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SHORT COMMUNICATION

INDUCTION OF GAMETOCYTOGENESIS IN *PLASMODIUM FALCIPARUM* BY THE CULTURE SUPERNATANT OF HYBRIDOMA CELLS PRODUCING ANTI-*P. FALCIPARUM* ANTIBODY

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There have been many unsuccessful attempts to induce gametocytogenesis in vitro. In the present experiment, however, we found that RPMI-CS medium and RPMI-FS medium prepared by dissolving powdered RPMI 1640 medium in the culture supernatants of hybridoma cells, hybrid line D21 and 219.5, respectively, that produce anti-*P. falciparum* antibody induced gametocytogenesis. Gametocytogenesis was consistently observed from 3 days after addition of these media. The culture supernatant of anti-*P. falciparum* antibody producing hybridoma cells did not induce gametocytogenesis in the absence of RPMI 1640 medium. RPMI-MS medium, prepared by dissolving powdered RPMI 1640 medium in the culture supernatant of myeloma cells, SP2/O-Ag 14, which was used as a control, induced a few gametocytes.

Many previous attempts to induce the differentiation of asexual stages of malaria parasites to sexual stages in culture conditions have been unsuccessful. In the present work, however, we found that the culture supernatants of hybridoma cells that produce anti-*P. falciparum* antibody could induce gametocytogenesis.

Plasmodium falciparum strain Falciparum-Vietnam-Oaknoll (FVO) and Falciparum-Uganda-PaloAlto (FUP) used in this study were provided by courtesy of Dr. W. A. Siddiqui, Department of Tropical Medicine, University of Hawaii. Strains FVO and FUP were isolated from patients in 1967 and 1966, respectively, and since 1977, they have been cultured by the method of Siddiqui (1979). Gametocyte is rarely observed in these cultured strains.

Hybridoma cells producing anti-*P. falciparum* monoclonal antibody were prepared as follows. Balb/c mice were immunized three times intraperitoneally with 100 μg of sonicated whole parasites, FCH-9 strain, in FCA. The

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mice were boosted with an intravenous injection of 5 μg of antigen four days before their spleen was removed. The two hybrid lines used, D21 and 219.5, were obtained by fusion of myeloma cell lines, SP2/0-Ag 14 and P3-X63-Ag 8.653, respectively, with spleen cells from the Balb/c mice immunized as described above. Hybrid D21 was prepared in the Department of Tropical Medicine, University of Hawaii during cooperative work with our laboratory in 1981, and hybrid 219.5 was provided by courtesy of Dr. Siddiqui in 1983. Since then these cell lines have been maintained in our laboratory in DMEM medium with 20% calf serum in a CO_2-incubator.

When the hybridoma cells grew to a level of $5–9\times10^5$ cells/ml in DMEM culture, the culture supernatant was separated by centrifugation at 3,000 rpm for 10 min. Hereafter, the culture supernatants of hybrid cell lines D21 and 219.5 are abbreviated as CS and FS solution, respectively. Powdered RPMI 1640 (GIBCO) was dissolved at a suitable concentration in CS solution or FS solution, and the media (abbreviated as RPMI-CS medium and PRMI-FS medium, respectively) were tested for ability to induce gametocytogenesis. The following two methods were used to test induction of gametocytogenesis.: (1) At the time of subculture, 1 ml of erythrocyte suspension infected with parasites was inoculated into medium (named PRMI-CS reactive medium) consisting of 4 ml of RPMI-CS medium, 4 ml of regular RPMI 1640 medium and 1 ml of human serum, and incubated at 37 C. The medium was changed to regular RPMI 1640 medium containing 10% human serum, after 48 h culture, and the latter medium was renewed every 24 h. (2) On day 4 or 5 of culture in regular RPMI 1640 medium containing 10% human serum, the medium was replaced by RPMI-CS reactive medium or RPMI-FS reactive medium. After culturing for 24 h, the RPMI-CS reactive medium or RPMI-FS reactive medium was replaced by regular PRMI 1640 medium containing 10% human serum. As a control, powdered RPMI 1640 medium was dissolved in the culture supernatants of myeloma cells SP2/O-Ag 14 and anti-erythrocyte antibody producing hybridoma cells (abbreviated as RPMI-MS medium and RPMI-ES medium, respectively), and cells were incubated with these media in the same way as with RPMI-CS reactive medium or RPMI-FS reactive medium. The effect of concanavalin A (10 μg/ml final concentration; Sigma Chemical Company) on gametocytogenesis induction was evaluated by adding it to the RPMI-CS reactive medium or the RPMI-FS medium. Induction of gametocytogenesis was judged by examining Giemsa-stained blood smears prepared daily from the cultures. The stages of gametocytes found in smears were classified on the basis of the standard reported by Hawking et al. (1971) and Carter and Miller (1979).

The results shown in Table 1 were obtained by method (1). Gametocytes were consistently found from day 3 after subculture in RPMI-CS reactive medium. Gametocytes were usually seen for 4 days. The small numbers of gametocytes seen in culture with RPMI-MS reactive medium and RPMI-ES reactive medium, were used as controls. The rate of gametocytogenesis of the FUP strain was less than that of the FVO strain. Stage I gametocytes are difficult to distinguish from asexual trophozoites, so in the present experiment gametocytes were classified into two groups, stage II and stage III or more, gametocytes of stage III or more were fewer in number than those of stage II.

The results shown in Table 2 were obtained by method (2). The abilities of RPMI-CS reactive medium and RPMI-FS reactive medium to induce gametocytogenesis were examined when parasitemia reached 5–8%, usually 5 days after subculture in regular RPMI 1640 medium. As shown in Table 2, the course of gametocytogenesis induction was similar to that of the first experiment (Table 1). Gametocytogenesis was consistently observed from 3 days after addition of

TABLE 1. *Induction of gametocytogenesis by specific RPMI 1640 media prepared from the culture supernatants of hybridoma cells or myeloma cells. Specific RPMI media were added at the beginning of parasite culture*

Strain	Inducer of gametocytogeneis	Days after addition of inducer of gametocytogenesis								
		1	2	3	4	5	6	7	8	9
FVO	RPMI-CS medium	—[a]	1.5	1.9	3.0	5.8	5.8	7.5	9.3	7.7[b]
		—	0	4	8	4	2	0	0	0[c]
		—	0	2	4	4	2	0	0	0[d]
FVO	RPMI-MS medium	—	1.5	2.0	3.4	2.5	4.1	7.0	10.0	11.0
		—	0	1	2	2	1	0	0	0
		—	0	0	1	3	0	0	0	0
FVO	RPMI-ES medium	—	1.5	1.7	3.0	2.9	5.5	8.0	9.3	10.2
		—	0	1	1	1	0	0	0	0
		—	0	0	0	0	0	0	0	0
FVO	—	—	1.0	2.1	5.6	10.1	8.1	12.3	15.0	9.0
		—	0	0	0	0	0	0	0	0
		—	0	0	0	0	0	0	0	0
FUP	RPMI-CS medium	—	1.3	1.8	1.4	2.6	3.0	7.8	8.3	9.0
		—	0	5	6	5	2	1	0	1
		—	0	0	2	0	3	0	0	0
FUP	RPMI-MS medium	—	1.0	1.3	1.5	2.2	3.4	5.0	7.0	9.2
		—	0	0	1	2	2	1	0	0
		—	0	0	1	2	0	0	0	0
FUP	—	—	1.3	3.7	5.0	6.7	6.3	9.8	11.9	14.5
		—	0	0	0	0	0	0	0	0
		—	0	0	0	0	0	0	0	0

RPMI-CS medium: Specific RPMI 1640 medium prepared by dissolving powdered RPMI 1640 medium in the culture supernatant of anti-*P. falciparum* antibody producing hybridoma cells D21.
RPMI-MS medium: Specific RPMI 1640 medium prepared by dissolving powdered RPMI 1640 medium in the culture supernatant of myeloma cells SP2/O-Ag 14.
RPMI-ES medium: Specific RPMI 1640 medium prepared by dissolving powdered RPMI 1640 medium in the culture supernatant of anti-erythrocyte antibody producing hybridoma cells D14.
[a]The medium was not changed on this day. [b]The first line shows parasitemia as the percentage of parasites in 400 erythrocytes examined. [c]The second line shows the number of stage II gametocytes among 300 parasites. [d]The third line shows the number of gametocytes of stage III or more among 300 parasites.

either RPMI-CS reactive medium, or RPMI-FS reactive medium. RPMI-MS reactive medium, used as a control, induced a few gametocytes. When the culture supernatant of hybridoma cells was used without the addition of powdered RPMI 1640 medium, no gametocytogenesis was observed. Table 2 also shows that cultivation of parasites in regular RPMI 1640 medium with concanavalin A did not induce gametocytogenesis. However, addition of 10 μg of concanavalin A to the RPMI-CS reactive medium or the RPMI-FS reactive medium enhanced the induction of gametocytogenesis.

For analysis of the interaction between the induction of gametocytogenesis and the rate

TABLE 2. *Induction of gametocytogenesis by specific RPMI 1640 media prepared from the culture supernatants of hybridoma cells and myeloma cells. Specific RPMI 1640 media were added on day 4 or 5 of parasite culture*

Strain	Inducer of gametocytogenesis	Con A[a]	Days after addition of inducer of gametocytogenesis								
			0[b]	1	2	3	4	5	6	7	8
FVO	RPMI-CS medium	−	6.1	5.2	6.0	7.0	8.5	9.0	8.9	6.8	6.4[c]
			0	0	1	7	7	6	3	5	1[d]
			0	0	0	3	5	4	5	4	0[e]
FVO	RPMI-CS medium	+	6.0	5.5	7.3	5.0	5.5	7.2	8.5	6.2	8.8
			0	0	0	10	10	8	10	4	2
			0	0	0	5	6	6	6	1	0
FVO	RPMI-FS medium	+	8.6	7.6	6.5	6.4	8.0	6.7	6.3	4.8	6.0
			0	0	0	11	9	8	6	6	0
			0	0	0	1	4	6	4	5	2
FVO	FS solution	+	5.9	6.0	5.3	6.3	8.0	11.0	9.5	7.0	7.0
			0	0	1	1	1	1	1	0	0
			0	0	0	0	0	0	0	0	0
FVO	RPMI-MS medium	+	8.5	9.6	9.8	8.0	7.0	7.8	8.7	5.7	7.5
			0	0	0	2	2	3	1	0	1
			0	0	0	0	1	1	1	0	0
FVO	MS solution	+	7.5	5.9	4.2	9.5	8.4	9.8	9.8	7.5	8.0
			0	0	1	1	0	0	1	1	0
			0	0	0	0	0	0	0	0	0
FVO	—	+	7.2	6.7	6.8	9.7	7.7	8.1	8.0	10.7	11.0
			0	0	0	0	1	1	0	0	0
			0	0	0	0	0	0	1	0	1

RPMI-CS medium: Specific RPMI 1640 medium prepared by dissolving powdered RPMI 1640 medium in the culture supernatant (CS solution) of anti-*P. falciparum* antibody producing hybridoma cells D21.
RPMI-FS medium: Specific RPMI 1640 medium prepared by dissolving powdered RPMI 1640 medium in the culture supernatant (FS solution) of anti-*P. falciparum* antibody producing hybridoma cells 219.5.
RPMI-MS medium: Specific RPMI 1640 medium prepared by dissolving powdered RPMI 1640 medium in the culture supernatant (MS solution) of myeloma cells SP2/O-Ag 14.
[a]Concanavalin A at 10 μg/ml final concentration. [b]Specific RPMI media or the culture supernatant of hybridoma cells or myeloma cells and concanavalin A were added on this day. [c]The first line shows parasitemia as the percentage of parasites in 400 erythrocytes examined. [d]The second line shows the number of stage II gametocytes among 300 parasites. [e]The third line shows the number of gametocytes of stage III or more among 300 parasites.

of appearance of parasites, parasitemia was examined daily after subculture. As shown in Table 1, when parasite cultures were started in regular RPMI 1640 medium, parasitemia increased day by day. However, when the parasites were cultured in RPMI-CS reactive medium, RPMI-MS reactive medium, or RPMI-ES reactive medium, no increase of parasitemia was observed until 4–5 days after subculture, regardless of gametocytogenesis. When RPMI-CS reactive medium or RPMI-FS reactive medium was used alone or in

combination with 10 μg/ml concanavalin A after 4–5 days subculture in regular RPMI 1640 medium, the number of parasites decreased (Table 2). Addition of the culture supernatant of hybridoma cells or myeloma cells to regular cultures resulted in a transient decrease in parasitemia.

Gametocytogenesis in cultured parasites was induced only by addition of culture medium prepared by adding powdered RPMI 1640 medium dissolved in the culture supernatant of hybridoma cells (RPMI-CS medium and RPMI-FS medium) in place of distilled water. Addition of the hybridoma cell culture supernatant only to cultures of parasites did not induce gametocytogenesis for some unknown reason.

In the present experiment, addition of RPMI-CS medium or RPMI-FS medium to parasite cultures did not result in increase of parasitemia. Addition of RPMI-MS medium or RPMI-ES medium, which have no ability to induce gametocytogenesis, also did not increase parasitemia. No direct relation between induction of gametocytogenesis and decrease of parasitemia was demonstrated. But, various conditions that have adverse effects on the growth of asexual parasites in culture probably affect gametocytogenesis. The usual method to elicite sexual development is to put parasites under stress by not replenishing the erythrocytes. However, as shown in Table 1, RPMI-CS medium induced the appearance of gametocytes from 3 days after subculture with fresh erythrocytes.

In the experiment shown in Table 1, RPMI-CS medium prepared from the culture supernatant of the hybridoma cell line D21 was used. RPMI-FS medium, prepared from the culture supernatant of the hybridoma cell line 219.5, was used in part in the experiment shown in Table 2. In the indirect immunofluorescence antibody test, antibody from hybrid D21 bound to all the erythrocytic stages of the parasites, while antibody from hybrid 219.5 bound to merozoites within erythrocytes and to merozoite-free erythrocytes (Ono and Nakabayashi and Siddiqui et al. unpublished data). Since RPMI-CS medium and the RPMI-FS medium had similar abilities to induce gametocytogenesis, hybridoma cells producing anti-ring form, anti-trophozoite form antibodies do not seem essential for induction of gametocytogenesis.

In the present experiment, gametocytes appeared for 4 to 5 days. However, we recently found that survival of gametocytes could be prolonged by a modification of the culture method and alteration of the culture medium. The present results should be helpful in determining the mechanism of sexual differentiation of malaria parasites.

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