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Biochemical markers for strain differentiation in malarial parasites

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Genetic differences between micro-organisms can be identified by studying variations in enzyme forms and DNA characteristics. Enzyme electrophoresis has revealed considerable genetic diversity in populations of malarial parasites and has enabled populations reproductively isolated from one another to be identified. Studies on rodent malarial parasites from four regions of Africa have shown that each species and subspecies can be distinguished by its pattern of enzyme forms. In subspecies in which enzyme polymorphism occurs, the frequencies of each combination of enzyme forms suggest an extensive degree of random mating in the population. Populations of Plasmodium falciparum *are also enzymically polymorphic, similar enzyme variants being found in isolates from several parts of the world.*

In recent years, biochemical methods have proved of considerable value in differentiating among genetically distinct groups of malarial parasites and other parasitic protozoa. This account is concerned mainly with the technique of enzyme electrophoresis, which has been used to identify individual gene differences between parasite strains and to examine gene frequencies in parasite populations. A second technique has involved examining the DNA characteristics of the organisms, since variations in DNA base composition reflect differences in genetic information.

Enzyme electrophoresis was first applied to rodent species of *Plasmodium* (*1–5*) and subsequently to *P. falciparum* (*6–8*), as well as to species infecting primates (*9*). Other parasitic protozoa examined include trypanosomes (*10–14*), *Leishmania* spp. (*15–16*), coccidia (*17–18*), and *Babesia* spp. (*19*). The technique is particularly suitable for taxonomic purposes, as the enzyme characteristics of a genetically homogeneous line are remarkable for their stability. As there is a direct relationship between genes and enzymes, variations in enzymes detected by these methods nearly always reflect gene differences. By examining the enzyme characteristics of a number of isolates from different parasite populations, one can obtain information on genetic diversity within and among the populations. Further, in organisms in which sexual reproduction occurs, such as malarial parasites and coccidia, it is possible to identify groups of parasites that are reproductively isolated from each other; clear demarcations can thus be made among species, subspecies, etc.

Characteristics of DNA have been examined in species of malarial parasite (*20–21*) as well as in *Leishmania* spp. (*22*) and trypanosomes (*23*). These studies involve measuring physical properties of the DNA of the organisms involved, such as its buoyant density and hybridization characteristics. The method reveals differences in the total genetic information of the organisms instead of differences in individual gene loci.

In this review, we shall use the terms " isolate ", " line ", and " clone " rather than " strain ". While it is conventional to refer to parasites derived from a specified source as a " strain ", the term is an imprecise one. For example, " strain 17X of *P.y. yoelii* " refers to parasites derived from a particular wild host specimen, while the " St Elizabeth strain of *P. vivax* " is frequently used to refer to a type of *P. vivax* found in several parts of the world. The studies described here show that there is considerable genetic diversity among organisms not only in a given region but also in infections in individual hosts. The terms " isolate ", " line ", and " clone " will be taken to have the following meanings:

Isolate refers to a sample of parasites, not necessarily genetically homogeneous, collected on a single

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occasion from a wild host and preserved in the laboratory either by passaging or by deep freezing.

Line refers to parasites that have undergone a particular laboratory passage, usually following a special treatment such as selection for drug resistance.

Clone refers to genetically identical organisms derived from a single cell by asexual reproduction; in malarial parasites, clones are usually derived from blood forms by means of a dilution technique (*24*).

ENZYME ELECTROPHORESIS—METHODS OF STUDY

Enzyme variation is most readily studied in malarial parasites by means of the blood forms. Preparations are made of infected blood or of parasites liberated from their host cells by methods such as immune lysis, saponin lysis, etc. The quantity of parasite material required varies according to the activity of the enzyme under investigation. Sufficient amounts can usually be obtained with little difficulty from parasites grown in laboratory animals. For *P. falciparum* recovered from human patients, it may be necessary to culture blood forms to provide adequate amounts. Placental blood, rich in mature trophozoites and schizonts, is also a good source of material of this species. Preparations of blood forms can be freeze-dried and stored in this condition for many years with little loss of enzyme activity.

For electrophoresis, samples are dissolved in water and inserted into slots along a line of origin in a starch gel block (Fig. 1). After electrophoresis for a set time, the position to which a particular enzyme has migrated is identified by applying to the gel a stain specific for the enzyme so that a band of stain is deposited on the gel at the position of the enzyme (Fig. 2). Enzymes of the parasite can be distinguished from those of the host by including samples of uninfected host blood on the gel.

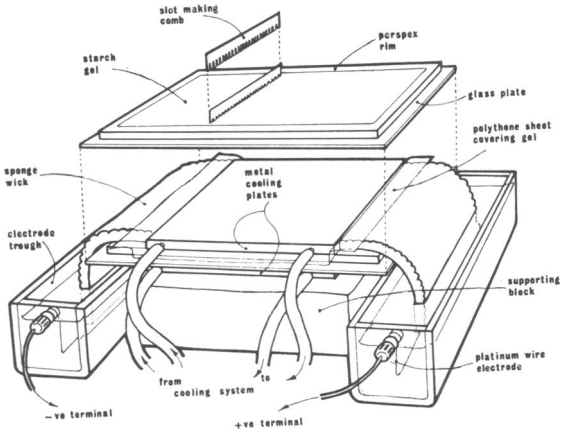

Fig. 1. Apparatus for starch gel electrophoresis.

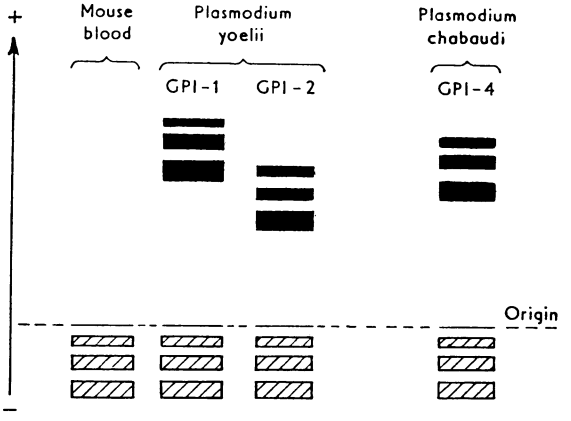

Fig. 2. Electrophoretic variants of glucose phosphate isomerase (GPI) in infected mouse blood. Dark bands represent parasite enzymes and hatched bands represent host forms.

Several enzymes have been studied electrophoretically in malarial parasites and four have been found to be of particular practical value: glucosephosphate isomerase (GPI) (EC 5.3.1.9), phosphogluconate dehydrogenase (PGD) (EC 1.1.1.43), lactate dehydrogenase (LDH) (EC 1.1.1.27), and NADP-dependent glutamate dehydrogenase (GDH) (EC 1.4.1.2).

Several electrophoretic forms of each enzyme have been discovered in the various malarial parasites examined. Each variant form is denoted by a number, e.g., GPI-1, GPI-2, etc. The numbers given have no special significance, but simply indicate the order of discovery. A similar system of enzyme notation has been adopted for the *Plasmodium* species infecting rodents and for *P. falciparum*, but it should be emphasized that there is no homology between enzyme forms with a similar number in the two groups; thus GPI-1 in the rodent malaria group is not the same variant form as GPI-1 in *P. falciparum*.

SURVEYS OF ENZYME VARIATION

Rodent malarial parasites

Malarial parasites occur in rodents in four regions of Africa—the Central African Empire, Nigeria, the

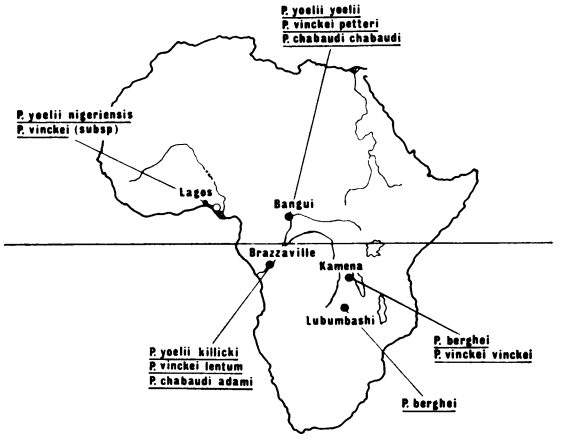

Fig. 3. Distribution of species and subspecies of rodent malarial parasites in Africa.

Congo, and Shaba (formerly Katanga region) in Zaire. Four species have been recognized—*P. berghei*, *P. yoelii*, *P. chabaudi*, and *P. vinckei*, the last three of which possess two or more subspecies. The distribution of the species and subspecies is illustrated in Fig. 3. Numerous isolates of each species have been derived from wild rodents and introduced into laboratory hosts, and clones of certain isolates have been established in order to produce lines of genetically identical parasites.

Table 1 shows the enzyme characteristics of a selection of isolates of each species. It can be seen that variations in enzymes occur between species, between subspecies, and between isolates of the same subspecies. The variation is most pronounced at the species level. Each of the four species can be distinguished by reference to any of the enzymes, with the exception of LDH which is similar in *P. berghei* and *P. yoelii*; it is possible that further studies under different conditions for electrophoresis may show differences in this enzyme also. Each subspecies can also be recognized enzymically, although here some sharing of enzyme forms occurs; for example, each of the three *P. yoelii* subspecies, *P.y. killicki*, *P.y. nigeriensis*, and *P.y. yoelii*, possesses similar forms of GPI (-1 and -2), PGD (-4) and LDH (-1), but differs in GDH (-1, -2, or -4). Variation within a single subspecies is seen most clearly in *P.c. chabaudi*; among isolates of this subspecies are three PGD variants (PGD-2, -3, and -7) and four LDH variants (LDH-2, -3, -4, and -5). *P. berghei*, on the other hand, is an example of a species in which no enzyme variation has been detected, at least among the isolates examined so far.

Table 1. Distribution of electrophoretic forms of enzymes among a selection of isolates of rodent malarial parasites

Species and subspecies	Isolate	Enzyme forms			
		GPI	PGD	LDH	GDH
P. berghei	ANKA	3	1	1	3
	NK65	3	1	1	3
P. yoelii yoelii	17X	1	4	1	4
	33X	2	4	1	4
	1AK	1	4	1	4
	2CN	1,10	4	1	4
P. y. nigeriensis	N67	2	4	1	2
P. y. killicki	194ZZ	1	4	1	1
P. chabaudi chabaudi	1AL	4	2	2	5
	1AM	4	2	3	5
	BJ	4	2	4	5
	3AC	4	2,3	2,4	5
	16AF	4	2	5	5
	40BE	4	3	2	5
	54X	4	3	3	5
	2CB	4	3	4	5
	2CQ	4	3	5	5
	1BK	4	7	2	5
P.c. adami	556KA	8	2	8	5
	408XZ	8	2	10	5
P. vinckei vinckei	v-52	7	6	6	6
P.v. petteri	2CE	5	5	7	6
	2CR	5,9	5	7	6
P.v. lentum	170L	6	5	7	6
	194ZZ	6	5	7	6
	408XZ	11	5	9	6
P.v. brucechwatti	N48	6	6	9	6

It will also be seen from Table 1 that certain isolates possess more than one variant of a given enzyme (e.g., isolate 3AC of *P.c. chabaudi* possesses PGD-2 and -3 and LDH-2 and -4). This is due to the simultaneous presence of more than one parasite clone; clones derived from such isolates possess only one form of each enzyme. This is discussed further in the section on the genetic basis of enzyme variation.

Table 2. Distribution of forms of GPI, PGD, LDH and GDH in populations of *P. falciparum*

Region of origin	Number of isolates examined	Enzyme forms			
		GPI	PGD	LDH	GDH
East Africa	30	1,2	1	1,2	NT
West Africa	177	1,2	1, (2, 3) [a]	1,2	1
South-East Asia	4	1	NT [b]	NT	1
Central America	2	1,2	NT	NT	1

[a] PGD-1 was present in all but four isolates from West Africa; two of these possessed PGD-2 and two PGD-3.
[b] NT = not tested.

Plasmodium falciparum

Enzyme variation in *P. falciparum* has been examined in isolates from West Africa (principally the Gambia) and East Africa (principally Tanzania), and in a few isolates from Central America and South-East Asia.

As in the rodent malarial species, variant enzyme forms are found, there being two forms of GPI (designated GPI-1 and -2), three of PGD (PGD-1, -2, and -3), and two of LDH (LDH-1 and -2). No variation in GDH has yet been found. Table 2 shows the distribution of enzyme forms in isolates from each region.

Other primate malarial parasites

A limited study of enzyme variation in some primate species of *Plasmodium* was carried out by Carter & Voller (*9*). The species investigated were *P. vivax hibernans*, *P. knowlesi*, *P. inui*, *P. fieldi*, *P. cynomolgi bastianellii*, *P. cynomolgi cynomolgi*, and *P. cynomolgi* (Langur strain). Only one strain of each species was examined, except for *P. knowlesi* and *P. inui*. Genetic differences between each species were demonstrated by the occurrence of specific enzyme forms. There was, however, a sharing of LDH type between *P. fieldi* and the *P. cynomolgi* subspecies, and between *P. knowlesi* and *P. vivax hibernans*. A more extensive survey, involving a study of more enzymes in a greater number of isolates, will be necessary to characterize these groups more precisely.

THE GENETIC BASIS OF ENZYME VARIATION

Studies on numerous organisms have established that enzyme forms of the type discussed here are under the control of nuclear genes that are inherited in a simple Mendelian fashion. In diploid heterozygotes, the enzyme forms characteristic of both parents are expressed, and this is demonstrated by the simultaneous presence of each parental band on gels following electrophoresis. The observation that clones of malarial parasites derived from wild isolates invariably exhibit only one form of each enzyme provides circumstantial evidence that the blood forms are haploid. Proof of this has been obtained in crosses between enzymically distinct lines of *P.c. chabaudi* (*25–26*). Crosses were made between two parent lines, one characterized by PGD-2 and LDH-3 and the other by PGD-3 and LDH-2. Clones derived from the progeny of the crosses were of four types—two like the parent lines and two recombinant types (PGD-2/LDH-2 and PGD-3/LDH-3). Each clone possessed only one form of each enzyme. It could be concluded, therefore, that the blood forms were genetically haploid, recombination and segregation (meiosis) having occurred between fertilization in the mosquito and the emergence of parasites into the blood.

GENETIC STRUCTURE OF PARASITE POPULATIONS

Mixed infections with more than one species or subspecies

In nature, animals may be infected simultaneously with more than one species or subspecies of parasite. This situation is exemplified by the parasites found in rodents in the Central African Empire, where three species (*P.y. yoelii*, *P.c. chabaudi*, and *P.v. petteri*) may coexist in the same host. Simultaneous infections can frequently be recognized by morphological differences between species, but this may not be immediately apparent if the morphology is similar. In these cases, a study of enzyme characteristics may provide the clearest evidence for a mixed infection.

An example is provided by a study of the species in the Central African Empire (*4*). Two species of *Plasmodium* were originally described in rodents of this region by Landau (*27*), i.e., *P. yoelii yoelii* and *P. chabaudi*, the latter being distinguished from the former mainly by its predilection for mature erythrocytes. Examination of a large number of cloned isolates for enzyme variation made it clear that the mature erythrocytes of these animals were infected with two reproductively separate groups of parasites, one group being characterized only by GPI-5 and -9,

Table 3. Approximate frequencies of variant forms of enzymes in natural populations of *P.y. yoelii*, *P.c. chabaudi*, and *P. falciparum*

	GPI	PGD	LDH	GDH
P.y. yoelii	1 (68 %)	4 (100 %)	1 (100 %)	4 (100 %)
Central African Empire	2 (28 %) 10 (4 %)			
P.c. chabaudi	4 (100 %)	2 (48 %)	2 (32 %)	5 (100 %)
Central African Empire		3 (48 %) 7 (4 %)	3 (28 %) 4 (24 %) 5 (16 %)	
P. falciparum	1 (64 %)	1 (96 %)	1 (78 %)	1 (100 %)
West Africa	2 (36 %)	2 (2 %) 3 (2 %)	2 (22 %)	

PGD-5, and LDH-7, and the other only by GPI-4, PGD-2, -3 and -7, and LDH-2, -3, -4, and -5. No sharing of enzyme forms between the two groups occurred. Further examination revealed that the morphology of the parasites in each group differed. It was thus concluded that each group comprised separate species, which were named, respectively, *P.v. petteri* and *P.c. chabaudi*.

A similar study carried out on parasites derived from rodents of the Congo has revealed a similar situation, in which the mature erythrocytes of these animals are infected by the two species *P.c. adami* and *P.v. lentum* (*5*).

Variation within a species or subspecies

When large numbers of isolates of a species or subspecies are examined, it becomes possible to calculate the frequencies of the variant forms of each enzyme in the parasite population. Table 3 shows the frequencies of each enzyme variant in *P.y. yoelii*, *P.c. chabaudi*, and *P. falciparum* from West Africa, the populations from which most isolates are available.

In *P.c. chabaudi*, three forms of PGD and four of LDH occur. Theoretically, with random mating and equal frequencies of the various alleles, twelve combinations of these alleles should occur. In practice, in the seventeen isolates exhibiting only one form of each enzyme, nine combinations are found (see Table 1). Only three combinations involving PGD-7 are lacking; this is not surprising in view of the rarity of this form in the population (see Table 3). There is evidence, therefore, for a considerable degree of random mating (panmixia) within the parasite population.

In *P. falciparum* in Africa, a similar situation is found in which all possible combinations of the variant forms of GPI and LDH occur in the population; the observed frequencies of each combination are similar to those predicted on the assumption of random assortment of forms among parasite clones (*8*). It is also of interest that the same variants of GPI, LDH, and PGD occur among the isolates from the east and west coasts of Africa, the frequencies of each being similar in each region. These findings suggest that the eastern and western populations may represent a single gene pool; there is no evidence for genetic divisions between the populations.

Preliminary studies of the enzyme forms in a small number of *P. falciparum* isolates from Central America and South-East Asia suggest a close genetic similarity between these isolates and those of Africa, the same forms of GPI and GDH being found in each region. Further isolates will need to be examined, however, to characterize these populations more precisely.

DNA STUDIES

Techniques for characterizing DNA have proved of value in differentiating species of trypanosomes (*23*) and *Leishmania* (*15*, *22*), but so far little comparative work has been carried out on species of *Plasmodium*. Chance & Warhurst (*21*) were able to distinguish the four rodent species by measurements of DNA buoyant density and hybridization properties, and Gutteridge & Trigg (*20*) found that the DNA of *P. falciparum* and *P. knowlesi* possessed similar buoyant densities. In *Leishmania* spp., the methods have been used in conjunction with enzyme studies to allocate isolates of uncertain taxonomic status to specific groups (*15*), and it seems possible that this approach would be valuable in similar situations with malarial parasites.

SUMMARY AND CONCLUSIONS

1. Enzyme forms revealed by electrophoretic techniques provide stable genetic markers for differentiating genetically distinct micro-organisms.

2. Enzyme studies on both rodent and human malarial parasites have demonstrated a high degree of genetic polymorphism in parasite populations.

3. Within populations of a single species or subspecies, there is evidence for a considerable degree of random mating (panmixia).

4. Among the *rodent malarial parasites*, it has been possible to identify populations that are reproductively isolated from one another, and thus to demarcate species and subspecies. In the past, morphological differences, together with geographical isolation, have been used to differentiate species and subspecies (*5*, *28*, *29*). Enzyme studies and DNA characterization have confirmed the taxonomic status of several groups and have clarified relationships between them; in some cases, e.g., the two subspecies of *P. chabaudi*, enzyme variation provides the only method of differentiation other than the geographical origin of each group. It seems clear that the geographical isolation of the populations of rodent malarial parasites in Africa has been sufficient to allow reproductively isolated and genetically distinct populations to evolve.

5. In *Plasmodium falciparum*, enzyme studies have provided no evidence so far of genetic isolation between geographically separate populations. Although isolates of *P. falciparum* are morphologically similar, there is evidence from immunological studies of differences between isolates from the same locality (*30*, *31*), from different regions of the same continent (*32*), and from different continents (*33*). It is also possible that the high incidence of chloroquine resistance in *P. falciparum* in South-East Asia, and its absence from Africa (*34*), could be explained by fundamental genetic differences between the parasites of these regions. The results of enzyme studies have provided no evidence yet of such differences, but it will be necessary to examine many more isolates from different parts of the world before firm conclusions can be drawn.

RÉSUMÉ

MARQUEURS BIOCHIMIQUES EN VUE DE LA DIFFÉRENCIATION DES SOUCHES PARMI LES PARASITES DU PALUDISME

Des différences génétiques entre les micro-organismes peuvent être décelées par l'étude des variations dans les formes de leurs enzymes et les caractéristiques de leur ADN. L'électrophorèse des enzymes a révélé une diversité génétique considérable dans des populations de plasmodies et a permis d'identifier des populations isolées les unes des autres au point de vue reproduction. Des études sur les plasmodies de rongeurs provenant de quatre régions d'Afrique ont montré que chaque espèce et sous-espèce peut être distinguée par les particularités de ses formes enzymatiques. Chez les sous-espèces dans lesquelles il existe un polymorphisme enzymatique, les fréquences de chaque combinaison de formes enzymatiques donnent à penser qu'il existe une proportion élevée d'accouplements aléatoires dans la population. Les populations de *Plasmodium falciparum* présentent elles aussi un polymorphisme enzymatique, des variants enzymatiques similaires étant trouvés dans des « isolements » provenant de plusieurs parties du monde, « isolement » désignant ici un échantillon de parasites, pas nécessairement homogène au point de vue génétique, recueilli en une fois à partir d'un hôte sauvage et conservé au laboratoire soit par passages, soit par congélation à très basse température.

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