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INDIRECT IMMUNOFLUORESCENCE TEST FOR ANTIMALARIAL ANTIBODIES. INFLUENCE OF TEMPERATURE, TIME OF INCUBATION, AND PRESENCE OF CELL MONOLAYERS ON THE ANTIGENIC CHARACTERISTICS OF PLASMODIUM FALCIPARUM MATURED "IN VITRO"

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SUMMARY

The influence of temperature, time of incubation, and the presence of eucaryotic cell monolayers, as well as the interaction of these factors, on the antigenic characteristics of **Plasmodium falciparum** matured in short-term cultures were studied by the indirect immunofluorescence test. Antigens obtained under different conditions of maturation were assayed with two batteries of sera, one consisting of homologous specimens from a hyperendemic area (Ghana) and another from a nonendemic area for **P. falciparum** (Peru). The simultaneous morphologic examination of each antigen indicated that the maximal sensitivity was not always associated with the schizont content, althoug antigens presenting these forms usually gave high titers for the homologous sera. Antigens which produced the highest titers were obtained by four different combinations of culture conditions, 28°C for 96 h, 33°C for 48 or 72 h, and 37°C for 24 h. Antigens from the original inoculum blood and from cultures kept at 41°C were much less reactive than from all other combinations. Although the inclusion of monolayer substrates in the culture flasks had no effect on the reactivity of antigens obtained at 28°C and 33°C, these nurse substrates improved the reactivity of **P. falciparum** cultivated at 37°C.

INTRODUCTION

The indirect immunofluorescence test (IIF) has been and remains one of the major serological tools for individual diagnosis of malaria as well as in the assessment of malaria prevalence in a given geographical area in serological surveys [2,14].

Although initially recognized as being genus rather than species specific [5], a given serum in the IIF test usually yields the highest titer when the homologous species antigen is used. The activity of an antigen of a given **Plasmodium** species, whether human or simian, may

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vary according to the most prevalent stage of the parasite present when the antigen was prepared. It was observed early that more mature forms of the parasites (schizonts) yielded better reactivity than trophozoites or younger forms [1,6,7], and Targett in 1970 [10] defined these differences in reactivity by comparing schizonts and trophozoites as sources of antigen in the IIF test.

Especially for **P. falciparum**, peripheral blood of infected individuals or monkeys seldom present the more reactive schizont stages in the circulation because there is a withdrawal of the parasite into deep tissues before schizogony [10]. The scarcity of schizonts in the antigen prepared from blood is a problem which has been overcome by allowing the young forms of the parasite in the infected blood to mature **in vitro** into more reactive forms which are then used for the preparation of the antigen slides [4,9,11,13]. The several maturation techniques presently available differ greatly in methodology, but all were demonstrated to yield schizonts at sufficient levels to give maximal reactivity of the antigen. These schizont antigens tend to yield higher homologous species reactions than less reactive younger form antigens. This increased reactivity exceeds, in general, the heterologous species reaction, therefore allowing a better resolution.

The advent of the continuous culture of **P. falciparum** [12] seemed to solve part of the problems of antigen supply. Many laboratories, however, do not have the resources or technical skills to carry out this procedure and the method is not adaptable for the cultivation of the other human malarial species. Therefore, serodiagnosis is at the case detection level only, since the antigens from the other species can not be included for diagnosis at the species level.

To overcome this problem, SULZER & LATORRE [9] described a maturation technique that has been successfully applied to **Plasmodium** species other than **P. falciparum.** These Authors employed both culture medium alone and cell monolayers as a nurse substrate for the short-term culture with incubation at 33°C instead of 37°C, the temperature commonly used for other maturation techniques. They found more consistent schizont yields when incubating at 33°C in the presence of cell monolayers, but they did not show quantitative data to demonstrate that antigen obtained from their cultures yielded better reactivity than when other temperatures of incubation, either in the presence or in the absence of nurse eucaryotic cell monolayer, were used.

Since optimal reactivity of antigens is of paramount importance for serodiagnosis, we investigated the influence on the **in vitro** maturation of **P. falciparum** of temperature, length of incubation, presence or absence of nurse cell monolayers, and the interaction of these factors for the production of antigen of high reactivity for use in the IIF test.

MATERIALS AND METHODS

Source of parasites — Owl monkeys (**Aotus trivirgatus** subspecies **trivirgatus**) were infected with **P. falciparum**, Walter Reed strain, by inoculation of 1.5cc of cryopreserved parasitized blood into the femoral vein. Previous parasitologic examination had indicated that the animals were free of malarial infection. Thin and thick blood films were made and examined daily until parasitemia approached the desired level of about 10%. At this time, 10cc of blood was aseptically drawn into a syringe containing 1.0cc of heparin (1,000 U/ml). The animals were then treated with chloroquine at the prescribed dose/weight ratio. Parasitized blood from human donors was also found equally satisfactory for maturation (unpublished data).

Short-term cultures — **In vitro** maturation of the parasites was done as previously described [9] with the following modifications. Washed infected erythrocytes in a 10% suspension in Eagle's minimum essential medium, supplemented with 10% fetal bovine serum, and antibiotics (200 U/ml Penicillin and 100 μg/ml streptomycin) were dispensed into 75 cm^2 tissue culture flasks, either with or without eucaryotic cell monolayers (MRC 5 human embryo lung fibroblasts derived from ATCC-CL-13), from the same passage line for all monolayer flasks. One or two flasks from each group were incubated at four different temperatures, 28°, 33°, 37°, and 41°C. After adjusting and calibrating the incubators, they were monitored regularly to ensure that temperature variation during the experiment did not exceed ± 0.5°C. Two independent experiments were performed, each with blood from a single monkey.

Morphologic examination — Every 24 h, for a period of 10 days, aliquots of 2.0cc were withdrawn from the resuspended erythrocytes of each flask and centrifuged to pack the cells. Slides prepared from the sediment were stained (Giemsa); the slides were assorted and coded before being examined microscopically. Percentages of parasitic stages, general aspects of the morphologic changes, and the ratio between the number of erythrocytes and parasites were recorded.

Preparation of antigens — After removing the smear sample, the sediments were washed three times in phosphate-buffered saline, pH 7.6. The washed, packed organisms and erythrocytes were resuspended in 0.3cc of the same buffer and distributed in microscope slide wells for use in the IIF tests [7].

IIF tests — Tests were done as previously described [7]. A gamma chain specific anti-human IgG (Tago Inc., Burlingame, Calif., lot 90-11-11) fluorescent conjugate was used. All slides were sorted and coded before being read in a Leitz Ortholux microscope equipped with a Ploem epiiluminator and oil immersion objectives. Staining patterns and intensity of reactions were recorded according to the parasitic stages present in the antigen. For comparison of titers, end point reactions were used.

Sera — Forty-eight positive specimens obtained from malarial endemic areas were titrated by the IIF test against all of the different antigens obtained in two sets of short-term cultures, each from a different monkey. Thirty of these sera originated from a **P. falciparum** endemic area of Ghana, West Africa. The other 18 were from Peru, Western South America; of these 14 came from a **P. vivax** endemic but **P. falciparum**-free village on the Pastazas river and the other 4 from a **P. vivax/P. malariae**-endemic, but **P. falciparum**-free village on the Ene river. In addition, two sera known to lack antimalarial activity were included in each test of the reactive sera. These negative controls were assorted and coded with the other specimens and all tests read "blind".

Different types of reactivity could be identified by using two antisera with special characteristics: one (≠ 474-A) displaying high titers for schizonts and highly reactive forms and poor or no reactivity with trophozoites, and the other (≠ 450 B) presenting equivalent high reactivity for both forms. Both of these specimens came from naturally infected patients.

Statistical analysis — Since the combination of four different temperatures of incubation either in the presence or absence of nurse monolayer was studied through harvesting time against 50 sera, the number of individual serum titers for all different antigens reached 1,694 in the two sets of cultures. A subset of such bulk data was evaluated by analysis of variance of the results. Values of F were obtained for the effects of time, temperature, nurse substrate, and specimens as well as their interactions.

RESULTS

For the two sets of cultures, the mean parasitemia for the inoculation of flasks was 7.8%. The mean percentages for the parasitic stages were 97.7% and 2.3%, respectively, for ring forms and trophozoites. No schizonts were observed in the samples used as inocula, although they had been seen occasionally during early stages of the infection. These forms were seen, however, after 24 h in cultures incubated at all temperatures except for 41°C. The highest percentages of schizonts occurred at 72 h for 28°C, 48 h for 33°C, and 24 h for 37°C, with a mean percentage of the total determinations of 19.6 and standard deviation of 4.6. The effect of eucaryotic cell monolayers was noted mainly after 48 h at the higher temperatures. Schizont yields were higher thereafter, when cell monolayer substrates were present; however, the erythrocyte-to-parasites ratio was lower in these flasks, which was caused by early lysis of erythrocytes. This effect was observed at all temperatures as early as 24 h. Percentages of ring forms decreased through time. Trophozoites increased in number, at the expense of rings, until 72 h, when they lost much of the distinctive shape and staining characteristics. Aberrant forms characterized by small and large rounded forms were noted in increasing number after 24 h at 41°C, 48 h at 37°C, 72 h at 33°C, and 96 h at 28°C, regardless of the presence of nurse substrate in the flasks.

Table I shows the results of the analysis of variance of the titers for two groups of sera obtained with different **in vitro** matured an-

tigens from parasitized blood from a single monkey. Titers decreased (Table II) with increase of both harvesting time and incubation temperature. However, the presence of cell substrate in the cultures yielded a more reactive antigen only for the homologous serum group. When temperatures were compared over time, the differences in reactivity of the resulting antigens were less evident for the flasks containing nurse cell monolayers. The only exception was 41°C which yielded antigens reacting similarly to antigen from the original inoculum, i.e., having reactions similar to rings or trophozoites.

TABLE I

F values[a] for isolated or associated maturation conditions in the preparation of **Plasmodium falciparum** indirect immunofluorescence antigen used in the study of serum samples from **P. falciparum** endemic and nonendemic areas

Conditions	Endemic (Ghana)[b]		Nonendemic (Peru)[c]	
	F	Levels of Significance	F	Levels of Significance
Time	48.31	< 0.01	6.78	< 0.01
Temperature	500.48	< 0.001	37.53	< 0.01
Cells	21.00	< 0.01	1.12	NS[d]
Time X Temp.	3.68	< 0.01	4.35	< 0.01
Time X Cells	2.89	< 0.05	1.50	NS
Temp. X Cells	22.16	< 0.01	1.12	NS
Time X Temp. X Cells	2.22	< 0.05	0.93	NS

a- Analysis of variance of log serum titers obtained with antigens from a single set of short-term culture.
b- n=18.
c- Pastazas river (n=6).
d- Nonsignificant at the level of 0.05.

High titers were obtained (4,096 to 16,384) with the schizont-specific serum (≠ 474-A) for antigens harvested until 10 days of maturation kept at 28°C, 33°C, and 37°C, regardless of the presence of cell monolayers. Antigens matured at 41°C either with or without nurse cells, however, exhibited poor reactivity with this schizont-specific serum (< 64) as did the antigen incubated at 37°C after 10 days of culture. A similar response was obtained with this serum when reacting with antigen prepared directly from the inoculum blood. No differences were found among antigens when reacting with the multistage specific serum (≠ 450-B). Titers obtained with this serum were high (4,096 to 16,384) even with the inoculum blood and the 41°C antigen, regardless of harvesting time.

DISCUSSION

The importance of the presence of an adequate number of schizonts in the malarial antigen used in the IIF test has been recognized since before the last decade, but it was proven by Targett only in 1970 in studies comparing the reactivity of antigen from short-term cultures and from the donor blood. Maturation of the parasite in short-term cultures especially for **P. falciparum** allows the enrichment of the resulting antigen with higher reactive forms, the schizonts, which otherwise are rarely seen in the circulation of the human or simian host [10].

The need for schizont enriched antigens was further confirmed in this study by correlating the findings of the morphologic exami-

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TABLE II

Indirect immunofluorescence geometric mean titers of 20 sera from a **Plasmodium falciparum** hyperendemic area (Ghana) obtained with homologous antigen A matured under different combinations of temperature, length of incubation, and presence of cell monolayers

Length of incubation (h)	Temperature during maturation							
	28		33		37		41	
	W/C	CML	W/C	CML	W/C	CML	W/C	CML
24	1896.2 (1.50)	2389.1 (1.55)	4597.6 (1.53)	3378.6 (1.51)	2580.3 (1.45)	3649.1 (1.52)	512.0 (1.23)	348.4 (1.46)
48	4965.7 (1.39)	2352.5 (1.53)	3511.3 (1.43)	3378.6 (1.44)	2482.8 (1.64)	3010.0 (1.44)	512.0 (1.35)	553.0 (1.40)
72	4256.8 (1.43)	3104.2 (1.32)	3941.3 (1.51)	3941.3 (1.51)	1625.5 (1.61)	2212.0 (1.55)	474.0 (1.40)	645.1 (1.37)
96	3941.3 (1.49)	4096.0 (1.43)	2786.9 (1.45)	2580.3 (1.45)	597.3 (1.38)	3010.0 (1.60)	298.6 (1.37)	276.5 (1.33)
120	2896.3 (1.54)	2702.4 (1.34)	2786.9 (1.42)	2786.9 (1.39)	812.7 (1.30)	2212.0 (1.50)	149.3 (1.35)	256.0 (1.43)
240	1505.0 (1.60)	ND	2048.0 (1.50)	1505.0 (1.37)	276.5 (1.44)	1194.5 (1.57)	69.1 (1.33)	101.6 (1.40)

Numbers in parentheses are the geometric standard errors (used to multiply and divide the geometric mean).
A- Mean titer with nonmatured antigen: 237.0 (1.41).
CML- antigen matured in flasks containing cell monolayers.
W/C- antigen matured in flasks containing medium alone.
ND - not done.

nation of the cultures and the resulting reactivities of the antigens. However, the titer of a given serum is not always determined by its schizont reactivity. For example, inclusion in this study of a serum with high antischizont, as compared to low antitrophozoite reactivity, revealed low reactivity with antigens produced under conditions yielding poor schizont production. On the other hand, many of the other homologous sera from a **P. falciparum** endemic area reacted at significant levels with the schizont-poor antigens produced at 41°C even after 10 days of culture as well as with antigen prepared from the original inoculum.

Our results show that maturation of rings and trophozoites to the more reactive forms proceeds in a rate directly proportional with temperature variation within certain limits. The analysis of variance of titers for all groups of sera and for all antigens showed that the optimally reactive antigens were produced under lower incubation temperatures combined with the nurse eucaryotic monolayer substrates and were harvested up to 72 h. Final reactivity and not only schizont frequency in the antigen were always associated with the dynamic combination of the above factors. Therefore, there was no single optimal combination for the production of a highly reactive antigen since this could be obtained by different ways, for example, by cultivating at 28°C for 96 h, at 33°C for 48 or 72 h, or even at 37°C for 24 h, but not as noted above at 41°C.

Although the inclusion of a monolayer of cells in the cultures, as suggested by SULZER & LATORRE[9], played no role in the final sensitivity of the antigens when reacted with heterologous sera from a malarious area nonendemic for **P. falciparum**, those antigens showed a much better reactivity when studied with anti-**P. falciparum** sera from a **P. falciparum** endemic area. Monolayers, at all temperatures, showed a deleterious effect on the survival of the donor erythrocytes, as measured by the decreased erythrocyte: parasite ratios as compared with cultures without monolayers. Despite

this effect, we recommended the inclusion of cells, since they seem to contribute to a better synchronism in the parasitic maturation process. The importance of monolayers was markedly evident at higher temperatures, especially at harvesting times longer than 72 h. In these combinations, titers were much higher than in the absence of monolayers. That the monolayer exerts a protective influence on the parasite when exposed to increased temperatures is obvious. The mechanism of this effect remains obscure and presents itself as a subject of further research.

The continuous culture of **P. falciparum** [12] is now a well standardized technique. It presents obvious advantages over the short-term maturation methods in the preparation of antigens for serological tests [3]. However, it has not been adapted for the other malaria species affecting man and involves laboratory equipment and skills not always available in endemic areas. Another disadvantage which sometimes obscures the usefulness of antigens obtained from this type of culture is the frequently observed nonspecific background reaction in some test procedures, probably associated with the incorporation in the parasite of serum globulins of human origin from the serum added to the medium. Despite the washing process after harvesting from the cultures, some of the parasitic stages present this undesirable phenomenon (unpublished data).

Both the unavailability for other human malaria species and the uncertain specificity of the **P. falciparum** antigen obtained from continuous culture can be overcome by the use of the short-term maturation method as described by SULZER & LATORRE [9]. Control of the time of production of the more reactive forms is much more convenient than awaiting schizont production by the host. If the donor is human, mandatory treatment prohibits waiting for schizonts to appear in the circulation. For other primate donors, such as **Aotus** monkeys, early bleeding for trophozoites, followed by immediate treatment, conserves a valuable animal for subsequent reuse.

This technique has been used in this laboratory in the last five years for the preparation of antigens from all human malaria species. A major advantage is the possibility of preparation of the complete set of antigens in the most reactive state necessary to assess infection at the species level, since parasitological findings are not always available.

The data presented show that the extra level of reactivity associated with the species-specific antibodies in the serum can be obtained only if there are enough highly reactive stages, such as the schizonts, which will provide in most cases a higher titer with the homologous serum.

In those situations where comparison of titers between antigen species is required, the life cycle stage used as antigen is of utmost importance. Titers which trophozoite antigens of one species may be less than titers with schizont antigens of a different species. If schizont antigens of both species are used, the picture would be different.

The possibility of synchronization of the maturation process by combining appropriately time and temperature of incubation, allows the preparation of antigens in the same time period, even if the parasitized blood obtained from the simian or human donors was not drawn in the same day. This might allow the practical preparation of the multi species antigen [8] which is indicated for maximal sensitivity in the IIF test.

RESUMO

Reação de imunofluorescência indireta em malária. Influência da temperatura, tempo de incubação e presença de monocamada celular nas características antigênicas de Plasmodium falciparum **cultivado "in vitro"**

Os Autores avaliaram a influência da temperatura, tempo de incubação, presença de "feeder layer" e associação destes fatores nas características antigênicas de **Plasmodium falciparum** cultivado por curtos períodos. Antígenos obtidos sob diferentes condições de maturação foram testados pela imunofluorescência indireta com duas baterias de soros. A primeira consistindo de espécimes homólogos oriundos de área hiperendêmica (Ghana) e a segunda obtida de área não endêmica para **P. falciparum** (Peru). O exame morfológico de cada antígeno, feito simultaneamente, indicou que máxima sensibilidade não estava sempre associada com

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abundância de esquizontes, embora antígenos apresentando estes estágios geralmente produziram altos títulos para soros homólogos. Os antígenos que forneceram os mais altos títulos foram obtidos por quatro diferentes combinações de condições de maturação; 28°C após 96 h, 33°C após 48 ou 72 h e 37°C por 24 h. Os antígenos preparados diretamente da amostra que servira como inóculo e de culturas mantidas a 41°C foram muito menos reativos que os das outras combinações. Embora a inclusão de "feeder layer" não tivesse exercido efeito na reatividade dos antígenos preparados a 28°C e 33°C, tais substratos melhoraram a reatividade dos parasitas mantidos a 37°C.

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