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Failure to Characterize the Isolates by Isoenzyme Variations in *Plasmodium falciparum* Isolates

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Abstract. Isoenzyme variation in the Sudanese isolates and the control isolates of *Plasmodium falciparum* Welch, 1897 was sought by starch gel electrophoresis and isoelectric focusing techniques. No isoenzyme differences were detected with Glucose Phosphate Isomerase (GPI), Lactate Dehydrogenase (LDH), Glutamate Dehydrogenase (GDH) and 6 Phosphogluconate Dehydrogenase (6 PGD). Starch gel electrophoresis revealed three parasite bands of GPI, isoelectric focusing revealed four bands of the same enzyme even in cloned material.

Introduction

Glucose Phosphate Isomerase (GPI) is a glycolytic enzyme which has been used with other enzymes for typing certain organisms because such enzymes posses different forms known as isoenzymes. Isoenzyme variation in rodent malaria was studied by Carter [1] and variation in *Plasmodium falciparum* was demonstrated by Carter and Voller [2], and Carter and McGregor [3]. A similar study on *Plasmodium vivax* was done by Myint-Oo [4]. In these works specificity of the parasite isoenzyme was evident from the absence of the particular enzyme band in the host material. Sarivastava *et al.* [5] were able to purify the specific parasite GPI isoenzymes and to raise specific antiserum, in BALB/C mice, which was inhibitory to the parasite encoded GPI activity but had no effect on the host enzyme. The common belief is that *Plasmodium falciparum* have two forms of the enzyme Glucose Phosphate Isomerase (GPI) called GPI-1 and GPI-2, and a particular strain either has GPI-1 or GPI-2, and this has lead Thaithong *et al.* [6] to conclude that *Plasmodium falciparum* isolates from different endemic areas constitute a single, world wide species, containing potentially interbreeding individual organisms. The last phrase of the conclusion explains the posses-

sion of the two forms of the enzyme by a single isolate, which can be explained further by the co-dominant inheritance like in human blood group AB. However Thaithong *et al.* [7] later discovered in isolates of *Plasmodium falciparum* a new variant of the enzyme GPI which they denoted GPI-3, but more recently Babiker *et al.* [8] did not find more than the usual two forms of the enzyme GPI in their Sudanese isolates.

In this study, isoenzyme variation in 23 Sudanese isolates, one Gambian (FCR3) and two Asian isolates (K1 and Indochina 1/CDC) were studied using starch gel electrophoresis and isoelectric focusing techniques. The enzyme studied were Glucose Phosphate Isomerase (GPI), Lactate Dehydrogenase (LDH), Glutamate Dehydrogenase (GDH) and 6-Phosphogluconate Dehydrogenase (6 PGD).

The aim of this study is to detect any variation among the different Sudanese Isolates of *Plasmodium falciparium* using the above mentioned techniques, and to relate such variation to the different geographical regions or to the degree of sensitivity of the concerned isoloates to the different antimalarials.

Materials and Methods

The isolates of *P. falciparum* used in this work fall into two groups, the first group consists of the strains which were already adapted for continuous culture in the laboratory and being used in different laboratories. The other group consists of wild strains isolated in the field and adapted in the laboratory for continuous culture.

The first group contains

(1) K1 strain which was isolated in Kancharaburi in Thailand on the 29th of January 1979 and tested by Thaithong and Beale [9] and found to be resistant to chloroquine and pyrimethanmine; this strain was provided to Liverpool School of Tropical Medicine by Doctor D. Walliker of Edinburgh University.

(2)FCR3 strain isolated in September 1976 from a patient in Gambia (Nguyen-Dinh and Trager [10]; this strain was provided by Professor M. Hommel, Head of Immunology Section, Liverpool School of Tropical Medicine.

(3) Indochina 1/CDC also provided by Professor Hommel. It is an isolate from an American physician working on the Thai-Kampuchea border in 1983, this parasite after adaptation for culture in the laboratory was maintained in *Aotus* monkey.

The second group isolated

In the field contains 23 isolates 20 of them were isolated from patients in El Geraif West area, south east of Khartoum Province, Sudan, the 20 isolates which

were called arbitrary S1 to S20 were sensitive to chloroquine *in vivo* and to six anti malarials *in vitro* namley, chloroqine, quinine, amodiaqunine , mefloquine, cycloguanil and pyrimethamine. Another isolate with a similar drug profile both *in vivo* and *in vitro* and also called arbitray S4-HT was isolated from a laboratory attendant who lives in the very north of Khartoum Province. The two remaining isolates also arbitrary called SD 19 and SD 23 were provided by F.M. Omer, Immunology Section, Liverpool School of Tropical Medicine, the isolates were from Gezira in the center of Sudan and both were sensitive to chloroquine *in vitro*.

The technique of culture was modified from the method of Jensen and Trager [11]. The culture was carried in tissue culture flasks, Nunclon, (Nunc). The medium used was RPMI - 1640 (Gibco, Biocult) supplemented with HEPES buffer 5.94 gram/liter, sodium bicarbonate 2 gram/liter and human AB positive serum at rate of 10 – 15 ml/100 ml medium, O positive human red blood cells were used as well as a gas mixture (Nitrogen 93%, Oxygen 3% and Carbon dioxide 4%), incubation was at 37 °C. The infected cells were washed in ice cold saline and were either used as a whole or the parasites were freed for electrophoresis, the method used to free the parasites was that described by Christopher and Fulton [12] using saponin. The material for electrophoresis (either infected red blood cells or freed parasites) was diluted in an equal volume of double distilled water or dilute Triton X-100 (1 or 5%), and the suspension was either used directly for electrophoresis or treated by one of the following methods:

a) Freezed in liquid nitrogen and thawed rapidly at 37 degrees water bath (repeated three times).
b) Sonicated with probe sonicater (Soniprobe type 7532 B, Dave).
c) Sonicated in a frequency sweep water bath sonicator (FS 100 Decon).

Freeze thawed and sonicated suspensions were spun in a chilspin (MSE) at 4 °C for half an hour, occasionally they were spun in a sorvall RC5B refrigerated super speed centrifuge (Du Pont) at 20000 g for half an hour, the supernatant was then pipetted carefully and either used immediately or stored frozen at minus 20 °C for use in the future. The product of similarly treated fresh washed human red blood cells was used as a control for host enzymes. The human blood was the same one used to maintain the parasite culture and it is obtained from the blood bank of Liverpool Royal Infirmary Hospital and stored at 4 °C after being divided aspetically under a sterile hood into aliquots of 20 ml in sterile screw capped plastic universal containers.

Starch gel was prepared according to Smith [13, pp. 153–184], 22 grams of electrostarch (Electro-starch Co., U.S.A.) were suspended in 250 ml of gel buffer and after cooking the gel was poured quickly but gently over a mould consisted of two perspex rims, one over the other and after cooling at room temperature the preparation was kept at 4 °C.

The electrophoresis run of the parasite material, which was always on ice whenever it was outside the refrigerator, was a horizontal one, on a surface cooled to

minus 10 °C with an antifreeze cooling system (Grant FH 15). The power pack was a Vokam power unit (Shandon) which was able to deliver a current up to 80 milli Amperes and a voltage up to 400 volts, and it could be set either at a constant current or a constant voltage.

After the electrophoresis run, the sliced gel was stained in a staining system similar to that of Carter [14], the gel was sliced between the two perspex rims with a cheese wire and the top portion of the gel was laid on a glass plate exposing the internal surface over which a perspex rim was laid. The staining chemicals were dissolved in some of the incubation buffer and the rest of the buffer was used to cool a 1% agar solution which was very rapidly mixed with the staining solution before it was poured on top surface of the sliced gel which was immediately incubated at 37 °C until the band or bands of the specific enzyme appeared. The warm pre-boiled agar solution was dissolved in the staining buffer to minimize the spread of the enzyme bands.

The electrophoresis and staining systems for each enzyme are shown in Table 1.

The gel used for isoelectric focusing was obtained ready made from LKB (LKB Ampholine PAG Plates pH range 3.5 to 9.5) and the tank used was Multiphor 2117 (LKB), connected to the Grant FH 15 cooling system, and the power pack was a stabilised power supply unit (Chandos), set at a maximum current of 50 mA with a maximum voltage of 1500 volts and a maximum power of 30 watts for 90 minutes. The isoelectrofocused gels were soaked in staining solutions each specific for the required enzyme.

Results

Glucose phosphate isomerase (GPI)

The initial four hours starch gel electrophoresis of the probe sonicated material from 20 Sudanese isolates (S1-S20) and the control fresh red blood cells, did not show satisfactory staining, as some of the isolates showed three closely related bands of the parasite enzyme migrating towards the anode and a single host enzyme migrating in the opposite direction, the remaining isolates either showed a faint staining or did not stain at all. A more concentarated extract of the isolates which failed to stain after the first run, showed the three bands when stained after a second four hours run. The same 20 Sudanese isolates showed the three enzyme bands distinctly separated when the starch electrophoresis was continued for six hours instead of four hours, and depending on the degree of host cells contamination, the host enzyme showed either one or two bands while the relatively pure preparations did not show any staining for the host enzyme. The three enzyme bands were also demonstrated in the extract of the other isolates (Indochina 1/CDC, K1, FCR3, SD 19, SD 23, S4-HT, S1 and S2.) when the run was continued for six hours. All The preparations from the fresh free FCR3 parasite material which were either probe sonicated, water bath sonicated or

Table 1. The electrophoresis and staining systems used for each enzyme

The enzyme	The gel buffer	The electrode buffer	The origin of the sample	The duration of the run	The type of power used	The staining buffer including the enzyme substrate and the staining chemicals
Glucose phosphate isomerase (GPI)	0.01 M Tris HCl pH8	0.05 M Tris MCl pH8	6.35 cm from the cathodal end	4 to 6 hours	Constant current 60 milli amperes	The electrode buffer plus 1mg disodium fructose -6- phosphate, 0.1 mg NADP, 0.1mg MATT, 0.4mg Mg chloride , 0.2 µL glucose -6-phosphate dehydrogenase and 10 µg phenothiazine metasulphate (PMS) All in 1 ml of buffer
Lactate dehydrogenase (LDH)	Tris citrate pH6.5 (0.065 M Tris 0.0222 M citric acid)	Tris citrate pH 6.2 (0.22 M Tris, 0.078 M citric acid)	7.62 cm from the cathodal end	14 hours (overnight)	Constant current 70 milli ampere	0.05 M Tris HCl pH8 plus 4mg Lithium lactate, 0.1 mg NAD, 0.1 mg MTT and 10 µg phenothiazine metasulphate (PMS). All in 1ml of buffer.
6-Phosphogluconate dehydrogenase (6PGD)	Tris citrate pH6.4 (0.175 M Tris 0.0063 M citric acid)	Tris citrate pH 6.2 (0.22 M Tris, 0.0785 M citric acid)	6.35 cm from the cathodal end	4 hours	Constant current 75 milli ampere	0.05 M Tris HCl pH8 plus 1mg barium-6 phosophogluconate, 0.1 mg NADP, 0.1 mg MTT and 10 µg phenothiazine metasulphate (PMS). All in 1ml of buffer.
Glutamate dehydrogenase (GDH)	Citrate phosphate pH7 (0.0054 M citric acid, 0.032M disodium hydrogen phosphate)	Citrate phosphate pH7 (0.013 M citric acid, 0.155M disodium hydrogen phosphate)	6.35 cm from the cathodal end	14 hours (overnight)	Constant current 75 milli ampere	0.05 M Tris HCl pH8 plus 2mg glutamic acid monosodium salt, 0.1mg NADP, 0.1mg MTT and 10 µg phenothiazine metasulphate (PMS). All in 1 ml of buffer.

1% Triton x-100 lysed and those from the liquid nitrogen frozen free FCR3 parasite which were either probe sonicated or 1% Triton x-100 lysed showed the three bands when specifically stained after a six hours run. The parasite in the liquid nitrogen was freed and freezed by a different worker (Miss. M. Percy).

After the isoelectric focusing of the extracts from the following isolates, Indochina 1/CDC, K1, FCR3, SD 19, SD 23, S4-HT, and S1 together with that of the control red blood cells, the stained gel showed that, the parasite enzyme in each isolate was focused at four different points which were almost identical in all the tested isolates, and the host enzyme was focused at three different sites. In K1 and S4-HT due to the highly diluted extract the stain was too faint.

In all the isolates the isoenzyme with the highest isoelectric point (IEP) was the most faintly stained one.

Lactate dehydrogenase (LDH)

In all the 26 isolates studied there was only one parasite enzyme band seen migrating towards the anode when the starch gel was stained after electrophoresis, and there were six host enzyme types demonstrated, five of them moving towards the anode while the sixth had an opposite direction. The stain after the isoelectric focusing of the seven isolates (Indochina 1/CDC, K1, FCR3, SD 19, SD 23, S4-Ht and S1) was not satisfactory and the resolution was very poor because fuzzy host enzyme bands were mixed with those of the parasite origin, and this was persistent all through the next four trials. In a separate trial a duplicate of the seven isolates was focused in a single gel and after the run the gel was divided into two halves each half containing the seven isolates, one half was stained for lactate dehydrogenase and the other half stained for glucose phosphate isomerase (GPI), and although the half stained for GPI showed distinct and sharply focused bands, the other half stained for lactate dehydrogenase showed the same confusing picture.

Glutamate dehydrogenase (GDH)

All the 26 isolates were tested for this enzyme and the starch gel showed a single form of this enzyme in all the isolates tested but failed to show any host enzyme activity. The stain after the isoelectric focusing of the seven selected isolates was able to demonstrate only the single parasite enzyme focused at an identical point in all the isolate.

6 Phosphogluconate dehydrogenase (6PGD)

All the 26 isolates were tested for this enzyme in starch gel in which the specific stain showed two enzyme bands in each isolate which migrated towards the anode, the slow moving band was demonstrable in the extract from the fresh uninfected red blood cells and so it was related to the host enzyme. The parasite band in all the isolates moved the same distance from the place of origin indicating the similarity of the

enzyme type. Isoelectric focusing was done four times for the seven selected isolates but the result of the stain in the four trials was inconclusive like that of lactate dehydrogenase.

Discussion

From the results obtained from the 26 isolates of *Plasmodium falciparum* employed in this work, it was evident that there was no enzyme variation between the Sudanese isolates, similarly there was no obvious variation between the Sudanese isolates and the reference isolates; namely FCR3, K1 and Indochina 1/CDC as detected by starch electrophoresis of the following enzymes, glucose phosphate isomerase (GPI) lactate dehydrogenase (LDH), glutamate dehyrogenase (GDH) and 6 phosphogluconate dehydrogenase (6PGD). Isoelectric focusing also showed that there was no variation between all the isolates in case of the GPI and GDH. Due to unexplained technical difficulties which resulted in persistently bad resolution of the bands, variation in LDH and 6PGD could neither be confirmed nor excluded.

Of the 26 isolates studied, one isolate FCR3 has been investigated by Sanderson *et al* [15] and it was found to have a single form of each of the four enzymes being investigated in this work, and has been classified a GPI-1 *P. falciparum*. They used starch gel electrophoresis for the demonstration of the enzymes, and as in the present study the FCR3 strain was found to have three forms of the enzyme GPI, using the same electrophoretic technique and with each of the different techniques of enzyme extraction, it was concluded that starch electrophoresis can sometimes fail to demonstrate more than a single form of the enzyme despite the actual presence of other isoenzyme variants. It was also clear that starch electrophoresis was inferior to isoelectric focusing which was able to demonstrate four forms of the enzyme GPI, in an isolate in which only a single form was demonstrated by other workers using starch electrophoresis. In the present study the FCR3 line was cloned and the current results cannot be explained as resulting from a mixed FCR3 with multiple variants each with individual isoenzymes. The unresolved question, therefore is how multiple forms of GPI occur within a cloned stock of the parasite?

That a similar isoenzyme pattern for GPI was found in the FCR3 stock and in each of the other 25 isolates studied further indicates that, this enzyme is of lesser value in parasite taxonomy than previously assumed. The possibility of an artifact was excluded as the result was repeated in all experiments with different types of preparations of the enzyme extract in fresh and forzen parasites.

Myint-Oo [4] found five forms of GPI in *Plasmodium vivax* three of which were not reported in *P. falciparum* and this may be explained by the fact that similar forms of GPI occur both in *P. falciparum* and *Plasmodium vivax* but some of them were difficult to demonstrate in *P. falciparum* due to their presence in small quantities while they are predominant in *P. vivax*.

The other 25 isolates have not been investigated by other workers to see if any of them possessed another variant of the other three enzymes, LDH, GDH, 6PGD, as more than a single form of these enzymes were demonstrable in other isolates, but not in FCR3 Sanderson *et al* [11].

In this work it was apparent that there was no demonstrable difference in four enzymes between *P. falciparum* isolates of African and Asian origin, including strains sensitive and resistant to a series of anti malarial drugs.

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إخفاق في تمييز المعزولات عن طريق اختلاف الأيزوإنزيمات وذلك بالنسبة لمعزولات المتصوّرة المنجليّة

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مستشفى الأمل بالرياض، صندوق بريد ٢٩٩٣٢ ،

الرياض ١١٤٦٧ ، المملكة العربية السعودية

ملخص البحث. تم البحث عن اختلاف الإيزوإنزيمات في المعزولات السودانّية والمرجعيّة بالنسبة للمتصوّرة المنجليّة *Plasmodium falciparum* بوساطة الرحلان الكهربائي في الهلام النشوي وبوساطة التبئير الكهرساوي (المتساوي التكهربي). لم يوجد أي اختلاف في مزامرة فوسفات الغلوكوز (GPI) ؛ نازعة هدروجين اللاكتات (LDH) ؛ نازعة هدروجين الغلوتمات (GDH) نازعة هدروجين الـ ٦ فوسفوغلوكونات (6PGD) . الرحلان الكهربائي في الهلام النشوي كشف عن ثلاثة أنواع من إنزيم مزامرة فوسفات الغلوكوز (GPI) والتبئير الكهرساوي كشف عن أربعة أنواع من الإنزيم نفسه وذلك حتى في معزولات منسلّة.