Cloning of Naturally Occurring Mixed Infections of Malaria Parasites

Abstract. *Clones have been established from a cultured isolate of* Plasmodium falciparum *characterized by two electrophoretic forms of glucose phosphate isomerase. Cultures initiated from diluted samples containing an estimated 1.0 or 0.5 parasite showed microscopically visible parasites after 21 days. Most of these cultures were characterized by only single enzyme forms.*

Malaria parasites in samples of blood taken from individual human patients in Africa and Southeast Asia have been shown to be heterogeneous by studies on electrophoretic variants of enzymes (*1, 2*). This heterogeneity is important because it implies that the parasites cultured in vitro for the purpose of studying their reactions may not, clinically, be the most important component of the original infection. It is necessary to demonstrate that natural infections are mixtures of genetically diverse clones of parasites, and to develop a method for the production of cultures of *Plasmodium falciparum* that are single clones. Such cloning can be achieved by diluting a culture of parasites showing two or more enzyme types and growing isolated samples into new cultures that exhibit only a single enzyme variant. This technique, previously used with rodent malaria parasites growing in mice (*3*), has now been applied to an isolate of *P. falciparum* from Thailand, and we show in this report that cultures of this species can be produced in vitro from single parasites.

The isolate used (T9) was obtained from a patient at Tak, Thailand, and established in continuous culture by Sodsri Thaithong at Chulalongkorn University, Bangkok, by the petri-dish method of Trager and Jensen (*4*). When first examined this culture was found to contain two forms of glucose phosphate isomerase (GPI-1 and GPI-2) (E.C. 5.3.1.9), and two forms of adenosine-deaminase (ADA-1 and ADA-2) (E.C. 3.5.4.4). However, after receipt of the culture in Edinburgh and growing it for 2 weeks in the laboratory, the band of enzyme activity for ADA-2 could no longer be detected, and after 5 weeks the band for GPI-2 had also disappeared. These observations suggested that the original isolate included several clones, and that under the conditions of culture in vitro some clones were selectively favored at the expense of others.

Individual clones were isolated by establishing a culture exhibiting good growth; by the time this was done, however, ADA-2 had disappeared and GPI-2 was present in reduced amount by comparison with GPI-1. The culture was diluted with RPMI 1640 medium containing 10 percent (by volume) freshly washed red blood cells to concentrations at which 0.1-ml samples contained an average of 1.0 or 0.5 parasite. These 0.1-ml samples were pipetted individually into wells of Falcon Microtest II plates that were then covered with lids and incubated at 37°C in a candle jar (*4*). After certain periods the parasites that grew in individual wells were typed in regard to the enzyme variants.

Since the cloning method depends on the isolation of single red cells infected with single parasites, it was important to reduce the proportion of cells infected with two or more parasites to a minimum. Unshaken cultures in vitro contain many multiply infected red cells. Therefore, the petri dishes containing the undiluted cultures were shaken (*5*) for 12-hour periods for 4 days. This reduced the proportion of cells containing multiple infections to about 5 percent, a percentage that does not significantly affect the results of the cloning experiments.

Two experiments were carried out:

1) With cultures having an initial hematocrit value of 10 percent that later decreased to 7 percent.

2) With cultures showing a 2 percent hematocrit value throughout (*6*).

In experiment 1 the supernatant in the wells was withdrawn every 24 hours and replaced with fresh medium, the red cells remaining at the bottom of the wells. After 5 days, following removal of the supernatant from each well, the red cell pellet was divided into two, one part being placed in a new well, the other remaining in the original well, and medium together with fresh uninfected red blood cells were then added to both wells. This procedure minimized loss of parasitized cells when only a very small number were present in individual wells. After another 4 or 5 days the medium and red blood cells were again replaced, but half the red cells from each well were discarded. In experiment 2, the medium was replaced every 48 hours and fresh red blood cells were added every 4 days.

Stained preparations of blood from the wells were made on day 21 in both experiments 1 and 2, and if parasites were seen, the well contents (0.1 ml)

Table 1. Enzyme types of cultures established from estimated 1.0 and 0.5 parasite. Figures in parentheses are calculated expected numbers, allowing for numbers not tested; figures in brackets are expected proportions of single clones in wells containing a single enzyme type. Parasites not tested were from contaminated cultures. In experiment 1 the cultures showed hematocrit values of 10 percent, decreasing to 7 percent; in experiment 2 the cultures showed 2 percent hematocrit values.

Calculated average number of parasites per well	Total number of wells inoculated	Number of wells		Enzyme types of parasites in individual wells			
		Developing parasites	Not developing parasites	GPI-1	GPI-2	GPI-1 and GPI-2	Not tested
				Experiment 1			
1	40	31	9 (14.7)	23 (14.4)*	6 (4.9)	1 (5.0)	1
0.5	40	7	33 (24.3)	4 (10.2)	1 (3.9)	2 (1.7)	0
				Experiment 2			
1	56	32	24 (20.6)	18 (18.3) [70 percent]	6 (6.3) [85 percent]	4 (6.4)	4
0.5	48	18	30 (29.1)	10 (10.9) [80 percent]	4 (4.2) [90 percent]	2 (1.8)	2

*The method of calculating expected numbers is as follows. Let m be the mean number of parasites inoculated into wells, p the frequency of GPI-1, and $(1 - p)$ the frequency of GPI-2. If one assumes independence of infection by parasites containing GPI-1 or GPI-2, the probability of zero parasites per well is $e^{-mp} \times e^{-m(1-p)} = e^{-m}$. The expected number of wells containing one or more clones of parasites with GPI-1 (no GPI-2) is $(1 - e^{-mp}) \times e^{-m(1-p)} = e^{-m(1-p)} - e^{-m}$; the expected number of wells containing one or more clones of parasites with GPI-2 (no GPI-1) is $e^{-mp} - e^{-m}$; and the expected number of wells containing GPI-1 + GPI-2 is $(1 - e^{-mp})(1 - e^{-m(1-p)})$. The probability that GPI-1–containing wells contain single clones is $pm/(e^{mp} - 1)$, or for small numbers, $\sim 1/(1 + mp/2)$; and the probability that GPI-2–containing wells contain single clones is $(1 - p)m/(e^{m(1-p)} - 1)$, or $1/1 + m(1 - p)/2$.

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were transferred to larger wells (volume of culture, 1 ml) and, after further growth, to petri dishes (volume of culture, 4 ml), to yield sufficient material for the starch-gel enzyme tests. These were carried out 24 to 38 days after the original isolation of the 0.1-ml samples, depending on the growth rates of the different clones. Parasites were prepared for the enzyme tests by the method of Carter (*7*).

Table 1 shows that the original culture contained, at the time of dilution, a mixture of parasites of which approximately 70 percent were of type GPI-1 and 30 percent of type GPI-2 (all being ADA-1). The samples isolated into wells contained GPI-1, GPI-2, or GPI-1 plus GPI-2, the last presumably originating either from the simultaneous isolation of two different parasitized cells in the original 0.1-ml sample, in accordance with the Poisson distribution, or from the isolation of doubly infected cells. Analysis of the data in Table 1 shows that the results of experiment 2 agree well with the calculated expectations, whereas those of experiment 1 deviate considerably. It is assumed that growth of parasites in medium with a 2 percent hematocrit value is more regular than with a 10 or 7 percent value. As shown in Table 1, it is expected that with the dilutions chosen, 70 to 80 percent of the GPI-1 cultures and 85 to 90 percent of the GPI-2 cultures contained single clones.

Among the isolates obtained by this dilution procedure, five took 8 to 15 days longer than the remainder to yield enough material for enzyme typing. These isolates had a markedly slower growth rate, indicating that, in addition to enzyme variation, the original T9 isolate was heterogeneous with respect to growth-rate determining factors. Thus this isolate may have contained a larger number of clones than are shown by enzyme typing alone.

Electrophoretic forms of enzymes are particularly suitable as markers in this work because they are genetically stable and because mixed infections can be identified easily. *Plasmodium falciparum* is also heterogeneous with regard to antigens (*8*), and isolates that exhibit multiple drug resistance (*9*) may, in fact, be mixed infections of parasites exhibiting resistance to each drug separately. Cloned material will be important for analyzing the genetic composition of such isolates, as well as for other types of research, for example, for epidemiological and immunological studies aimed at the production of an antimalarial vaccine.

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References and Notes

1. R. Carter and A. Voller, *Trans. R. Soc. Trop. Med. Hyg.* **69**, 371 (1975).
2. S. Thaithong, T. Sueblinwong, G. H. Beale, *ibid.*, in press.
3. D. Walliker, R. Carter, S. Morgan, *Parasitology* **66**, 309 (1973).
4. W. Trager and J. Jensen, *Science* **193**, 674 (1976).
5. G. A. Butcher, *WHO Bull.* **57** (Suppl. 1), 17 (1979).
6. P. Nguyen-Dinh and W. Trager, *Am. J. Trop. Med. Hyg.* **29**, 339 (1980).
7. R. Carter, *Parasitology* **76**, 241 (1978).
8. R. J. M. Wilson, *Nature (London)* **284**, 451 (1980).
9. *WHO Tech. Rep. Ser.* **529**, 30 (1973).
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Polyphenolic Substance of *Mytilus edulis:* Novel Adhesive Containing L-Dopa and Hydroxyproline

Abstract. *The fouling marine mussel* Mytilus edulis *attaches itself to various substrates by spinning byssal threads, the adhesive discs of which are rich in the amino acid 3,4-dihydroxyphenylalanine (dopa). An acid-soluble protein was extracted and purified from the phenol gland located in the byssus-secreting foot of the animal. This protein is highly basic and contains large amounts of lysine, dopa, and 3- and 4-hydroxyproline. The composition of this protein and its sticky tendencies in vitro strongly suggest that it contributes to byssal adhesion.*

The marine mussel *Mytilus edulis* successfully inhabits many of the most turbulent and inhospitable niches in the intertidal zone. This success is in part related to the animal's use of the byssus to glue itself securely to a wide variety of substrates (*1*). The byssus consists of a bunch of collagenous threads secreted by exocrine glands in the foot of the mussel (*2*). The adhesive ends of these threads are rich in a polyphenolic substance that is derived from the phenol gland located near the tip of the foot (*3–5*). The remarkably high tensile strength of the bond between the byssal adhesive and its substrate (*6*) has drawn the attention of an increasingly interdisciplinary research effort in recent years. Although the identification of the polyphenolic substance has been a prime target of research, the insoluble and sclerotized condition of the byssus has frustrated attempts to extract individual protein components from the structure. We have isolated the soluble polyphenolic substance from the phenol gland and report that it is a protein rich in 3,4-dihydroxyphenylalanine (dopa) and hydroxyproline.

In a study of structural proteins in *M. edulis*, a soluble polyphenolic substance resembling dopa was extracted from the phenol gland with dilute acid (*3*). The presence of dopa in hydrolysates of byssal discs as well as in acid-soluble protein from the phenol gland has now been confirmed (*7*). We dissected phenol glands from locally collected mussels, extracted them with neutral salt buffer, and homogenized the salt-insoluble material in 5 percent acetic acid. The acid-soluble proteins usually contained 25 to 40 μg of dopa per milligram of protein; dopa was associated with two polypeptide bands as judged by electrophoresis at acid *p*H (Fig. 1). The acid-soluble dopa proteins have a tendency to become adsorbed to chromatographic materials and to precipitate irreversibly at high ionic strength and neutral *p*H, during sample concentration, and in the presence of sodium dodecyl sulfate (SDS). For these reasons, we adopted the following procedures for purification: The dopa-containing material was applied to a column of SP-Sephadex. Using a linear gradient of guanidine hydrochloride we eluted a dopa-containing peak at about 8 percent guanidine hydrochloride (*8*). Peak fractions were pooled and fractionated on phenyl Sepharose 4B-CL and Sephadex G-200, respectively. Acid gel electrophoresis of the G-200 peak showed two bands (Fig. 1, bands a and b), both of which stained for protein and dopa. Since SDS gel electrophoresis proved useless for this protein, we turned to electrophoresis in the presence of cetylpyridinium chloride to estimate molecular weights. Bands a and b of Fig. 1 exhibit apparent molecular weights of 135,000 and 125,000, respectively, migrating between the α and β chains of type I collagen.

The presence of two polyphenolic proteins with similar mobilities on gel filtration, ion-exchange chromatography, and gel electrophoresis, at first suggested to us a two-subunit composition such as the α_1 and α_2 chains of type I collagen. However, upon repeated extraction of the polyphenolic protein from the phenol

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