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Clonal diversity in a single isolate of the malaria parasite *Plasmodium falciparum*

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Summary

Clones of an isolate of *Plasmodium falciparum* from Mae Sod (Thailand) were prepared by a dilution procedure. Some of the parasite cultures thus obtained have been typed for the following characters: (i) electrophoretic variants of three enzymes; (ii) susceptibility to chloroquine and pyrimethamine; (iii) antigen diversities recognized by ten strain-specific monoclonal antibodies; (iv) presence or absence of knobs on infected erythrocytes and (v) two-dimensional PAGE variants of seven proteins. Amongst the clones there was variation involving each of these five characters. At least seven different types of clones were found in ten cultures produced by dilution. The amount of phenotypic variation within a single isolate has thus been shown to be surprisingly great. Variations in drug susceptibility and antigens are considered to be particularly important in view of their relevance to anti-malarial treatments.

Introduction

Isolates of *Plasmodium falciparum* taken from individual patients have previously been shown to contain several different genetic types of parasites. CARTER & MCGREGOR (1973) showed that West African (Gambian) isolates often exhibited more than one electrophoretic form of one or more enzymes. ROSARIO (1981) prepared clones from a single isolate (T9) of *P. falciparum* from Thailand, and showed that while the original uncloned material exhibited a mixture of several enzyme variants, single clones showed only a single form of each enzyme. Surveys of parasites collected in different parts of the world have frequently revealed that isolates contained mixtures of enzyme types, and were therefore assumed to comprise mixtures of genetically diverse clones (SANDERSON *et al.*, 1981; THAITHONG *et al.*, 1981). Moreover TRAGER *et al.* (1981) prepared clones from a Gambian isolate (RFCR-3) by micromanipulation, and showed that there was clonal variation involving presence or absence of knobs on infected erythrocytes, and susceptibility to chloroquine.

In the present paper we summarize an analysis of ten cultures derived by dilution from the above-mentioned Thai isolate T9. A preliminary report on enzymes and drug susceptibility of some of these cultures has been published by THAITHONG (1983). We now present fuller data on five types of variant involving: (i) enzymes, (ii) drug susceptibility, (iii) antigens, (iv) knobs and (v) proteins distinguished by two-dimensional electrophoresis. More extensive accounts of each type of variant will be published elsewhere. Here we wish to stress the extent of the genetic diversity which can exist within a single isolate, or "strain", of these parasites.

Material and Methods

Source of the parasites

The isolate T9 was collected on 7th April 1980 from a malaria patient at Mae Sod (near Tak) 543 km north of Bangkok, Thailand. Unfortunately a detailed history of the patient could not be obtained and it was therefore not known exactly where the infection was acquired. The blood sample was immediately taken to Bangkok, cultured by the Petri-dish method of TRAGER & JENSEN (1976) and in due course preserved in liquid nitrogen. When required, samples were thawed out and characterized as described below.

Cloning techniques

Two of the cloned cultures studied here (Nos. 94 and 96) were prepared in 1980, as previously described by ROSARIO (1981), while the remainder (Nos. 97-107) were obtained in the summer of 1982 by Thaithong, using basically the same technique. Diluted samples of cultured parasites, estimated to contain an average of 0·5 parasites per 100 µl sample were grown up in microtitre wells, and the cultures thus obtained were transferred to Petri dishes after varying periods depending on the growth rates of individual cultures. As will be seen below, the technique did not succeed in producing pure clones exclusively, but most of the cultures appeared to contain only a single type of parasite. Theoretically a higher proportion of pure clones could have been obtained by diluting the original culture more extremely, but this would have involved growing up many more diluted samples. It should be added that the red cells in the original 100 µl samples included about 5% with two or more parasites per cell. Cultures which were obtained by the dilution technique, but which were not homogeneous as regards all the characters studied, will be denoted here as "clonal mixtures", and marked with a star (e.g. Nos. 97* and 105*) (see Table I).

Characterization methods

Variant forms of three enzymes—GPI, ADA, PEP (see Table I)—were identified by cellulose acetate electrophoresis of parasite lysates. The band positions of the enzyme variants observed by this technique are essentially similar to those described by SANDERSON *et al.* (1981) who used starch gel electrophoresis. The enzyme variants are denoted GPI/1 or GPI/2, ADA/1 or ADA/2, and PEP/1 or PEP/2.

Drug susceptibility was measured by the method of THAITHONG & BEALE (1981). Parasites in microtitre wells were exposed for 72 hours to chloroquine or pyrimethamine at various concentrations, with daily changes of drug-containing medium. The results were recorded from microscope observations of thin films, and expressed as "mini-

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Table I—Characterization of clones produced by dilution of isolate T9 of *P. falciparum*

		Original uncloned isolate (T9)	Reference nos. of cultures produced by dilution of original isolates: 102	106	100	101	98	97*	96	107	94	105*
Enzyme types	GPI	1/2	1	1	1	1	2	1	2	2	1	1
	ADA	1/2	1	1	1	2	1	2	1	1	1	1
	PEP	1/2	1	1	1	1	2	1	2	2	1	1
Drug susceptibility (MIC)	Chloroquine	10^{-6}	10^{-6}	10^{-6}	10^{-6}	10^{-6}	10^{-7}	10^{-6}	10^{-7}	10^{-8}	5×10^{-7}	10^{-6}
	Pyrimethamine	5×10^{-5}	5×10^{-5}	5×10^{-5}	5×10^{-5}	5×10^{-5}	5×10^{-5}	5×10^{-5}	10^{-9}	10^{-9}	10^{-9}	10^{-5}
Antigens (Monoclonal antibodies)	5·1	+	−	−	−	−	+	−	+	+	+	+
	7·3	+	−	−	−	−	−	−	−	−	+	+
	9·2	+	+	+	+	+	+	+	+	+	−	−/+
	9·5	+	−	−	−	+	−	+	−	−	−	−
	12·2	+	−	−	−	−	+	−	+	+	−	−
	12·1	+	+	+	+	−	+	−/+	+	+	−	−/+
	10·4	+	+	+	NT	+	NT	+	+	+	−	−/+
	9·4	+	+	+	NT	+	−	+	−	−	−	−/+
	12·3	+	+	+	+	+	+	+	+	+	+	−/+
	9·21	+	+	+	+	+	+	+	+	+	−	+
Knobs		+/−	+	+	+	+	+	+	+	+	−	+
Proteins by 2-D electrophoresis	A	2/4	1	1	1	1	1	1	2	2	4	2/4
	C	1/2/3	2	2	2	2	2	2	1	1	2	2
	D	2/4/5	5	5	5	4	5	4	4	4	5	4
	E	2/3/4	4	4	4	4	1	4	3	4	2	2
	F	1/2/3	2	2	2	2	2	2	2	2	3	1
	G	1/2	2	2	2	2	2	2	2	2	2	1
	K	3/5/6	6	6	6	6	3	6	3	3	6	5
Inferred clone type			I	I	I	II	III	II(+I)	IV	V	VI	VII(+?)

1. Reference nos. of cultures are given as they appear in the dilution-cloning experiments. *indicates mixed clones.
2. Enzymes shown are glucose phosphate isomerase (GPI) (EC5.3.1.9); adenosine deaminase (ADA) (EC3.5.4.4); peptidase (PEP) (EC3.4.11 or 13). Numbers refer to variants of each enzyme.
3. Drug susceptibility values refer to minimum inhibitory concentration (MIC) in molar (M) units.
4. Antigen diversity is shown by indirect immunofluorescence tests with ten monoclonal antibodies. Presence or absence of reaction is indicated by + or − respectively. −/+ indicates mixtures containing a small (ca) 2% proportion of + parasites.
5. Knobs: presence or absence is indicated by + or − respectively.
6. Proteins by 2D. A-K are seven selected protein spots on 2D gels; numbers refer to variants (see Text).
7. Inferred "clone-types". A roman numeral indicates clones which are alike in all characters observed.
8. NT = not tested.

mum inhibitory concentrations" (MIC) of drug which killed all or nearly all the parasites. The MIC values are given as molar (M) concentrations of the drugs.

Antigenic diversity was investigated by the use of strain-specific monoclonal antibodies in indirect immunofluorescence tests, as described by McBRIDE *et al.* (1982), and McBride (unpublished). In this paper we present selected data based on the reactions of parasites with ten antibodies denoted 5.1, 7.3, 9.2, 9.4, 9.5, 9.21, 10.4, 12.1, 12.2 and 12.3. Each of these antibodies recognizes a different antigenic specificity. Approximately 10^4 schizonts were examined in each test. Mixtures in which most of the parasites did not react, while a minority did, could be detected, but the technique does not permit detection of a small minority of non-reactive organisms amongst a majority of reactive ones.

Presence of knobs on infected erythrocytes was recorded from observations of cells under a differential interference contrast microscope (Olympus, BH2-NIC), using essentially the same procedure as that of TRAGER *et al.* (1982), though without a video screen.

Two-dimensional SDS-polyacrylamide gel electrophoresis of proteins was carried out by methods described by TAIT (1981) and Walker and Tait (in preparation). In the results presented here, seven proteins—denoted A, C, D, E, F and K—each showing two or more forms in different parasite clones, are recorded. The protein variants are denoted by numbers—thus protein A exhibits variants A/1, A/2 or A/4, while protein C exhibits variants C/1 or C/2 and so on. The seven proteins referred to here were selected out of more than 100 seen on the gels, on a basis of relative ease of identification and presence of variants in different clones. It should be added, however, that the homology of variants of proteins indicated by a given letter cannot be taken as certain, in view of the absence of information about the biochemical specificities of the proteins, or of their controlling genes.

Results

The results are summarized in Table I. It will be seen that at least two of the cultures (Nos. 97* and 105*) are clonal mixtures as shown by data on antigens, and—in the case of No. 105*—on 2D protein analysis. The remaining cultures shown in Table I appear to be homogeneous, but this statement is subject to the reservation that some may contain small amounts of a second clone which would be undetectable by most of the characterization tests used.

Considering first the enzyme data, we note the occurrence, in different clones, of three combinations of variants ((i) GPI/1, ADA/1, PEP/1; (ii) GPI/1, ADA/2, PEP/1 and (iii) GPI/2, ADA/1, PEP/2), out of a possible maximum of eight combinations of two variants of each of three enzymes.

As regards pyrimethamine susceptibility, two distinct classes exist: most clones are highly resistant (MIC = 5×10^{-5}M), while a minority (Nos. 94, 96, 107) are sensitive (MIC = 10^{-9}M). Susceptibility to chloroquine also shows considerable variation in different clones (MIC ranging from 10^{-6}M to 10^{-8}M).

Considerable antigen diversity has been found, as shown in the reaction of the parasites to the ten different monoclonal antibodies. In different clones at least five different combinations of antigen markers can be recognized.

Knobs are present in most clones, but absent from one (No. 94), confirming the findings of TRAGER *et al.*, (1981) with another isolate of *P. falciparum*. It may be added that we have also noticed appreciable variation in the size and number of knobs per red cell, in different clones.

The 2D analysis of seven proteins (A-K) shows at least seven different combinations of variants.

In general therefore the results indicate a large amount of diversity amongst the clones which have been studied from isolate T9. Seven "clone types" (denoted I to VII) are indicated at the bottom of Table I, in the ten cultures studied from the cloning experiments. Only three of the cultures (Nos. 100, 102, 106) appear to be alike in all the characters studied and, of these, No. 100 has not been completely analysed for antigens.

Discussion

In considering these results, it should be borne in mind that the analysis has been conducted entirely at a phenotypic level, since formal genetic analysis of *P. falciparum* has so far not been achieved. However, from genetic experiments with rodent *Plasmodium* species (BEALE *et al.*, 1978), it may be assumed that variants in electrophoretic properties of enzymes, and in resistance to chloroquine and pyrimethamine, are gene controlled in *P. falciparum* also. The other characters studied here have been found to be stable in cloned cultures grown *in vitro* for considerable periods, and it is therefore reasonable to believe that variants of all these characters are under gene control, although the details of the genetic mechanisms are unknown at present.

It is therefore clear that a large amount of genetic diversity exists within the population of parasites of a single isolate. Variations in the growth rates of different clones have also been noted, and it is therefore to be expected that cultures derived from an uncloned isolate may change their character during long periods of *in vitro* cultivation. Thus some "strains" of *P. falciparum* might well undergo changes in such characters as drug-resistance or antigenic phenotype, when grown under laboratory conditions.

The clonal diversities in drug susceptibility and antigens are of particular interest. Most isolates of *P. falciparum* from Thailand—and elsewhere in SE Asia—have been found in recent years to be resistant both to chloroquine and pyrimethamine, as shown both by *in vivo* (WERNSDORFER, 1982; PINICHPONGSE *et al.*, 1982) and *in vitro* tests (THAITHONG *et al.*, 1983). However the data obtained in this study now show that these "resistant" isolates may contain a proportion of sensitive parasites, which would not be noticed in *in vivo* drug-resistance tests, nor in routine *in vitro* tests of uncloned material. Were drug-treatment to be relaxed for a period, it is conceivable that the drug-sensitive parasites might increase in frequency and even outgrow the resistant ones, though this is of course only a speculative suggestion.

With regard to clonal diversity in antigens, it should be stated that at present it is unknown whether any of the antigens recognized by the monoclonal antibodies used here have any role in protection against infection by malaria parasites. It is, however, significant how much antigenic diversity is revealed by this panel of monoclonal antibodies, and it would

not be surprising to find that antigens involved in immune protection were also variable in different clones or isolates.

Variations involving the knobs may also be significant in relation to the severity of malarial attacks, though nothing is known with certainty about this at present.

We think that the implications of these findings should be borne in mind when future methods of malaria treatment, either by chemotherapy or by vaccination, are being planned, since it is clear that the parasites, even within a relatively small area, contain a very large amount of incipient variability, and give ample scope for future changes in drug resistance, antigens and other characters, even in the absence of further mutations, when therapeutic or prophylactic measures are applied.

Finally, it should be pointed out that the characterization methods described here can be used to check the identity of any cultures which have been maintained for long periods in the laboratory.

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