Plasma Replacement for in vitro Culture of Plasmodium knowlesi

Abstract. *Of six fractions of human plasma tested, only Cohn's fraction IV-4 was effective for the replacement of whole plasma from monkey* (Macaca mulatta) *for the in vitro culture of* Plasmodium knowlesi. *The effects observed on multiplication and morphology of parasites suggest a specific role of some substance or substances in fraction IV-4.*

The appearance of strains of human falciparum malaria resistant to chloroquine (*1, 2*) has stimulated investigations on the mode of action of this drug and on the mechanism of resistance to it. To obtain a better understanding of malarial parasites and their metabolism, pathogenesis, immunity, and susceptibility to chemotherapeutic agents, it is imperative to continue intensive biological and biochemical in vitro studies of known pathogenic malarial parasites. Extensive work has been done in vitro by Trager (*3*) with *Plasmodium lophurae* in duck red blood cells, by Anderson (*4*) with *P. gallinaceum* in chick red blood cells, and by Geiman *et al.* (*5*) with *P. knowlesi* in monkey red blood cells. The choice of *P. knowlesi* in the latter studies, that started in 1942, was fortunate because the species has a 24-hour asexual cycle, is highly pathogenic for monkeys (killing them in approximately 5 to 8 days with a falciparum-like syndrome), and will produce clinical malaria in man. Recently *P. knowlesi* has also been found as a natural infection in man (*6*).

A medium was developed (*5*) in which suspensions of monkey red blood cells supported intraerythrocytic growth and multiplication of *P. knowlesi*. Under these conditions the requirements of the parasites for glucose, *p*-aminobenzoic acid (*7*), and methionine (*8*) were demonstrated. However, a precise definition of essential and nonessential components of this medium was not achieved; this may have been due, in part, to unknown factors in plasma or to the masking effect of plasma components of the whole blood under study (*7*). Growth experiments were performed with red cells that had been washed with a modified Ringer solution and resuspended in a complete synthetic medium containing purified serum albumin at a final concentration of 1 to 5 percent (*7*). Results indicated that growth and multiplication of parasites were less in a plasma-free medium even when supplemented with albumin, except for one experiment in which 1 percent albumin did enhance growth. McGhee and Trager (*9*), working with *P. lophurae* in chick red blood cells, found adverse effects on growth and multiplication of parasites when chick plasma was replaced by bovine albumin. In this report we describe the successful replacement of monkey (*Macaca mulatta*) plasma by Cohn's fraction IV-4 of human plasma for the intracellular cultivation of *P. knowlesi* in vitro.

Plasmodium knowlesi (*10*) was maintained by weekly intravenous inoculation of erythrocytic stages into monkeys (*Macaca mulatta*). The techniques for culturing, counting, and evaluating the parasites were based on those previously described (*5*). The rocker-dilution technique was modified by increasing the ratio of synthetic medium to blood from 3 : 1 to 6 : 1. The synthetic medium was identical to that described by Anfinsen *et al.* (*7*) except for addition of 0.005*M* glycylglycine (final concentration). Blood containing the parasites was diluted with normal blood to give a starting parasitemia of 1 to 3 percent. Blood was centrifuged to remove plasma; erythrocytes were then washed twice with cold modified Ringer solution (*11*) and resuspended in synthetic medium. Measurement of protein (biuret method) in the supernatant from the second wash indicated that more than 90 percent of plasma protein was removed by this washing procedure.

Human plasma fraction IV-4 (*12*) was suspended in 0.5 percent $NaHCO_3$ at a concentration of 300 mg/ml. Loss of protein from the final medium, upon sterilization by filtration, indicated that all of the material did not dissolve.

In a series of six experiments conducted to test the effect of Cohn's fraction IV-4 on growth and multiplication of *P. knowlesi* in a plasma-free medium, two sets of controls were used—one without and the other with plasma. Tubes in all experiments were run in duplicate. Results (Table 1) indicate that growth and multiplication of parasites in tubes containing synthetic medium without plasma were very poor in comparison with those of parasites in tubes containing plasma and synthetic medium. However, in tubes in which synthetic medium was supplemented with fraction IV-4 growth and multiplication of parasites was as good as in control tubes with plasma. Experiment No. 36 demonstrated that 1 percent albumin was ineffective as a replacement of plasma.

The percentage of extracellular and degenerate forms of parasites was higher in tubes containing plasma-free syn-

Table 1. Cohn's fraction IV-4 as plasma replacement for in vitro cultivation of *Plasmodium knowlesi*. The synthetic medium is identical to that described by Anfinsen *et al.* (*7*), except for the addition of glycylglycine so that its final concentration was 0.005*M*. For these experiments 9.0 ml of synthetic medium and 1.5 ml of infected blood were added to each vessel. Cells were washed twice with cold modified Ringer solution. Measurement of protein by the biuret method demonstrated that 90 percent or more of the plasma protein was removed by this washing procedure. Cohn's fraction IV-4 of human plasma was added in the amount of 15 mg per milliliter of medium (before filtration). In experiments No. 37 and No. 41, 80 to 90 percent of the parasites were at the early ring stage, while in the rest of the experiments most of the parasites were at mature trophic and early schizont stages.

Exp. No.	Additions to culture medium	Parasites per 100 RBC		Ratio 24/0 hr	Degenerate and extracellular forms (%)
		0 hr	24 hr		
36	None	1.9	2.8	1.5	14
	1% Albumin	1.9	2.0	1.05	12
	Fraction IV-4	1.9	7.1	3.7	4
	Plasma	2.5	7.1	2.8	< 1
37	None	2.2	2.1	1.0	8
	Fraction IV-4	2.2	3.05	1.4	2
	Plasma	1.9	3.6	1.9	2
38	None	1.5	2.3	1.5	19
	Fraction IV-4	1.5	8.0	5.3	2
	Plasma	1.5	8.7	5.8	< 1
39	None	1.4	0.9	0.65	30
	Fraction IV-4	1.4	3.6	2.6	2
	Plasma	1.3	3.4	2.8	3
40	None	2.3	2.2	1.0	39
	Fraction IV-4	2.3	9.4	4.1	6
	Plasma	4.0	15.2	3.8	1
41	None	2.4	2.9	1.2	19
	Fraction IV-4	2.4	5.0	2.1	7
	Plasma	2.7	6.3	2.3	4

thetic medium than in the control tubes. When the synthetic medium was supplemented with fraction IV-4 the percentage of these forms was approximately as low as in the control tubes with plasma. Differential counts made at the end of 24 hours indicated that in experiments No. 37 and No. 41 (in which 80 to 90 percent of the parasites were in the early ring stage when the experiment was started) 90 to 94 percent of parasites in plasma-free medium developed only to the mature trophic stage, whereas in the plasma-free medium supplemented with fraction IV-4, 61 to 82 percent developed to schizont and segmenter stages similar to those that developed in vitro in the presence of plasma, and in vivo. Such results suggest that a substance or substances in whole plasma and in fraction IV-4 are required for intracellular asexual development of this strain of *P. knowlesi*.

The beneficial effect of plasma on in vitro cultivation of cells and tissues was interpreted as being a nonspecific "physical" property of plasma macromolecules (*13*). Anfinsen *et al.* (*7*), in discussing the results of their experiments in which albumin was substituted for plasma, concluded that "the albumin does not appear to act as a nutritional factor but rather in a physical manner." Although no assignment of a physiological role to a substance or substances in fraction IV-4 of human plasma can be made, the role of this fraction appears to be more than a nonspecific one for the following reasons: (i) Other fractions of human plasma, gamma globulin fraction II, globulin beta fraction III, beta lipoprotein fraction III-0, albumin fraction V, and crystalline albumin (*14*) were also tested individually in the same manner as fraction IV-4, but growth and multiplication of *P. knowlesi* were as poor in these as in medium without plasma. If the effect of a plasma fraction is just a nonspecific one, such as buffer action or osmotic effects, one or more of these plasma fractions should have supported growth of the parasite. (ii) Concentration of fraction IV-4 in the medium is critical in its effect on growth and multiplication of the parasite. Fraction IV-4 was tested at concentrations of 3.5, 7.0, 15.0, and 18.5 mg per milliliter of medium, and the best result was obtained in tubes having a concentration of 15.0 mg/ml. While a satisfactory result was obtained with 7.0 mg/ml, an adverse effect on growth and multiplication of the parasite and an increase in percentage of degenerate and extracellular forms were observed with the lowest and highest concentrations of fraction IV-4. (iii) Glucose utilization during the 24-hour growth period in tubes with fraction IV-4 was approximately equal to that in tubes with plasma and 80 to 140 percent higher than in tubes without plasma or fraction IV-4. (iv) Chang *et al.* (*15*), while attempting to characterize the protein or proteins of human plasma essential for maintenance and growth of human conjunctival cells in culture, stated that "growth-promoting activity is associated chiefly with Fraction IV-4."

Results presented in Table 1 clearly demonstrate that fraction IV-4 can replace whole monkey plasma when added to the synthetic medium for cultivation of parasites. While this is significant progress toward our goal of attaining a truly chemically defined growth medium, it must be emphasized that fraction IV-4 is a complex mixture of known and unknown materials (*16, 17*).

QUENTIN M. GEIMAN
WASIM A. SIDDIQUI
JEROME V. SCHNELL
Department of Preventive Medicine, Stanford University School of Medicine, Palo Alto, California

References and Notes

1. M. D. Young and D. V. Moore, *Amer. J. Trop. Med. Hyg.* **10**, 317 (1961).
2. R. L. Degowin and R. D. Powell, *ibid.* **14**, 519 (1965).
3. W. Trager, *J. Exp. Med.* **74**, 441 (1941).
4. C. R. Anderson, *Amer. J. Trop. Med. Hyg.* **2**, 234 (1953).
5. Q. M. Geiman, C. B. Anfinsen, R. W. McKee, R. A. Ormsbee, E. G. Ball, *J. Exp. Med.* **84**, 583 (1946).
6. W. Chin, P. G. Contacos, C. R. Coatney, H. R. Kimball, *Science* **149**, 865 (1965).
7. C. B. Anfinsen, Q. M. Geiman, R. W. McKee, R. A. Ormsbee, E. G. Ball, *J. Exp. Med.* **84**, 607 (1946).
8. R. W. McKee and Q. M. Geiman, *Federation Proc.* **7**, 172 (1948).
9. R. B. McGhee and W. Trager, *J. Parasitol.* **36**, 123 (1950).
10. The strain used for these studies was originally isolated from a monkey (*Macaca irus*) from Malaya and was given to us in 1965 by E. H. Sadun of Walter Reed Army Institute of Research, Washington, D.C.
11. Composition of modified Ringer solution used for washing red blood cells: NaCl, 8.21 g; KCl, 0.30 g; $CaCl_2$, 0.20 g; $MgCl_2$, 0.10 g; and H_2O, 1000 ml.
12. Human plasma IV-4 is prepared by Cohn's method No. 6 [E. J. Cohn, L. E. Strong, W. L. Hughes, Jr., D. J. Mulford, J. N. Ashworth, M. Melin, H. L. Taylor, *J. Amer. Chem. Soc.* **68**, 459 (1946)] and was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio.
13. J. H. Woodliff, *Blood and Bone Marrow Cell Culture* (Lippincott, Philadelphia, 1964), p. 72.
14. Obtained from Nutritional Biochemical Corporation.
15. R. S. Chang, R. B. Pennell, W. Keller, L. Wheaton, H. Liepens, *Proc. Soc. Exp. Biol. Med.* **102**, 213 (1959).
16. Since fraction IV-4 is rich in alpha globulin, it is relevant to cite the results of A. Tella and B. G. Maegraith, *Ann. Trop. Med. Parasitol.* **59**, 153 (1965), which indicated that there was an increase in $alpha_1$ and $alpha_2$ globulin during the course of *P. knowlesi* infection in rhesus monkeys.
17. R. B. Pennell, in *Plasma Proteins*, F. W. Putnam, Ed. (Academic Press, New York, 1960), vol. 1, p. 48.
18. We thank W. F. Argall, Rochelle Golenberg, and M. J. Meagher for technical assistance. Supported by contract No. DA-49-193-MD-2587, Department of the Army and U.S. Army Medical Research and Development Command. This paper is contribution No. 81 from the Army Research Program on Malaria.

23 June 1966

Methylene-C^{14}-Dioxyphenyl Compounds: Metabolism in Relation to Their Synergistic Action

Abstract. *The methylene-C^{14} group is hydroxylated yielding formate-C^{14} in the microsome-reduced nicotinamide-adenine dinucleotide phosphate system in vitro and yielding expired $C^{14}O_2$ in living mice and houseflies. Methylenedioxyphenyl compounds apparently serve as alternate substrates for this enzymatic hydroxylation system of microsomes, and thus reduce the rate of metabolism and prolong the action of certain drugs and insecticide chemicals.*

The duration of action and toxicity of many drugs in mammals and of many insecticide chemicals in insects are greatly increased by joint treatment with certain compounds containing the methylenedioxyphenyl (1,3-benzodioxole) group. Such compounds prolong barbiturate-induced sleep in mammals (*1*), act as co-carcinogens with benzo-[*a*]-pyrene (*2*), and increase the toxicity of certain insecticidal chemicals (*3*). They synergize the insecticidal activity of compounds within almost all classes of insecticide chemicals [pyrethroids, chlorinated hydrocarbons, organophosphorus compounds, carbamates, and others (*4*)]. The published information on structural requirements for synergistic activity is largely restricted to that on the enhancement of the insecticidal activity of pyrethrum and carbamates. Of interest here is the finding that compounds having a planar methylenedioxyphenyl ring system are optimally synergistic to these insecticides, and that only slight reduction in activity results from incorporation of one sulfur atom or a deutero-