HUMAN-HUMAN HYBRIDOMAS SECRETING MONOCLONAL ANTIBODIES TO THE M_r 195,000 *PLASMODIUM FALCIPARUM* BLOOD STAGE ANTIGEN

By RUPERT SCHMIDT-ULLRICH,* JAMES BROWN,‡ HILTON WHITTLE,‡ AND PECK-SUN LIN*

*From the *Department of Therapeutic Radiology, Radiobiology Division, Tufts–New England Medical Center, Boston, Massachusetts 02111; and the ‡Medical Research Council Laboratories, Fajara, near Banjul, The Gambia, West Africa*

Erythrocytes infected with *Plasmodium* schizonts, as well as the merozoites released by these cells, bear surface proteins of M_r 195,000–250,000, depending on the parasite species (1–9). These proteins and their degradation products (1, 7, 10, 11) are immunogenic during natural malarial infections, and there is evidence that antibodies against these antigens interfere with parasite multiplication during erythrocytic schizogony, both in vivo and in vitro (12–14). Antibodies against the *P. falciparum* (Pf)[1] M_r 195,000 and the *P. chabaudi* (Pch) M_r 250,000 components correlate positively with in vivo protection of human and murine hosts, respectively (4, 6). Murine mAb against the M_r 230,000 protein of *P. yoelii* (Py) that can confer passive protection in mice (2), and murine mAb against the M_r 230,000 component of *P. knowlesi* (Pk) inhibit the in vitro growth of this parasite by interference with merozoite invasion (15). The concentrations of mouse mAb required to inhibit the growth of primate *Plasmodium* are unphysiologically high, >0.1 mg/ml, relative to the expected antibody concentrations in sera of the protected hosts (15). However, human IgG from EBV-transformed human B lymphocytes of Gambian adults immune to Pf malaria contain antibody to the Pf M_r 195,000 component that inhibit in vitro parasite growth at concentrations <100 ng/ml (16). The most direct evidence that this category of high-M_r *Plasmodium* components may induce a protective immune response in vivo is derived from vaccination trials in three compatible host-parasite systems. Repeated injection of Pf M_r 195,000 and Py M_r 230,000 with CFA into aotus monkeys and BALB/c mice, respectively, yielded partial but effective protection against potentially lethal parasite challenges (10, 17). A similar protection of rhesus monkeys across different Pk strains was induced using a Pk M_r 74,000 glycoprotein derived from the Pk M_r 230,000 component (7). The close correlation between antibody-mediated growth inhibition and

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[1] *Abbreviations used in this paper:* LCL, lymphoblastoid cell line; Pch, *Plasmodium chabaudi*; Pf, *P. falciparum*; Pk, *P. knowlesi*; Py, *P. yoelii*.

protective immunity make human mAb an ideal reagent to examine the mechanism of protection against Pf in man.

For this reason, we have used recent advances in the production of human mAb by the hydridoma technique (18–24) and fused PBL of Gambian adults immune to Pf malaria with GM4672 cells, a human lymphoblastoid cell line (LCL) (20, 21). Hybridoma-derived mAb were screened for immune fluorescence reaction with Pf schizonts, for in vitro parasite growth inhibition, and for immune precipitation reaction with the Pf M_r 195,000 protein.

Materials and Methods

Reagents. Reagents of highest purity available were obtained from the following suppliers: PEG (of 1,500 mol wt), from J. T. Baker Chemical Co. (Phillipsburg, NJ); PWM, nonessential amino acids, L-glutamine, and sodium pyruvate from Gibco (Grand Island, NY); FBS, (lot 110463) from HyClone Laboratories (Logan, UT); NCTC 109 medium from Whittaker M. A. Bioproducts (Walkersville, MD); Ficoll-Paque from Pharmacia Fine Chemicals (Piscataway, NJ); hypoxanthine (H), thymidine (T), aminopterin (A), glycine, Hepes, 6-mercaptopurine, oxaloacetic acid, bovine insulin, alkaline phosphatase–derivatized goat IgG against human IgG and IgM, and paranitrophenyl phosphate tablets, all from Sigma Chemical Co. (St. Louis, MO); chromatographically purified human IgG and IgM, goat IgG against human IgG and IgM from Cooper Biochemical, Inc. (Malvern, PA); mAb specific for human IgG subclasses and light chains from Bethesda Research Laboratories, Inc. (Gaithersburg, MD); [^{35}S]methionine from New England Nuclear (Boston, MA).

Media. The medium used for PWM-stimulation of PBL and for maintenance of the human LCL was RPMI 1640 (Gibco) containing 0.01 Hepes, 0.001 M sodium pyruvate, 0.004 M L-glutamine, four times the concentration of nonessential amino acids, and 11.5% FBS; this medium is referred to as RPMI/Hepes (21). The hybridoma selection medium is RPMI/Hepes medium containing 10% (vol/vol) NCTC 109 medium, 0.003 M oxaloacetic acid 0.0025 IU/ml insulin, 3.6×10^{-4} M H, 3.9×10^{-5} M T, and 3.5×10^{-7} M A.

Cells. GM 4672 cells containing a small number of EBV DNA copies (based on hybridization with EBV DNA [David Thorley-Lawson, personal communication]; the cells had previously tested negative for EBV nuclear antigen) were used as fusion partner (18, 21). For one week every month and one week before fusion, the cells were grown in RPMI/Hepes containing 10^{-4} M 6-mercaptopurine.

PBL from three Gambian adults (K. D., K. J., and S. R.), solidly immune of Pf malaria, were isolated from freshly drawn heparinized blood. 4 ml of blood was layered atop 3 ml of Ficoll-Paque and centrifuged at 10^4 *g*. After removal of plasma and platelets, the PBL were washed twice in RPMI/Hepes and then cultured in this medium containing PWM (1:1,000 dilution) at a cell density of 10^6 cells/ml, at 37°C and 5% CO_2/95% air for 5–7 d.

Fusion. Before fusion, 10^7 cells of each PWM-stimulated PBL and GM4672 cells were washed twice in serum-free RPMI/Hepes and pelleted together. The cell pellet was overlaid with 0.5 ml of 44% PEG (21), suspended, and pelleted at 300 *g* for 3 min. The PEG was diluted by dropwise addition of 10 ml of serum-free RPMI/Hepes over a period of 5 min, and the cells were pelleted again at 500 *g* for 5 min. The supernatant fluid containing PEG was removed, the cells suspended in RPMI/Hepes maintained at 37°C, 5% CO_2/95% air for 24 h, washed, suspended in RPMI/Hepes/HAT, and plated into microtiter plates at a cell density of 2×10^5 cells/well (19). The cells were refed twice weekly, and monitored for growth of colonies once a week.

Cloning. Proliferating cells producing antiplasmodial Ig (see below), were first expanded into several macrotiter wells and then cloned twice by limiting dilution into microtiter flat-bottom plates at densities of 30 and 0.3 cells/well. The cells were screened and refed once a week.

Plasmodium Culture. The Gambian K1 isolate of Pf was maintained in O Rh$^+$ eryth-

rocytes and serum as in Trager and Jensen (25), and synchronized weekly using gelatin (26). Metabolic labeling employed RPMI containing 20% of the normal methionine concentration plus [^{35}S]methionine at a concentration of 0.1 mCi/ml.

Screening for Antiplasmodial Antibodies. The antibody concentration of supernatants was screened using an ELISA, in polystyrene 96-well microtiter plates (Dynatech Laboratories, Inc.; Alexandria, VA). The plates were coated with human IgG or IgM, 2.0 μg/ml each, solubilized in 0.05 M sodium borate, pH 8.5. The equilibration with culture supernatants and development with alkaline phosphatase–derivatized antibodies and *p*-nitrophenyl phosphate as substrate was as in Schoenfeld et al. (21). The same ELISA was used to determine the Ig isotype and IgG subclass of mAb.

Antiplasmodial antibodies in culture supernatants that tested positive by ELISA were identified using indirect immune fluorescence. Acetone-fixed blood films of Pf cultures (1% hematocrit) at ~10% parasitemia were preincubated with goat serum at a dilution of 1:50, washed, incubated with the hybridoma supernatants for 30 min at 37°C, and washed again. Fluorescein-labeled goat IgG against human IgG and IgM was incubated for 30 min at 37°C. After extensive washing in PBS, the slides were counterstained with Evans Blue and evaluated using a Zeiss epifluorescence microscope.

Supernatants of cloned hybridomas were used to assess the in vitro growth inhibition of Pf by the antiplasmodial antibodies. Gelatin-synchronized Pf cultures (26) were initiated at a parasitemia of ≤1% and carried for 72 h in RPMI 1640 medium, refeeding daily as in Trager and Jensen (25). Each supernatant/serum was tested in triplicate. The metabolic labeling was done between 36 and 72 h of the culture, using RPMI 1640 containing 20% of the normal methionine concentration and 0.1 mCi/ml [^{35}S]methionine. The degree of inhibition was expressed relative to parasite growth obtained with hybridoma supernatants not producing antiplasmodial antibodies and Ig isolated from the sera of the immune PBL donors and of nonimmune individuals (27).

Immunochemical Techniques. To test for monoclonality of antibodies secreted by hybridomas, we selected only supernatants that, by ELISA, tested positive 10-fold above the background of the GM4672 cells. To metabolically label the IgG/IgM, the hybridomas were incubated during exponential growth in presence of [^{35}S]methionine, 0.05 mCi/ml, in methionine-free RPMI/Hepes. The Ig was purified on affinity columns using Sepharose 4 B–immobilized goat anti–human Ig. Specifically bound human Ig was eluted with 0.2 M glycine, pH 2.4. The Ig was dialysed against 0.15 M NaCl, concentrated, and subjected to bidimensional IEF and SDS-PAGE (28). Heavy and light chains were identified by autoradiography.

[^{35}S]methionine-labeled Pf antigens reacting with human mAb were identified by immune precipitation as previously described (7). Preabsorption of the antigen, the specific immune deposition with mAb, and fractionation of precipitated Pf antigens by SDS-PAGE was also as previously described (7).

Results

Donor Lymphocytes. 30 ml of blood from each of the Gambian adults S. R., K. J., and K. D. yielded 2×10^7, 3.5×10^7, and 4×10^7 PBL, respectively. After 7 d of PWM-stimulation, we recovered 60–80% of the PBL for fusion with GM4672 cells. The majority of PBL formed large clumps of blast cells with pseudopod extrusions typical for B lymphocytes. The PBL were relatively depleted in macrophages by leaving adherent cells in the culture flasks.

Hybridomas. 21–60 d after fusion and maintenance of the fused cells in HAT medium, 29.5, 23.9, and 13.6% of wells of fusions K. J., K. D., and S. R., respectively, contained proliferating hybrid cells producing, by ELISA, IgG and/or IgM 10 times higher than levels found in spent GM4672 culture supernatants (Table I). This corresponds to a minimum fusion frequency of 6.8×10^{-5}–1.5×10^{-6}. Each fusion yielded equivalent numbers of IgG and IgM at a

TABLE I

Ig-producing Human-Human Hybridomas from Human PBL and GM 4672 Lymphoblastoid Cells

Donor	Wells plated	Wells positive for Ig	Fusion frequency	IgG	IgM	IgG/IgM
K. J.	200	59 (29.5%)	6.8×10^{-5}	21	31	7
K. D.	264	63 (23.9%)	8.4×10^{-5}	34	25	4
S. R.	154	21 (13.6%)	1.5×10^{-6}	11	8	2

TABLE II

Human-Human Hybridoma IgG and/or IgM Reacting with Pf Antigens and Inhibiting In Vitro Parasite Growth

Donor	Wells positive for Ig	FA* reaction	IgG		IgM		IgG/IgM	
			Number	GI‡	Number	GI‡	Number	GI‡
K. J.	59	9	5	3	4	1	—	—
K. D.	63	5	3	1	1	1	1	1
S. R.	21	1	—	—	1	1	—	—

* FA, fluorescent antibody reaction.

‡ GI, in vitro growth inhibition ($p < 0.05$ relative to control using FA negative hybridoma supernatant fluids).

similar frequency, with a smaller number of wells containing hybridomas producing both IgG and IgM (Table I).

Hybridomas Producing Antiplasmodial Antibodies. Of the 143 Ig-yielding hybridomas, 16 produced IgG/IgM against plasmodial antigens (Table II), according to immune fluorescence. Positive reactions consisted of diffuse fluorescence of trophozoites and schizonts typical for immune sera (Fig. 1*A*) or fluorescence concentrated at the periphery of trophozoites, mature schizonts, and merozoites (Fig. 1*B*).

Four of seven hybridomas that produced Ig exhibiting significant in vitro inhibition of parasite growth could be cloned at a density of 0.3 cells/microtiter well (Table III). Preseeding of wells with a feeder layer of peripheral blood mononuclear cells did not increase the cloning efficiency.

Human mAb. Two consecutive affinity adsorptions on Sepharose 4 B–immobilized goat anti–human IgG allowed the purification of human Ig from cell supernatants with good yields. The [^{35}S]methionine-labeled human antibodies of the cloned hybridomas, KJ7-2C11D, KD3-7D3F, KD5-7A8E, and KD8-2B2D, showed only one heavy and light chain each, by IEF/SDS-PAGE, indicating their monoclonality. All antibodies inhibited the growth of Pf in vitro (Table III), reacted strongly with trophozoite/schizont/merozoite surfaces by immune fluorescence (Fig. 1, *A* and *B*), and produced, except for KJ7-2C11D, heavy and light chains different from those of the GM4672 parent line (Table III).

Inhibition of Pf Growth In Vitro by Human mAb. Inhibition of growth was assessed by [^{35}S]methionine metabolically incorporated by synchronized Pf cultures over a period of 36 h during schizont maturation and merozoite reinvasion; only inhibition of 45% or more was significant, applying the double-blind *t* test.

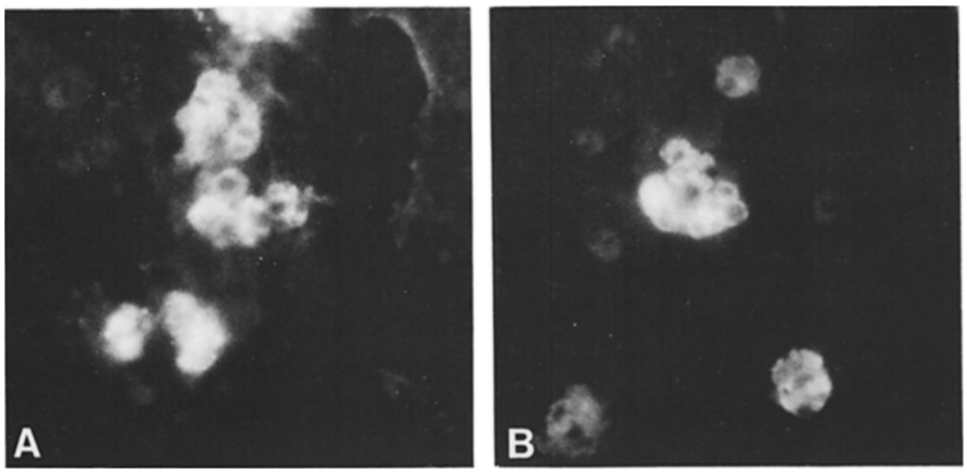

FIGURE 1. mAb from human-human hybridomas. Fluorescent antibody reaction with cultured Pf parasites. Acetone-fixed cells were reacted with primary human antibody and then with goat anti-human IgG or IgM. *A*, K. D. (Gambian adult) serum (1:20 dilution). *B*, mAb KJ7-2C11D from culture supernatant.

TABLE III
Pf-reactive Human Monoclonal Ig and Their Isotypes

Donor	Clones	Ig produced	Ig*	Inhibition‡
			μg/ml	%
K. J.	KJ3-1B3G	μ/κ	3.5	—
	KJ7-2C11D	γ_2/κ	0.5	90
K. D.	KD3-7D3F	μ/λ	1.2	45
	KD5-7A8E	γ_2/λ	4.7	92
	KD5-8C10B	γ_2/λ	15.2	—
	KD8-2B2D	γ_1/κ	2.3	95
S. R.	SR3-1D4B	γ_1/λ	0.9	—
GM4672§		γ_2/κ	0.02	—

* Based on ELISA; cell density of 10^6 cells/ml.
‡ At Ig concentration of 1 μg/ml. Tested using the Gambian K1 strain.
§ GM4672 is the human lymphoblastoid cell line used as fusion partner.

Although growth inhibition of human mAb was normalized to 1 μg Ig per milliliter of medium (Table III), one mAb, KJ7-2C11D (IgG2) gave 70% growth inhibition at a concentration of <50 ng/ml.

Antigens Recognized by Inhibitory mAb. Probably due to selection by the inhibition assay, three of four inhibiting mAb precipitated the Pf M_r 195,000 component. Two antibodies also deposited M_r 195,000 protein degradation products with M_r near 110,000 and 80,000; and one mAb gave only a very weak reaction with the M_r 80,000 protein (Fig. 2, lane 4).

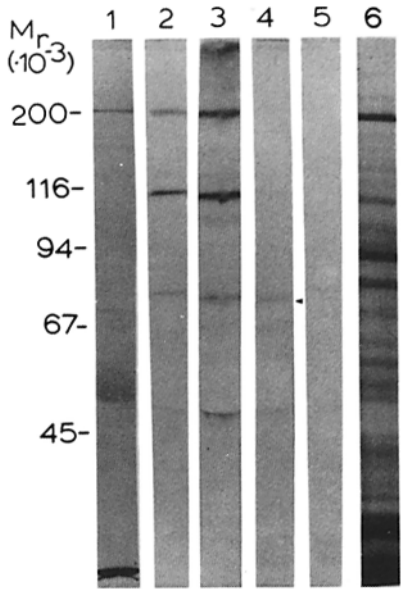

FIGURE 2. Immune precipitation of [^{35}S]methionine-labeled Pf antigens by human mAb. SDS-PAGE analysis followed by [^{35}S]methionine autoradiography. As source for mAb, we used affinity-purified Ig from culture supernatants. In all experiments, 5×10^5 cpm of [^{35}S]-methionine-labeled antigen were reacted with 0.03 ml of human mAb at a concentration of 5 μg/ml. Lane 1, KD5-7A8E; lane 2, KD3-7D3F; lane 3, KJ7-2C11D; lane 4, KD8-2B2D, M_r 80,000 component (labeled with an arrow); lane 5, human control serum (no malaria exposure); lane 6, [^{35}S]methionine-labeled *Pf* antigen.

Discussion

Our results show that one can produce human mAb to Pf antigens using hybridomas from human LCL and PBL of individuals immune to Pf malaria, and obtain a high yield of Ig-producing hybridomas relative to previously reported (21) experiences with the GM4672 line. The high frequency of IgG-secreting hybrid cells may be due to (*a*) stimulation of the PBL for 6–7 d, at which time predominantly IgG-producing lymphocytes elicit a proliferative response (29), and (*b*) increased susceptibility to mitogen stimulation and/or fusion with human LCL of PBL from individuals chronically exposed to malaria and other infections.

Since the experiments in man testing the protective capacity of immune IgG from individuals immune to Pf malaria (30), much research has been devoted to the humoral immune response to the malarial parasite and the identification of potentially protective *Plasmodium* blood stage antigens. Although polyspecific immune sera have allowed the identification of certain candidate antigens (13, 16, 31), no unambiguous correlation between protection and antibodies to a defined antigen has been found to date (13, 14).

The complexity of the immune response to *Plasmodium* antigens may be due to the fact that a given antigen may contain epitopes of very different immunogenicity. Evidence for this is available from gene cloning and DNA-sequencing experiments that provided the amino acid sequences of certain *Plasmodium* proteins. These proteins contain repetitive amino acid sequences (32, 33) that

are highly immunogenic, but antibodies against them do not necessarily provide antiplasmodial activity (34, 35). In fact, the function of these peptides may be to divert the hosts' immune response. At least one category of antigens bearing repetitive amino acid sequences, the circumsporozoite (CS) antigen, contains, in addition to the tandem repeat amino acid sequences (36), portions that are conserved throughout different *Plasmodium* species (37, 38). Similar conserved interspecies peptides were found for the Pk M_r 230,000 and Pf M_r 195,000 protein (Schmidt-Ullrich and Monroe, unpublished results).

It is reasonable to assume that similar proteins fulfill similar functions in different *Plasmodium* species, and that conserved sequences carry out these functions. Since there is evidence that binding of antibody to these moieties correlates with damage to the parasite, mAb that truly reflect the immune status of the host should be valuable reagents to identify functionally relevant epitopes of protective antigens. For Pf malaria, this can currently only be achieved with human mAb. An attractive Pf test antigen is the M_r 195,000 protein. This component has been shown to be a protective antigen in Saimiri monkeys, and to be the target antigen for antibodies that inhibit Pf growth in vitro (13, 16). The differences of in vitro growth inhibition by M_r 195,000 antigen-reactive mAb, produced in our laboratory, support the concept that recognition of certain epitopes on this antigen is essential for the antibody's biological activity. The very effective in vitro growth inhibition of Pf by our human mAb (see also 16) relative to some murine mAb against the analogous M_r 230,000 protein of Pk (15) suggests that short term immunization with incompatible *Plasmodium* parasites does not necessarily lead to the production of antibodies with similar epitope specificities as of those antibodies produced by the natural host after exposure to malaria over a long time.

The biological activity of our human mAb and of IgG to the Pf M_r 195,000 protein in one other study (16), together with the demonstration that this category of proteins is protective in three host-parasite systems (7, 10, 17) makes the M_r 195,000 component a potential candidate antigen for use as a vaccine against Pf malaria. One can expect that human mAb against this protein will become valuable in three respects: (*a*) the identification, characterization, and isolation of antigenic peptides of high-M_r schizont/merozoite antigens of human *Plasmodium*, (*b*) identification of portions of the Pf genome encoding these peptides, and (*c*) their use as therapeutic reagents for severe cases of drug-resistant malaria.

Summary

Using the human lymphoblastoid cell line, GM 4672, and PBL of Gambian adults immune to *Plasmodium falciparum* (Pf) malaria, we have produced human-human hybridomas and selected those that produce mAb against *Pf* antigens. The fusion frequency, using PWM-stimulated donor lymphocytes was between 6.8×10^{-5} and 1.5×10^{-6}. Using immune fluorescence, immune precipitation, and Pf in vitro growth inhibition, we cloned four hybridomas that reacted with the Pf M_r 195,000 schizont/merozoite protein. The differences in proteins immune precipitated and in growth inhibition indicate that, during development of protective immunity against Pf malaria, a spectrum of antibodies is produced

reacting with different epitopes on the same antigen. Only a portion of these antibodies exhibits biological activity, suggesting that the recognition of certain epitopes is required for the development of a protective immune response.

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