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Plasmodium falciparum: characterization of defined antigens by monoclonal antibodies

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SUMMARY

Monoclonal antibodies directed against *Plasmodium falciparum* detect stage-specific, species-specific and common antigenic determinants of Plasmodia. These antibodies provide new tools for purification and characterization of *Plasmodium falciparum* antigens in relation to future procedures for immunoprophylaxis.

INTRODUCTION

Malaria is a major health and socio-economic problem in tropical areas. There are more than 200 million cases annually throughout the world and at least one million deaths in Africa alone (WHO, 1977). Immunological research in this disease ultimately aims at the development of malaria vaccines and the improvement of immuno-diagnostic test systems (Wernsdorfer, 1979). Due to the development of continuous *in vitro* culture of *P. falciparum*, there is an increasing possibility of reaching these goals (Traeger & Jensen, 1976). Large amounts of antibodies of known specificities are required for the purification of malaria antigens now available and the development of new immuno-diagnostic tests.

The development of hybridomas secreting specific antibodies against *P. falciparum* would be of great help in approaching these problems. The generation of hybrid cells producing monoclonal antibodies directed against defined antigenic determinants has been made possible by progress in somatic cell hybridization techniques (Kohler & Milstein, 1975). Antigens which have been used for immunization and which are recognized by antibodies secreted by hybridomas include cell surface antigens and defined soluble antigens (Melcher, Potter & Warner, 1978; Kohler & Milstein, 1975; Lemke *et al.*, 1978). Such monoclonal antibodies directed against viral polypeptides have already been used for the characterization of antigenic determinants of a number of viruses (Gerhard *et al.*, 1978).

In this study we report some characteristics of monoclonal antibodies produced against antigens present in erythrocytic forms of *P. falciparum*.

MATERIALS AND METHODS

Culture of P. falciparum. P. falciparum parasitized RBC were obtained from a European patient on his return from Senegal. This isolate (SGE1) was adapted to *in vitro* culture and cultivated

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according to Traeger & Jensen (1976) for more than 6 months. Parasitaemia of 12 to 20% was found regularly every fourth day of culture. In some experiments, inhibition of *in vitro* growth of *P. falciparum* by various reagents was checked by a modification of the technique of Wilson & Phillips (1976). In brief, 200 μl of a suspension of 5% human RBC with an initial parasitaemia of 1·5% were incubated in ninety-six flat-bottomed wells on Falcon 3040 plates with culture medium (Traeger & Jensen, 1976) supplemented with 25% of either serum from individuals with high levels of antibodies against *P. falciparum* or monoclonal antibodies. The 'supplemented' culture medium was changed every 12 hr and final parasitaemia was determined on thin blood smears after 84 hr of incubation.

Internal labelling of P. falciparum. Five millitres of a 5% human RBC suspension with 20% parasitaemia were incubated in MEM (Eagle's) free of methionine (GIBCO) supplemented with glutamine, bicarbonate and 10% NHS. The parasites were labelled for 5 hr with ^{35}S-methionine (20 μCi/ml, sp. act. 600 Ci/mmol). The infected cells were then pelleted, washed three times with cold phosphate-buffered saline (PBS) and disrupted with 1 ml of 0·5% Nonidet P40 (NP40) in TNE (0·01 M Tris, pH 7·4, 0·1 M NaCl, 0·005 M EDTA). The lysate was cleared by centrifugation at 30,000 *g* for 30 min.

Immunoprecipitation and SDS polyacrylamide gel. Fifty microlitres of ^{35}S-methionine *P. falciparum* lysate provided by 5×10^6 parasitized RBC containing 300,000 c.p.m. were incubated with 10 μl of either hybrid supernate concentrated ten times by ammonium sulphate (precipitation at 50% final concentration and dialysis against PBS) or ascitic fluid. The mixture was incubated for 1 hr at room temperature. An optimal amount of rabbit anti-mouse Ig was then added and the mixture was incubated overnight. The precipitates were washed five times with 2 ml of TNE supplemented with 0·05% NP40. The precipitates were then dissolved in sample buffer and heated at 80°C for 5 min prior to analysis on 7·5% SDS polyacrylamide slab gels (Magazin & Allet, 1978). The gels were dried, impregnated for fluorometry (Bonner & Laskey, 1974) and exposed for autoradiography using Kodirex X-ray film (Eastman Kodak Co.).

Immunization schedule. BALB/c mice were immunized with either a *P. falciparum* antigenic preparation (gift from Dr I. McGregor, MRC Laboratories, The Gambia) obtained from placentae of individuals heavily infected with *P. falciparum*, or by merozoites and schizonts purified by plasma gel sedimentation from a *P. falciparum* isolate adapted to *in vitro* culture (isolate SGE 1) (Reese *et al.*, 1978). BALB/c mice were immunized three times intraperitoneally with 100 μg of antigenic preparation in Freund's incomplete adjuvant at 2-weekly intervals. Three days before the isolation of spleen cells, the mice were reimmunized by an intravenous injection of 5 μg of the same preparation.

Fusion procedure. Immune spleen cells were fused by polyethylene glycol with either P3X63 Ag8 myeloma or a non-secreting clone, FO, derived from P3X63 (gift from Dr Fasekas, Basel Institute of Immunology). Conditions for fusion were essentially as described by Galfre *et al.* (1977). Some of the positive hybrids were clone-purified on macrophage feeding layer by limiting dilution. Two of them were also injected i.p. into mice in order to obtain ascitic fluid.

Assay for monoclonal antibodies. Indirect immunofluorescence (IF) was used to assay hybrid supernates using *P. falciparum* parasitized RBC grown in culture and containing various stages of erythrocytic forms of the parasites as antigenic preparation, and fluoresceinated rabbit anti-mouse Ig as second antibody. The spot technique used allows for testing 100 samples at a time (O'Neill & Johnson, 1970). Some of the hybrid supernates positive for *P. falciparum* were also tested on *P. berghei* (a Plasmodium species grown in mice) and on *P. cynomolgi* (a Plasmodium species close to *P. vivax* and grown in rhesus monkeys) parasitized RBC. The immunoglobulin class of the concentrated hybrid supernates and the ascitic fluid class were identified by immunodiffusion in agar using antisera specific for immunoglobulin class heavy chain (Meloy Laboratories).

RESULTS

The results of nine successful fusion experiments are reported in Table 1. Approximately half of the wells showed proliferation of hybrid cells and of over 544 different supernates tested, thirty reacted with *P. falciparum* and forty-five with normal or parasitized RBC membrane. Some supernates also

Table 1. Production of anti-*Plasmodium falciparum* antibodies by hybrid cell cultures

Expt	Myeloma cells	*P. falciparum* AG	Hybrid cultures/total no. of cultures	No. of wells supernate with antibodies specific for: *P. falciparum*	NRBC	WBC	Plasma proteins
Hbl	P3X63 Ag8	Placental ext.	94/96	4	5	—	4
Hb3	P3X63 Ag8	Placental ext.	25/48	4	5	—	2
Hb5	P3X63 Ag8	Placental ext.	50/96	2	4	—	3
Hb6	P3X63 Ag8	Placental ext.	45/96	—	3	2	5
Hb7	P3X63 Ag8	Merozoites enriched	54/96	3	2	—	3
Hb9	P3X63 Ag8	Merozoites enriched	60/96	4	5	1	2
Hb12	FO	Merozoites enriched	26/72	3	4	—	—
Hb13	FO	Merozoites enriched	65/72	5	8	1	3
Hb15	FO	Merozoites enriched	72/72	5	9	1	5

reacted with white blood cells (WBC) or plasma protein (background staining with culture medium). Both antigenic preparations used for immunization and both types of myeloma cells gave positive results.

Fig. 1 shows the immunofluorescence pattern obtained using the supernate of hybrid 3A7 specific for *P. falciparum* antigens on slides coated with parasitzed RBC. The specificity of various

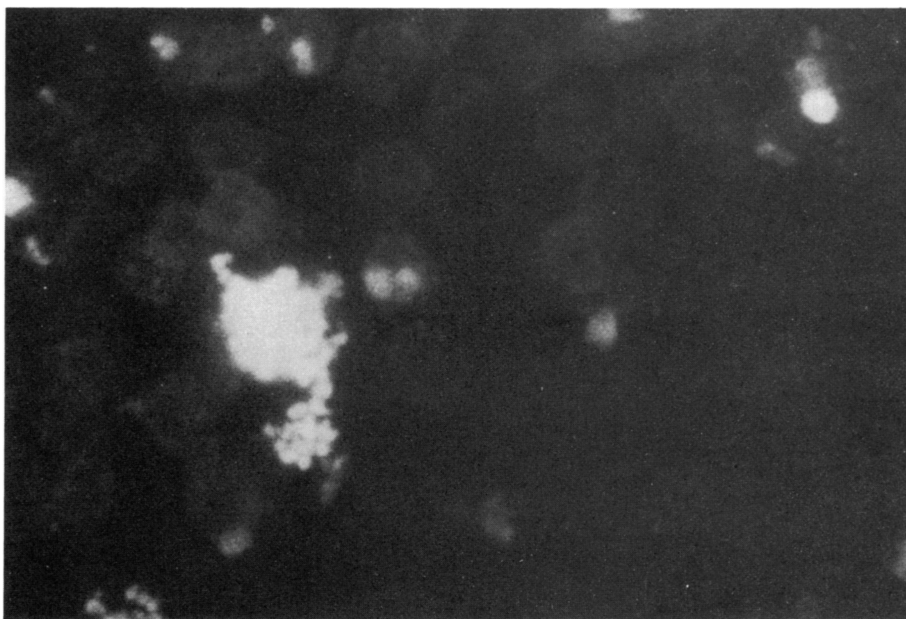

Fig. 1. Indirect immunfluorescence: *P. falciparum* parasitized red cells were incubated with supernate of hybrid 3A7 reacting with *P. falciparum* and then with fluorescein-conjugated rabbit anti-mouse Ig. Slides were examined in a Leitz Orthoplan microscope at a magnification ×630. For routine checking a magnification ×100 without oil was satisfactory. The antibodies present in this supernate react with ring forms (top right and left corners of the photograph) and with schizonts (on the left part one can see an aggregate of schizonts or merozoites and below this an aggregate of 15–20 merozoites probably still inside an RBC). Numerous controls were carried out using normal mouse serum, monoclonal antibodies specific for lymphocyte surface as first antibodies, and other sources of antigen such as normal RBC and trypanosomes instead of *P. falciparum* infected RBC: all proved to be negative.

supernates was further investigated. Hybrid supernates positive for *P. falciparum* were completely negative on preparation of normal human RBC. Supernates of twelve hybrid cultures, selected on the basis of their reactivity with *P. falciparum*, were also tested by indirect IF on *P. berghei* and *P. cynomolgi* parasitzed RBC. Only one hybrid supernate reacted with the three Plasmodium species, three supernates reacted with both *P. falciparum* and *P. cynomolgi* and eight supernates reacted with *P. falciparum* only. All the supernates positive for parasitized RBC from the isolate SGE1 also reacted with another isolate of *P. falciparum*: FCR3 (a gift from Dr Traeger, Rockefeller University). When the supernates were tested against either ring forms or mature forms of *P. falciparum* purified by plasma gel sedimentation (Reese *et al.*, 1978), only one supernate reacted with both ring and mature forms of *P. falciparum*, while the other supernates only reacted with mature forms of *P. falciparum* (old trophozoites, schizonts and merozoites).

Hybrids from six positive supernates were clone-purified twice on mouse peritoneal cell feeding layers by limiting dilution. One clone from each of them was selected according to positivity in indirect IF upon dilution and to growth curve: six of them were used for mass culture and tested after concentration by ammonium sulphate precipitation. Five of these clones secreted monoclonal antibodies of the IgM class and one secreted monoclonal antibodies of the IgG2a class. Two of these clones were injected intraperitoneally into six BALB/c mice. The two ascitic fluid pools had a titre of 1/20,000 and 1/40,000 respectively by indirect IF.

Supernates of the six clone-purified hybrids and two ascitic fluid pools were tested in double immunoprecipitation for their ability to precipitate ^{35}S-methionine-labelled proteins of *P. falciparum*. One of them precipitated a protein of approximately 195,000 daltons (Fig. 2).

Supernates from four clones and two ascitic fluid pools were also checked for their ability to

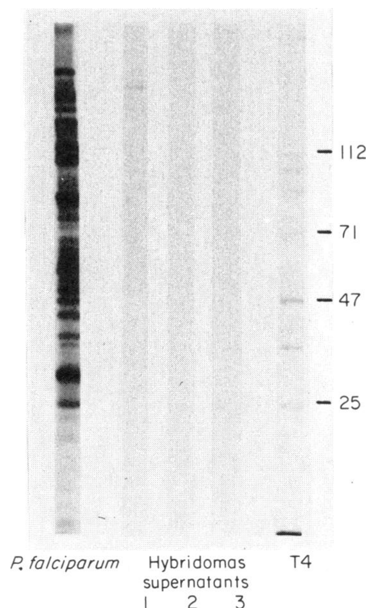

Fig. 2. Autoradiography of an SDS polyacrylamide slab gel containing five different samples. Lysate of *P. falciparum* was applied to the first column, immunoprecipitates of three different clones were applied to columns 1, 2 and 3; bacteriophage T4 protein labelled with ^{35}S-methionine was used as molecular weight marker and applied on the last column. Hybridoma supernate 2B6 (column 1) precipitated one of the radiolabelled proteins of *P. falciparum* while the others were negative (columns 2 and 3 presented analysis of two samples out of a series of eight different samples analysed).

inhibit *in vitro* growth of *P. falciparum* culture. None of the supernates or ascitic fluids was inhibitory. The parasitaemias (mean of three culture wells per test) were: 15·3% in the medium control supplemented with 2% mouse serum, 14–16% in medium supplemented with the various clone supernates or ascitic fluids and 9·1% in a parallel culture of medium supplemented with the serum of one individual from an endemic area and containing anti-*P. falciparum* antibodies.

DISCUSSION

This study demonstrates that it is possible to produce monoclonal antibodies which react with *P. falciparum* antigens and that indirect immunofluorescence can be used to check the specificity of hybrid products in this system. Indirect fluorescence allows for the direct visualization of the structure detected by the antibodies secreted by the hybrid cells. For example, it was easy to distinguish antibodies reacting with *P. falciparum* from antibodies reacting with RBC, WBC or soluble proteins. In addition, it was possible to identify the developmental stage of the parasite recognized by the antibodies or the species specificity detected by them. Indeed, some of the monoclonal antibodies show stage specificity (reacting only with mature forms of the parasites), some detect species-specific antigens while others react with common antigens present on various species of Plasmodia.

The complexity of the life cycle of *P. falciparum* and the difficulties in obtaining parasites free of host material (Kreier, 1977) have been major obstacles in the isolation and purification of antigens of Plasmodia. Monoclonal antibodies can be used in the purification of malaria antigens from the presently available antigenic mixtures. In this respect, one of the cloned hybrids secreted antibodies reacting with a single protein of *P. falciparum* of 195,000 daltons. Purification of malaria antigens by affinity chromatography using monoclonal antibodies will avoid the problem of contamination of antigenic preparations by host proteins. The preparation of such defined antigens is an essential prerequisite for the analysis of the role of individual antigens in the induction to protective immunity.

Antibody-mediated host protective immunity has been demonstrated in rodent and human malaria (Hamburger & Kreier, 1978; Cohen, McGregor & Carrington, 1961). The serum antibodies used in the latter studies reacted with a number of malaria antigens, making it difficult to identify the antigen(s) responsible for the induction of protective antibodies. The use of purified antigens would be valuable in the analysis of this immune response. In this respect, monoclonal antibodies can be used for the purification of antigens of Plasmodia and also in transfer experiments and/or in experiments of *in vitro* growth inhibition of Plasmodia (Wilson & Phillips, 1976). In this latter way, the antibody specificities involved in protection of the host or in experiments of *in vitro* growth inhibition could be defined. The monoclonal antibodies characterized in this report failed to inhibit growth of *P. falciparum in vitro*. They are probably not directed against relevant antigen(s) present on the surfaces of merozoites (Cohen & Butcher, 1970) and/or monoclonal antibodies of more than one specificity may be needed in this system (Howard *et al.*, 1978). Experiments are in progress using additional monoclonal antibodies and mixtures of monoclonal antibodies.

In humans suffering from acute malaria, malaria antigens are detectable in plasma and parasitizied RBC (McGregor *et al.*, 1968), but the techniques used for their detection are laborious and difficult to apply in large epidemiologic surveys. Monoclonal antibodies of well defined and restricted specificity may be used to detect malaria antigens by immunoassays which have already been developed for a murine model of malaria using hyperimmune sera (Mackey *et al.*, 1980). The use of monoclonal antibodies reacting with different species-specific malaria antigens described in this study is of potential value in the immunodiagnosis of malaria.

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