experiments not using cyclical transmission indicate that the use of certain drug combinations can greatly delay the development of drug resistance. It is important to test whether this observation applies under conditions of cyclical transmission.

The methodology is essentially the same as that used in No. 4. Controls will include lines treated exclusively with a single compound as well as lines left untreated. Two drugs that should be looked at in this context are mefloquine and one or other of the artemisinin analogues. It is also essential to look both at combinations which have been found to be synergistic and at others which are essentially additive. It is important to be aware that certain drug combinations may be antagonistic. Experiments suggested under proposals No. 4 and No. 5 would be run concur-

rently. Computer modelling should also be extended in an attempt to forecast the effect of using drug combinations in field conditions.

6. *The effect of resistance to one antimalarial on the selection of resistance to other antimalarials.* Field observation of *P. falciparum* and evidence from selection experiments in the laboratory indicate that resistance to pyrimethamine may predispose a parasite to becoming chloroquine-resistant. Much more information on this subject is required, with particular emphasis on new drugs. Initially the possible influence of pyrimethamine should be further investigated. Two types of model can be utilized:

(*a*) *P. falciparum in vitro.* Clones characterized with respect to their response to pyrimethamine and other drugs are required: starting with pyrimethamine-sensitive and resistant clones in continuous culture, passages are made with increasing selection pressure of other compounds (e.g., chloroquine, mefloquine, artemisinin); the response to pyrimethamine and the other drugs is monitored in successive passages of each line by such parameters as growth and reproduction rate, and classical *in vitro* micro testing.

(*b*) *Rodent Plasmodium in mice.* Essentially the same procedure is followed, starting with clones of, for example, *P. berghei* defined as regards their response *in vivo* to pyrimethamine and other relevant compounds.

7. *Dosage, pharmacokinetics and the development of drug resistance* (see also No. 17 below). The objective is to determine to what extent the rate at which resistance to a given drug develops depends on its dose and pharmacokinetic properties. It has been suggested that low, rather than high, drug doses favour the emergence of resistant forms. It is also possible that drugs with a long half-life and repository preparations may facilitate the emergence of such mutants.

The most suitable model with relevance to the field situation is a mixture of cloned, drug-sensitive and drug-resistant lines of rodent malaria *in vivo*. The choice of drug should include one for which a relatively high frequency of resistant genes has been demonstrated (e.g., pyrimethamine) and a second drug for which a lower frequency is suspected (e.g., chloroquine). Groups of male mice of the same genetic background and weight are infected with equal aliquots of parasitized donor blood. When the parasitaemia reaches a preset high level, animals of one group receive a single dose of drug equivalent to the maximum tolerated dose (MTD) (e.g., 60 mg/kg subcutaneously of chloroquine). Parasitaemia levels are monitored daily and blood is passaged to clean animals if and when the parasitaemia recrudesces. When the level reaches an adequate figure in the recipients, they are treated with the same drug dose, and so on in subsequent passages. Infected blood from mice of each passage is used to infect additional animals in which a standard "2% test" is carried out to monitor the level of drug response with the same drug. The same procedure is followed but using one third and one tenth of the MTD in other series of mice. The dynamics of the acquisition of resistance to the test drug(s) are compared between the three series.

The relative fitness of resistant and sensitive parasites in the presence of drugs

8. *The effect of antimalarial drugs on sensitive and resistant* P. falciparum *clones in terms of gametocyte production, infectivity to vectors, and sporogonic development.* The objectives are to: (*a*) establish the baseline gametocyte conversion rates of sensitive and resistant *P. falciparum* clones; (*b*) determine the effect of antimalarial drugs, particularly sulfadoxine/pyrimethamine and mefloquine, on the gametocyte conversion rates; (*c*) establish the baseline susceptibility of a cytotyped isoline of the anopheline vector for sensitive and resistant clones; (*d*) establish the infectivity of gametocytes to a vector following treatment of both the sensitive and the resistant clones with antimalarial drugs; (*e*) quantify sporozoite production in vectors fed upon the sensitive and resistant clones before and after treatment; (*f*) determine the viability of sporozoites obtained from infective feeds on the sensitive and resistant clones following treatment with an antimalarial drug.

Justification: (*a*) it was shown in Thailand that treatment of *P. falciparum* patients with a combination of sulfadoxine and pyrimethamine greatly increased the frequency and density of gametocytaemia and that the gametocytes were infective; (*b*) the above studies were done in 1975 and 1976 when sulfadoxine/pyrimethamine cured over 90% of the *P. falciparum* patients, while earlier treatment results indicated widespread resistance to pyrimethamine when used alone; (*c*) it was suggested that widespread use of sulfadoxine/pyrimethamine might increase the levels of gametocytes in the human population, potentially infecting more vectors and thereby indirectly enlarging the reservoir of disease in an area; (*d*) it is important to ascertain if *P. falciparum* parasites that are resistant to sulfadoxine/pyrimethamine will respond in the same way as sensitive parasites; (*e*) similarly, the sporontocidal effects (if any) of mefloquine need elucidation.

Methodology: (*a*) establish a cytogenetically identified isoline of a malaria vector species that occurs in an area where *P. falciparum* parasites resistant to sulfadoxine/pyrimethamine are known to occur;

(*b*) establish cloned cultures of *P. falciparum*, some that are susceptible to sulfadoxine/pyrimethamine and some that are resistant to this drug combination; (*c*) establish baseline gametocyte conversion rates; (*d*) feed mosquitos on gametocytes contained in membrane feeders and determine the degree of susceptibility of the vector; (*e*) feed mosquitos on cultures that have been treated with sulfadoxine/pyrimethamine or mefloquine and determine infectivity levels by dissection; (*f*) quantify sporozoite levels in salivary glands using the sporozoite ELISA kit; (*g*) determine the infectivity of the sporozoites by inoculation of cultured hepatocytes according to the Hollingdale technique.

9. *The effect of antimalarial drugs on the sporogony of resistant parasites*. There is some experimental evidence that chloroquine stimulates the development of chloroquine-resistant parasites in the mosquito. The validity of this observation needs to be confirmed, as it would have important implications for the spread and maintenance of resistance genes. In addition, the possibility that resistant mutants could be selected in the mosquito should be investigated.

This work will be best carried out with the rodent malaria species, but gametocyte-producing lines of *P. falciparum* should also be studied. Cloned drug-sensitive lines, and resistant clones derived from them should be compared for their infectivity to mosquitos, both in the presence and absence of the drug concerned. Oocyst and sporozoite rates in the mosquitos would be examined. Using rodent malarias, the parasites developing from the resulting sporozoites would also be examined for alterations in drug response.

The relative fitness of resistant and sensitive parasites in the absence of drugs (see also No. 25 and 26 below)

10. *The fitness of drug-resistant malaria parasites* (see also No. 11 below). The objective is to examine whether resistance to an antimalarial drug can be associated with a biological advantage for the resistant parasites. Field results show that chloroquine resistance in *P. falciparum* has spread remarkably fast. There is also some evidence from rodent species of plasmodium that chloroquine-resistant mutants can outgrow sensitive forms. So far, however, information on this subject has been limited to only a few experiments, and further research is needed.

This subject requires careful consideration as to the laboratory model most appropriate to field conditions. Ideally a series of independently collected, cloned parasite lines exhibiting resistance to a given drug should be obtained, and the selective advantage or disadvantage of each resistant gene examined in different genetic backgrounds. Studies would involve making deliberate mixtures of appropriate resistant and sensitive clones and following their respective fate in blood infections, and following mosquito transmission. The rodent malaria species will provide the most appropriate material for this work. Studies should also be made using resistant and sensitive clones of *P. falciparum* mixed in different proportions in culture. It would be particularly informative to compare the fitness of parasites exhibiting resistance to drugs of contrasting type, e.g., pyrimethamine and chloroquine.

11. *Studies on the possible biological advantage of resistant parasites* (see also No. 10 above). The spread of chloroquine-resistant parasites makes it desirable to undertake studies on the possible biological advantages of these strains over sensitive ones. Using a *P. falciparum* model, it is feasible to study: (*a*) the erythrocytic cycle of resistant and sensitive clones *in vitro*; (*b*) the production of gametocytes of these clones in culture, and then infecting mosquitos with them, using different vectors; (*c*) the results of mixing known proportions of both resistant and sensitive clones and following them in culture, in order to detect any possible advantage of one type of parasite over the other; this may involve, in addition to the drug response itself, the use of other characterization methods.

Using a rodent malaria model, 5–15 continuous mosquito transmissions would be undertaken using at the beginning 3 different proportions of R vs S parasites (10% R, 90% S; 50% R, 50% S; 90% R, 10% S). During mosquito transmission, clones from infected mice will be compared to evaluate proportions of resistant vs sensitive parasites.

12. *The susceptibility of vectors from different geographical areas to isolates of drug-susceptible and resistant* P. falciparum *from their own and other areas* (see also No. 13 below). The objectives are to: (*a*) determine the susceptibility of anopheline species (including sibling species where they are known to exist) to local drug-susceptible and resistant clones of *P. falciparum*; (*b*) determine the susceptibility of the main vector species in a designated area to imported chloroquine-susceptible and resistant clones from other selected geographical areas; (*c*) compare the longevity of the various vector species used when uninfected and infected with either the susceptible or resistant clones of the parasite.

Justification: there are three basic hypotheses with regard to occurrence and spread of resistance, namely (*a*) introduction of resistant parasites from a long distance; (*b*) diffusion from a central focus; (*c*) multifocal emergence of drug-resistant mutants; vector

susceptibility studies are relevant for the investigation of these hypotheses. As an additional complication there are indications that different members of a group of sibling vector species may vary in their ability to transmit *P. falciparum*, or one or other of the drug-resistant and susceptible variants. This may also have a bearing on spread and containment of drug-resistant malaria.

Methods and measurements: (*a*) characterization of the relevant vector species/sibling species by an appropriate taxonomic method and establishment of isolines; (*b*) isolation of local *P. falciparum* and production and cultivation of susceptible and chloroquine-resistant clones; (*c*) transmission/exchange of vector and parasite material among the institutions involved; (*d*) determination of the gametocyte conversion rate of each clone used so that they can be calibrated if necessary; (*e*) membrane feeding of the characterized vector species on each of the clones; (*f*) maintenance of the vector species for the period of the gonotrophic cycle, with periodic sampling to determine production of oocysts and sporozoites; (*g*) estimation of sporozoite production from each vector/clone combination by ELISA, or other improved method when available; (*h*) estimation of the longevity of the vector in each combination, compared to an uninfected control group.

13. *Relative fitness of resistant and susceptible genotypes in the sporogonic cycle* (see also No. 12 above). The objective is to determine whether or not the resistant parasites are more efficiently transmitted by susceptible mosquito populations, and subsequently to investigate whether resistance will eventually be spread more quickly in areas of high intensity of transmission. The information gathered from experimental models or laboratory work may suggest certain measures that might be undertaken to reduce malaria transmission in areas where resistance has recently been introduced.

(*a*) The efficiency of successive steps in sporogonic development can be assessed by counting the gametocytes, ookinetes, oocysts and sporozoites in salivary glands by the ELISA method, and by ascertaining the minimum number of sporozoites to establish infections in mice or counting EE schizonts in cultured hepatocytes. Attempts will be made to detect significant differences between resistant and sensitive parasites at any one of these steps.

(*b*) Experimental procedure with *P. berghei* and *P. chabaudi* in mice or rats, using as vectors *Anopheles stephensi* or *A. atroparvus*: (i) cloning from a resistant line could provide R and S clones; (ii) S clones could also be made resistant by the classical methods, eventually first to pyrimethamine and then to chloroquine, after prior characterization for gametocyte production and infectivity for mosquitos; (iii) R and S clones have to be characterized separately for virulence, gametocyte conversion rate, and infectivity to the mosquito; (iv) preparation of mixtures of resistant and susceptible parasites for feeding mosquitos may be made by either inoculating a group of mice with one R and one S clone, comparable from the point of view of virulence and gametocyte production, or by mixing *in vitro* parasitized blood from S clone and R clone, ensuring equal numbers of female gametocytes from the two clones.

Observations to be made on the resulting sporogonic cycle, comparing S, R and mixed clones; (i) counting the oocysts; (ii) counting the sporozoites; (iii) producing sporozoite-induced infections in a group of mice with the mixed sporozoite populations either by feeding infected mosquitos on mice or by dissection of salivary glands and inoculations of batches of sporozoites into mice; (iv) cloning from mice infected, as indicated above, and testing the clones for their drug resistance.

(*c*) Experimental procedure with *P. falciparum* in culture or in simian hosts. Possible vectors include *A. stephensi*, *A. gambiae* and *A. freeborni*. The parasitic material consists of isolates and clones of cultured *P. falciparum* that is sensitive, naturally resistant, or artificially made resistant to chloroquine, and mixtures of clones of R and S material, characterized for gametocyte production. Observations to be made include determinations of the number of oocysts, number of sporozoites, percentage of viable sporozoites worked out after inoculations into culture of hepatocytes or monkey hosts, and also measurement of resistance in a series of clones issued from the resulting mixed populations.

Effect of the parasite's environment: host factors

14. *The effect of antimalarials in the absence of an immune response*. The objective is to examine whether an antimalarial drug by itself can eliminate parasitaemia in the absence of an immune response, in order to resolve the question whether host immunity plays a role in the elimination of parasitaemia by drugs. This relates to the importance of the level of endemicity in relation to the rate at which resistance emerges.

This is also to some degree an extension of the experiments previously reported. A rodent malaria model is clearly optimal for this purpose, although consideration could be given also to an avian model such as *P. gallinaceum*. The course of treated and untreated infections should be compared in intact and immunodeprived albino mice in which humoral or cell-mediated responses, or both, have been eliminated by appropriate techniques. Drugs used should

include different classes as regards both modes of action and chemical structures. Evidence for surviving infection in any animals that appear to become free of patent parasitaemia for at least one month can be sought by splenectomy (of non-immunodeprived animals), subinoculation of blood into clean splenectomized animals, and challenge with homologous parasites. The drug response of any surviving parasites to the compound used initially should be monitored to detect if drug-resistant organisms have emerged in the course of the experiments.

15. *Studies on possible changes in expression of drug resistance and gametocyte production due to host genetic factors.* Chloroquine resistance has not developed in a similar manner in different parts of the world and this may be due to causes related to parasite differences or factors related to vectors and transmission. Interference of host factors may also be involved. Studies of some of these factors are proposed, in particular concerning the effect of red blood cells and/or serum from donors from different regions upon: (*a*) the expression of resistance by *P. falciparum* in *in vitro* drug assays; (*b*) the production of gametocytes and/or infectivity to mosquitos of resistant parasites.

Clones of *P. falciparum*, well characterized for chloroquine resistance and sensitivity and gametocyte production are used. It is necessary throughout the study to do drug assays periodically and ensure gametocyte production. This work should be carried out in situations where donation of blood/serum from required donors would be available on a continuous basis.

Effect of the parasite's environment: insecticides and drugs

16. *Influence of insecticide stress on the sporogony of drug-resistant and drug-sensitive* P. falciparum. The objectives are to: (*a*) determine whether exposure of a vector to sublethal concentrations of insecticide (DDT) enhances sporogony of *P. falciparum* (if so, is such enhancement affected by insecticide resistance or drug-resistance?); (*b*) determine the dose–effect relationships of such exposure (are DDT-residues, even in areas no longer treated with DDT, still high enough to induce this effect?); (*c*) determine the biochemical/physiological bases for this phenomenon.

Justification: (*a*) exposure of organisms to sublethal chemical stress may in certain cases affect the rate of growth or differentiation of the organism; in mosquitos, exposure of late-fourth instar larvae to an insecticide (DDT, temephos, etc.) accelerates the pupation rate; in house flies, the exposure of females to dieldrin results in increased egg production; (*b*) consideration must also be given to the possibility of dissimilar influences by the insecticide on drug-resistant and drug-sensitive *P. falciparum*.

Methodology: the study can be conducted on anopheline species of which DDT-resistant and DDT-sensitive strains are available such as *A. gambiae*, *A. arabiensis*, *A. albimanus*, *A. stephensi*. It involves the following: (*a*) determine the susceptibility of the mosquito to DDT and define the appropriate sublethal exposure for the DDT-sensitive and DDT-resistant strains; (*b*) determine the susceptibility of the DDT-resistant and DDT-sensitive vectors to *P. falciparum*; (*c*) expose the two mosquito strains (sensitive and resistant) to DDT immediately after infective feeding and at different times thereafter; (*d*) assess the effect of these exposures on the sporogonic cycle of the treated and control groups and compare these according to insecticide resistance and drug resistance.

17. *The relation between the terminal elimination period of an antimalarial drug, given for treatment, and minimal inhibitory concentrations preventing parasite multiplication—implications for the selection of resistance* (see also No. 7 above). A promotion of resistance to various antimalarials by subcurative dosages has been postulated. A selection of less susceptible parasites might also occur when patients recently treated for malaria are reinfected at a time when a substantial amount of the treatment drugs remains in the body. In order to study this possible mechanism of selection of drug resistance, baseline data on the MICs and the terminal elimination half-life of the treatment drug must be known. Unfortunately the minimum inhibiting concentrations for most antimalarials are poorly established *in vivo*. In addition, the terminal elimination phase of antimalarial drugs with long elimination times has not been fully described.

Starting approximately one week after medication, blood samples for drug analysis and thick films are collected every three to four days. At the time of recrudescence or patent reinfection, the drug concentrations are obviously not inhibitory any longer. Provided that the transmission is high and the drug elimination time is long the observed concentrations will, however, be close to the MICs. Appropriate measurements of drug concentrations during the study can easily be used to determine terminal elimination times.

Observational studies of P. falciparum *in human populations: descriptive studies*

18. *Baseline geographical distribution of the response of malaria parasites to new antimalarials.* This information is important to plan strategies for the deployment of new drugs (especially mefloquine and artemisinin) on a rational basis, as well as to provide

baseline data for monitoring the development and spread of resistance. Among the newly developed drugs, only the response of *P. falciparum* to mefloquine is currently being tested on a relatively large scale.

The baseline prevalence of resistance to new drugs should be determined by appropriate *in vitro* susceptibility tests and drug resistance probes (if and when such are developed) prior to the introduction of these drugs operationally. Such studies could be carried out within the context and extension of the WHO global monitoring system and could require in the case of artemisinin and its derivatives the development of appropriate *in vitro* susceptibility tests.

19. *Characterization of original and recrudescent parasites.* Some studies have shown that recrudescent parasites may be genetically different from the original isolates from which they were selected by a certain drug treatment. It is proposed that studies should be carried out using biological/biochemical markers to compare parasites from the original and the recrudescent population. These studies should include production of gametocytes and infectivity to mosquitos.

These studies should preferably be grafted onto standardized drug trials in which there is assurance that one is dealing with recrudescences and not with new infections. They will involve the following: (*a*) to cryopreserve isolates at the beginning of each trial; (*b*) to undertake characterization studies with both uncloned and cloned material from both the original and each recrudescent population; (*c*) to produce gametocytes and membrane-fed mosquitos with uncloned/cloned material both from the original isolate and the recrudescence; (*d*) for a later stage, to compare studies on the data obtained from analysis of recrudescent parasite populations obtained from *different* geographical origins but receiving identical treatment; this will enable ascertainment of any common pattern of identification, other than drug resistance.

20. *The effect of using mefloquine or mefloquine/sulfadoxine/pyrimethamine on the development of resistance.* The objective is to monitor the response of *P. falciparum* in patients treated with mefloquine and mefloquine/sulfadoxine/pyrimethamine where they are being used operationally, in order to assess the continued usefulness of these drugs, and to compare the speed with which resistance may arise in each instance.

Observational studies of P. falciparum *in human populations: analytic studies*

Analytic studies aiming at the identification of the factors determining the emergence and development of drug resistance, and at the evaluation of their relative importance and of their interactions, are confronted with serious constraints, including the multiplicity and interdependence of the factors involved and the largely unpredictable rate at which resistance emerges and develops. The four proposals in this section illustrate different possible approaches.

21. *Analysis of the existing information on the evolution of drug resistance.* It is suggested to utilize all currently available information and data, including that not yet collated, on the phenomenon of the susceptibility of malaria parasites to antimalarial drugs, for the purpose of forming a data base for analysing past events, predicting evolutionary trends, and providing better information for the development of rational drug policies.

This would involve the following; (*a*) to review all known references to drug resistance; (*b*) to collate all reported data (additional to the WHO Global Monitoring Programme inputs) related to *in vivo* and *in vitro* tests for susceptibility to chloroquine and other antimalarial drugs; (*c*) to continue the collation and analysis of country *in vivo* and *in vitro* drug-susceptibility test reports, submitted under the Global Monitoring Programme (in particular, to monitor the status of current antimalarial drug regimens with a view to predicting possible future developments); (*d*) to introduce into the global data base on the susceptibility of malaria parasites to antimalarial drugs other relevant data from village-based studies, such as drug use in the community and local transmission, which may provide indicators clarifying the relationship between the status of a drug and the life-style of the host and contacts; (*e*) to analyse the available data in order to determine if indices of drug susceptibility can be developed which are simpler to use and/or more reliably predictive of current and future drug susceptibility trends than those currently used.

Relevant sources include: publications in scientific journals, society records and books; reports and records of national and international agencies; WHO Global Monitoring Programme records; complementary observations from local investigators.

Relevant methods of analysis include: spatial analyses, comparison by means of evolutionary/dynamic trends, and new methods in computer analysis of maps.

22. *Prospective studies on the role of mobility of the population in the spread of drug resistance.* Resistance might propagate from a single or a limited number of foci or from multiple foci. In the first instance, population movements may be of greater importance than in the second instance. Each of the two mechanisms may require different measures for the prevention or containment of resistance.

The hypothesis is that if population movements are the main determinant of the spread of resistance, decreases in drug sensitivity should occur preferably in sites situated along the main routes of migrations, and less in areas situated far from these routes. Some observations made in Zaire near Lake Kivu are compatible with this hypothesis. The objective is to determine the levels of drug sensitivity in two areas situated respectively along and away from migration routes and sites and to follow up longitudinally their inhabitants. The two areas will need to be similar concerning the vector species, parasite species, levels of malaria transmission, the transmission season, the structure and coverage of health services, and the consumption of drugs.

Measurement methods should include; (*a*) standard parasitological, entomological, and serological measurements; (*b*) use of health services and of other providers of drugs; quantity and pattern of drug use; levels of the most commonly used antimalarials (especially chloroquine) in urines; (*c*) drug response *in vivo* and *in vitro*; (*d*) circulation of the population (number of people coming and leaving, short-term movements, length of stay in the area, origin of the travellers, and drug sensitivity of *P. falciparum* in the area of origin); (*e*) characteristics of the parasites.

Particular attention should be paid to the sampling procedures so that they are representative of all communities and are adequate to indicate variations in the parameters. The minimum duration of the observations will be 3 years. Interpretation will be based on differential development of resistance between the two areas, not explainable by variation in the other independent variables, such as malaria transmission and drug pressure.

23. *Longitudinal studies of factors associated with the emergence and spread of chloroquine-resistant* P. falciparum. The objectives are: (*a*) to examine factors prospectively which could be related to the development of chloroquine resistance in *P. falciparum* under natural conditions; (*b*) to continue the descriptive epidemiological studies of these factors before, during and after the emergence of chloroquine resistance; (*c*) to determine potential associations between the factors considered and the emergence of chloroquine resistance as a basis of causal hypotheses.

The situations and the complexity of factors leading to the selection and spread of chloroquine-resistant *P. falciparum* parasites in malarious areas are not well understood. Descriptive epidemiological studies of a number of relevant variables done prior to and during the emergence of drug resistance in an endemic area would be useful in enhancing our knowledge. Factors to consider include: (*a*) population movements; (*b*) low blood levels of chloroquine in the population and their relationship to the use of "subcurative" doses; (*c*) drug consumption patterns; (*d*) sources of antimalarials; (*e*) the presence of a large parasite reservoir and an efficient vector facilitating recombination; (*f*) the *in vivo* and *in vitro* response of *P. falciparum* to chloroquine.

Isolates of *P. falciparum* can be collected, cloned and examined genetically to provide some evidence regarding the "diffusion theory" (geographical spread). It will be possible to evaluate methods for drug pressure, blood levels of antimalarials, drug utilization, population movement and *in vivo* and *in vitro* tests.

Population groups may be studied in West African countries where *P. falciparum* is still susceptible to chloroquine, but is likely to show resistance in the near future. Places where there is known migration to and from other countries having resistance should be considered particularly.

The following are relevant methods; (*a*) census-taking; (*b*) establishing transmission levels, using immunological techniques; (*c*) establishing baseline data and subsequently monitoring the sensitivity of *P. falciparum* to chloroquine and quinine (*in vivo* and *in vitro*); (*d*) establishing drug utilization patterns in individuals by questionnaire (sources of antimalarials, and rural, periurban and urban distribution differences to be considered); (*e*) determining population movements including international travel; (*f*) undertaking case studies of individuals who are found to show chloroquine-resistant isolates, retrospectively, through routine interviews to establish factors under which the infection was acquired; (*g*) determining blood or urine levels of chloroquine by age in order to estimate drug pressure; (*h*) cloning and genetic characterization of selected isolates.

24. *Prospective study on* P. falciparum *chloroquine sensitivity in a country or area where chloroquine resistance has not yet been detected* (see also No. 33 below). The objective is to ascertain if *P. falciparum* chloroquine sensitivity is retained in the relevant area, and whether this situation should be altered to elucidate the epidemiological factors that might have been responsible.

In many countries, longitudinal studies on the degree and spread of resistance have been carried out after the discovery of the resistance phenomenon. It is desirable to conduct a study with regard to *P. falciparum* chloroquine resistance in a country where such a resistance has not yet been detected, in order to identify the epidemiological and other factors that might contribute to or might be responsible for the parasites no longer responding to the drug. The ideal country should be one in which *P. falciparum* sensitivity to drugs is regularly monitored, where chloroquine resistance has not yet been detected, and

where malaria control activities are carried out by a relatively effective primary health care delivery system.

Methods and measurements: (*a*) collection of existing data on the types of antimalarials in use and their dosages, their distribution and their patterns of utilization by the population; this information may be incomplete and there may be a need to organize and conduct special surveys (special questionnaires will have to be developed and tested); information may also be available or will have to be collected on levels of transmission in different ecological areas and their seasonality as well as on vector control activities; (*b*) study of the immunological status of the local inhabitants (urban and rural) in representative geographical areas, and of migrants; tests for cell-mediated immunity in addition to antibody assays may be utilized; (*c*) detection of drug failures through sentinel posts; (*d*) confirmation that drug failure has really occurred, repeating the *in vivo* studies if necessary; (*e*) performance of *in vitro* micro tests in and around the resistant case(s) to delimit the extent of the problem, and an epidemiological investigation in and around the case(s), including investigations concerning the intensity of transmission, immunity status, drug consumption, population movement, and associations between those variables and the emergence of resistance, both at the community and individual levels; (*f*) treatment of chloroquine-resistant case(s) with a second-line antimalarial; (*g*) specialized immunological studies on any case not responding to chloroquine treatment; (*h*) in addition, the possibility should be explored of using parasite characterization methods to test whether resistant mutants are more likely to be of local or of foreign origin (see No. 33 below).

The effect of specific interventions on the drug response of P. falciparum *in human populations*

In this area, intervention studies, let alone experimental studies, are faced with logistic and ethical constraints, including in particular the question of controls and the likelihood that "rationalization" of current drug practices call for the simultaneous introduction of several changes (e.g., changes concerning targetting, dosage, detection and differential treatment of non-responders). We should probably try to obtain a maximum of information from the careful follow-up of the drug response after changes introduced on their own merit (i.e., not specifically for research). In certain cases it may be feasible to introduce the changes on a trial basis in part of a country, leaving others as controls; it would indeed be worth knowing whether a "package" of changes towards "rationalization" of drug use were associated with a delay in the selection of resistance, even without direct proof of a causal relationship or without the possibility of distinguishing the effects of individual components of the package. Incidentally, this is an area where mathematical and computer simulations could be of great help, at least for the clarification of our thoughts (see the pioneering paper of Curtis & Otoo).[u]

25. *Drug resistance after withdrawal of drug pressure* (see also No. 26 below). The objective is to investigate whether changes occur in the frequency of resistant genes in a parasite population, once drug pressure is withdrawn. It is possible that if drug pressure is withdrawn, some resistant genes will be at a disadvantage and be selected against or that high-level resistance to a given drug will break down (see No. 1 above). In these instances a drug to which resistance has developed may become effective again in the future.

Chloroquine resistance should be monitored in a selected region in which the drug has ceased to be widely used. *In vitro* micro tests could be used to examine parasites from a large sample of patients at periodic intervals.

26. *Observations on changes in the level of chloroquine resistance in* P. falciparum *relative to the reduction of chloroquine utilization and the associated changes in the utilization of other antimalarials* (see also No. 25 above). The objectives are to determine, in an area where resistance of *P. falciparum* to chloroquine is well established at the RIII and RII levels: (*a*) if a reduction or cessation in the utilization of chloroquine for malaria treatment in general, or for the treatment of *P. falciparum* only, will result in a change in the degree of sensitivity of *P. falciparum* to chloroquine and under what conditions this might occur; and (*b*) if there is any relationship between an observed change in the sensitivity of *P. falciparum* to chloroquine and the different alternative drugs used.

There are laboratory observations that suggest that in the absence of chloroquine pressure, and in the presence of mefloquine pressure, some reduction in the level of resistance of *P. falciparum* to chloroquine may occur. From preliminary observations *in vitro* through the WHO Global Monitoring Programme, it would appear that in Thailand and Hainan Island, China, chloroquine-resistant *P. falciparum* may have become less resistant to this drug as a result of a reduction in its use.

Since chloroquine is the cheapest antimalarial that can be safely used by community health workers it is essential to prolong its effectiveness for as long as possible. Chloroquine could continue to be useful for the clinical treatment of *P. falciparum* cases in certain

[u] CURTIS, C. F. & OTOO, L. N. A simple model of the build-up of resistance to mixtures of antimalarial drugs. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **80**: 889–892 (1986).

situations, even when *P. falciparum* is resistant to it at a low level. Thus, for the development of drug policies it is essential to know whether and under what conditions a reduction of the resistance level might occur. The results of such a study are of immediate practical importance as well as contributing to a better understanding of the processes involved in the development and maintenance of drug resistance.

Two study areas are proposed: Hainan Island, China, and a province in south-east Thailand, possibly Chantaburi. These offer different situations with respect to the amount of chloroquine continuing to be used, the alternative drugs being used, and the availability and use of other drugs in general.

Measurements: (*a*) chloroquine consumption (see also No. 29 below); (*b*) *P. falciparum* resistance to chloroquine; initially monitoring only by *in vitro* tests would be justified until a significant change towards sensitivity is reached (if at all), at which stage *in vivo* testing might be considered in hospitals where blood levels of chloroquine would be determined; the characterization of the *P. falciparum* isolates tested by other biochemical markers may not be part of this study, but this opportunity may be utilized by other investigators, in which event some isolates should be appropriately preserved during the course of this study; (*c*) consumption of other antimalarials, either schizonticides or gametocytocides; reasons for monitoring the use of other antimalarial drugs is related to the laboratory findings with mefloquine (see working paper by V. E. do Rosario & S. Thaithong)[v] and also to the different pharmacological relationship between chloroquine and mefloquine and between chloroquine and quinghaosu (artemisinin); as part of the same monitoring surveys it will be important to have some idea of general drug usage in the population, especially other drugs that may have an antimalarial activity such as sulfonamides and tetracyclines; (*d*) levels of malaria transmission, morbidity and mortality, relative abundance of different parasite species, seasonality of transmission, and malaria control measures in use; (*e*) *P. falciparum* resistance to other antimalarials: it would be equally important to monitor simultaneously the *in vitro* response to other drugs, including drugs *not* in use.

27. *Application of a single dose of primaquine as an additive to "second-line" treatment for chloroquine-resistant* P. falciparum. The objective is to study whether the addition of primaquine (8-AQ) to the "second-line" treatment of chloroquine-(4-AQ)-resistant *P. falciparum* should delay (or reverse) the further development of manifest chloroquine resistance.[w]

The concomitant use of 4-AQ and 8-AQ for treatment of *P. falciparum* has been advocated for the purpose of reducing both the transmission and selection of resistance in areas where this resistance is manifest. The systematic addition of an 8-AQ to the first-line treatment might however accelerate the selection of resistance. The latter hypothesis is based on the expectation that the 4-AQ will eliminate susceptible asexual parasites while the 8-AQ will eliminate mature gametocytes from both susceptible and resistant genotypes, and while the recrudescence of resistant parasites will produce new gametocytes that are carrying the mutations for 4-AQ resistance. If 4-AQ-susceptible parasites on the other hand were only treated with 4-AQ, the mature gametocytes would not be affected and thus could still "dilute" the resistance already at hand as they would also be transferred by subsequent mosquito bites; this dilution of resistance could be enhanced if the 8-AQ could be used only or preferentially against resistant parasites; hence the suggestion to add the 8-AQ to the second-line treatment of non-responders, rather than to the first-line treatment.

The study could take place in areas where there is a fair amount of resistance to 4-AQ drugs, in which primaquine is not part of the current first-line treatment. It should preferably be conducted in relatively closed communities with little migration and little access to drugs outside the primary health care system. For the inclusion of 8-AQ in the second-line treatment to have any noticeable impact, a large fraction of the population with 4-AQ-resistant *P. falciparum* must be treated with the second-line drug schedule which calls for well-functioning primary health care facilities.

The study, which will comprise 4-AQ only as a first-line treatment of *P. falciparum* and sulfadoxine/pyrimethamine plus primaquine for second-line treatment, will also require a control area, preferably one where the epidemiological characteristics are the same and the drug schedule is the same, except for the primaquine.

Measurements: before the study, data on sporozoite rate, (if possible, *P. falciparum*-specific) parasite rate, and *in vivo* and *in vitro* resistance to 4-AQ are collected; the same data can be looked for every year and at the end of the study; during the whole study period, the number of tablets consumed and the persons who receive treatment with first-line or second-line drugs should be recorded at the primary health care centres.

28. *A prospective study comparing the evolution of* P. falciparum *chloroquine sensitivity under treatment with either a "suboptimal" dose (10 mg/kg) or the "optimal" dose (25 mg/kg in three days) of chloroquine.* The objective is to assess the relative rate and

[v] See p. 799.

[w] 8-AQ, 8-aminoquinolines; 4-AQ, 4-aminoquinolines.

intensity of change in sensitivity of *P. falciparum* to chloroquine in populations receiving two different chloroquine regimens. In some areas chloroquine is being used in "suboptimal" doses because it is thought to be effective, easier to administer, cheaper and may not contribute to the selection of resistance as much as higher doses. However, there are those who believe that the regimen using 10 mg of chloroquine base per kg may accelerate the selection of resistant mutants.

In a country with a policy of administering the 10 mg/kg dose, and in which chloroquine resistance is not yet a problem, two large areas having similar high transmission levels and not affected by large population movements may be selected. In one area the "suboptimal" treatment regimen (10 mg base/kg) will be continued, while the "optimal" treatment regimen (25 mg base/kg) will be administered in the second area.

Methods and measurements: (*a*) assessing the availability and usage of antimalarial drugs and acquiring basic information on malaria endemicity and epidemiological patterns in the study areas; (*b*) developing a distribution network for the administration of 25 mg dose/kg of chloroquine or 10 mg dose/kg, as indicated by the situation in the selected country, and monitoring the compliance; (*c*) establishing sentinel posts for evaluating the sensitivity of *P. falciparum* infections by *in vivo* and *in vitro* studies; (*d*) monitoring the chloroquine sensitivity in *P. falciparum* at regular intervals in the two populations.

Methodological research

29. *Antimalarial drug utilization in populations* (see also No. 30 below). The objectives are to obtain information of the actual drug consumption and to relate this "drug pressure" to (*a*) the import of drugs to the community and (*b*) the emergence of drug resistance.

Justification: (*a*) information on drugs consumed will aid in investigations of how resistance develops, in evaluation of current programmes of chemotherapy and chemoprophylaxis, and in formulation of realistic suggestions for their improvement; self-medication is common, and more needs to be known about its efficacy; it may constitute a basis upon which to build; (*b*) information on the use of inappropriate drugs may require national action; (*c*) this information may indicate the need for refresher courses for all ranks of health workers, pharmacists, and short training courses for small traders; (*d*) the fact that a substantial part of semi-immune people have been shown to have ingested chloroquine rather recently, despite their statements that they did not, necessitates a simultaneous approach with both questionnaires and chemical tests.

Study populations should include samples from a variety of areas differing in access to drugs and/or transmission of malaria. Such areas include urban, peri-urban and rural areas, the latter with high and low transmission. A representative sample should include both males and females and adults and children.

Methods and measurements: (*a*) providers to be sampled include health workers (government and private), pharmacists, traders, midwives, and other traditional practitioners; (*b*) consumers: sample size will be much larger; (*c*) see suggested questionnaires in the working paper by C. P. MacCormack;[x] (*d*) urine determinations of antimalarials.

Studies such as this are of great potential practical relevance and should be coordinated or integrated with epidemiological investigations oriented towards the planning of malaria control.

30. *Development of an indicator of "chloroquine pressure" and application to the problem of chloroquine-resistant* P. falciparum (see also No. 29 above). It is essential to establish the possible relation between recommendations for use of antimalarial drugs, their actual consumption, and emergence and/or spread of *P. falciparum* resistance to them under different endemic conditions. Drug use or "drug pressure" needs to be estimated in terms of compliance and actual drug concentrations in different age groups. The methodology needs to be simple and easily adapted to the field if such estimations are to be made under the prevailing and widely differing conditions in terms of endemicity, drug susceptibility and drug accessibility. An initial study should focus on chloroquine.

The objectives are to obtain information on the actual consumption of chloroquine under defined conditions, by assessing the concentration of chloroquine in blood or urine in the population; and to define a reliable indicator of "chloroquine pressure".

This study, which should be complementary to an ongoing drug-sensitivity survey, involves use of the following materials and methods: (*a*) study areas should preferably include areas with varying malaria endemicity and access to chloroquine; in a second step, areas may be selected with varying degrees of drug (chloroquine) resistance; sampling should include both sexes and all age groups; (*b*) malaria endemicity, estimated by prevalences of asexual parasitaemia and anti-sporozoite seroreactivity; (*c*) drug provision — local recommendations for treatment and prophylaxis with chloroquine are recorded and inquiries about the supply of this drug are made in local clinics and from pharmacists, traders

[x] See p. 803.

and other local distributors; (*d*) alleged drug (chloroquine) consumption is assessed by questionnaires directed to the study population; (*e*) actual drug consumption (drug pressure) is ascertained by individual determination of urine samples performed by a semi-quantitative method for chloroquine in urine; chloroquine determination of the same urine sample concomitantly performed by an immunoassay; in addition, selected urine or blood samples are pooled to be assayed by a quantitative method (HPLC); these methodologies are finally compared in terms of their feasibility and reliability, questionnaires being given to providers and/or consumers, and correlated with results from the drug susceptibility surveys.

31. *Modification of the* in vivo *drug-sensitivity test.* *In vivo* chemosensitivity assays are particularly relevant to a given field situation because they involve the host component of the drug effect (metabolism of the drug, host resistance). They are however difficult to perform owing to the long-term follow-up required.

Since *in vivo* Grade I resistance is manifested by a decrease of parasitaemia below levels detectable by microscopic methods, and thereafter a secondary increase, and since several novel assays are being developed which allow the detection of levels of parasitaemia as low as one infected RBC per one to twenty million, a simplification of the standard *in vivo* assays can be envisaged. Preliminary results (using FITC-labelled antibody, P^{32}-labelled DNA probes, and I^{125}-labelled monoclonal antibodies) suggest that persisting parasites can be detected up to 10–15 days following successful treatment, and that the pattern of decrease differs in RI resistant cases, with a plateau at levels higher than in sensitive isolates, yet below the level of thick smear detection. Therefore if further research confirms the above, follow-ups as short as 4–7 days should be sufficient with such techniques for detection of the RI level of resistance. Such an assay would also permit discrimination between drug treatment failure and reinfection.

The immunoradiometric assay with monoclonal antibodies directed to blood-stage antigen appears to be the most sensitive available means of detection. Labelled DNA-repetitive probes, which are currently under development, may subsequently be employed for the same evaluation purpose.

32. *Improvement of* in vitro *assays. P. falciparum* drug resistance has now been recorded in all continents and drug use is inducing fast changes in the pattern of resistance, which requires closer monitoring. Owing to the relatively small number of drug sensitivity tests currently performed with the available resources, it would appear essential to develop more rapid tests for monitoring in all the affected and potentially affected areas. These tests would detect changes in susceptibility and thus provide, in good time, information to update the drug policies.

It is also apparent that the malaria parasite carries a number of foreign proteins, enzymes and degraded host proteins (i.e., pigment) which provide numerous targets for simple biochemical means of detection. Several approaches can be envisaged: (*a*) to develop DNA probes specific for drug resistance; (*b*) to measure parasite response in the micro test by means other than microscopic examination, such as parasite enzyme assays, monoclonal antibodies, parasite-specific DNA probes, and parasite pigment assays; (*c*) to determine maturation of resistant parasites in a semi-solid medium in the presence of test drugs (as in testing bacterial sensitivity to antibiotics).

33. *Identification of markers of the geographical origin of populations of* P. falciparum (see also No. 24 above). The objective is to evaluate available characterization assays which may reflect differences between *P. falciparum* populations including their susceptibility to drugs or their geographical origin and would therefore be helpful markers to monitor the spread of drug resistance. The relative importance of geographical diffusion of resistant mutants versus independent mutations in different geographical locations is not known, but the selection of control strategies should depend on this relative importance.

Methods: (*a*) the assays require large numbers of parasites and the markers should be first searched in cloned lines of *P. falciparum* and thereafter in isolates, or strains, from different geographical areas; (*b*) assays must include *in vitro* drug-sensitivity micro assay (with as many drugs as feasible), chromosome-sized DNA patterns, restriction electrophoretic patterns and hybridization with DNA probes, isoenzyme patterns, 2-dimensional-gel peptide patterns, and identification of polymorphic antigens; if preliminary analysis suggests the existence of a correlation between one of these markers and geographical origin and/or drug sensitivity, this will need to be further investigated in additional isolates.

Postscript (added in proof, October 1987). Many papers concerning drug resistance of malaria parasites have appeared since the above was written. Perhaps the most significant finding is the reversal, *in vitro*, of resistance of *P. falciparum* to chloroquine by verapamil (Martin, S. K. et al., *Science*, **235**: 899–901 (1987).

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