Characterisation of Monoclonal Antibodies Against Blood Forms of *Plasmodium falciparum* by the Immunofluorescent Test*

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SUMMARY **Successful production of monoclonal antibodies (MABs) against the blood forms of *Plasmodium falciparum* was made after a single fusion of SP2/0 myeloma cells with the spleen cells from an immunised BALB/c mouse. From 326 hybrid clones obtained, 125 clones (38%) produced malaria specific antibodies, which could be divided into 5 groups based on their immunofluorescent staining patterns. Group I MABs showed bright generalised staining of all blood stages including gametocytes. Group II MABs reacted strongly with the merozoites but weakly with the other stages. Group III MABs reacted intensely around the perimeter of individual merozoites and their reactions with the intraschizont merozoites took a form of a cluster of grapes. Group IV MABs reacted more intensely with the schizont producing an appearance of a cluster of small balls and group V MABs showed a mottling surface appearance. The numbers of group I to group V MABs were 39, 33, 33, 15 and 5 respectively. The potential use of MABs was discussed in relation to the development of malaria vaccine, diagnosis of low grade parasitaemia and for distinquishing between parasite isolates.**

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Malaria remains to be increasing in incidence and prevalence in most tropical areas and still be a major health problem impeding the economic growth of many tropical developing countries.[1] For many obvious reasons, the present strategy of the malaria control programme has failed, and there is a need for introduction of alternative control measures for malaria, one of which is through vaccination, which has been proved possible in aortus monkeys immunised with merozoites or mature segmenters incorporated in adjuvants against challenge with *Plasmodium falciparum*.[2-4] Ideally the future vaccine should comprise only the 'protective' antigens, which are still poorly defined. A logical approach which could lead to proper identification of the 'protective' antigen is through the study of monoclonal antibodies which react alone or in combination with effector cells causing parasite death. The success in characterisation of such 'protective' antigen(s) in combination with the noval recombinant DNA technology will in principle lead to the success of production of future malaria vaccine.

The objective of this study is to raise monoclonal antibodies against well-defined antigenic determinants of blood stages of *P. falciparum* and to characterise them by the immunofluorescent test. The success of this study will be followed by a series of investigations to analyse the functional properties of these monoclonal antibodies hoping eventually to a successful identification of 'protective antigen'. These monoclonal antibodies will also be used for typing of strains of *P. falciparum* similar to the approach of McBride.[5]

MATERIALS AND METHODS

Parasites

The SO strain of *P. falciparum* was used. The parasites were grown in RPMI-1640 medium in petri dishes according to the technique of Trager and Jensen.[7] Their growth was synchronised by sorbitol treatment according to the technique of Lambros and

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Vanderberg[8] and the concentrated schizonts were prepared by centrifugation in percoll gradient according to the method of Schoffield et al.[9] The parasitised cells were washed 3 times in physiological saline by centrifugation at 1,000 xg at 4°C for 10 minutes.

Myeloma cells

The SP2/0 myeloma cells resistant to 8-azaguanine[10] kindly provided by Dr. J. Kadouche (Saint Louis Hospital, Paris, France) were used. The cells were grown in RPMI-1640 in the presence of 10 per cent foetal bovine serum (Flow Laboratories) and 6 $x10^{-3}$ mM 8-azaguanine (Sigma). Cell viability determined at the time of fusion by the trypan blue exclusion test was more than 90 per cent.

Indirect fluorescent antibody test

Blood smears were made from unwashed, synchronously grown SO strain of *P. falciparum* mostly in schizont and late trophozoite stages. IFA antibody of supernatants from hybrid cultures was determined using the technique modified from that described earlier,[11] in that goat anti-mouse IgG was used in place of goat antihuman Ig (G,A,M), the time allowed for the culture supernatant to react with the parasitised cells was two hours, and the time of applying fluorescein labelled secondary antibody was one hour instead of 30 minutes for each step.

Immunisation

An eight week old BALB/c mouse was immunised by three intraperitoneal injections of washed $1x10^8$ erythrocytic forms of *P. falciparum* composing of not less than 40 per cent schizonts with an equal volume of Freund's complete adjuvant initially and incomplete adjuvant subsequently at an intervals of one to two weeks. A week after the third immunising dose, the indirect fluorescent antibody titre was determined and the titre of 1:2560 was obtained. The mouse was then given an intravenous injection of $1x10^8$ washed parasites in physiological saline one week after the third immunising dose. Fusion was done three days later.

Cell fusion and selection of hybrids

Spleen cells from the immunised mouse were fused with myeloma cells using 4,000 M.W. polythylene glycol (PEG, Sigma) according to the technique modified from that of Galfre *et al*[12] with spleen cells to myeloma cells ratio of 10:1. After a brief PEG treatment (90 seconds), the cell suspension was diluted by warm RPMI without serum followed by centrifugation at 220 x g for 10 minutes at room temperature. The supernatant was then removed and the cell sediment resuspended in a selective medium comprising RPMI, $5x10^{-2}$ mM hypoxanthine (Sigma) and $1x10^{-2}$ mM azaserine (Sigma), so that the final cell concentration was $1.0x10^6$ /ml. Two hundred and twenty µl of the cell suspension was then dispensed into each well of the 96-well plate (Costar, Mass., U.S.A.) and the cells were incubated at 37°C in 5% CO_2 in air in a humidified CO_2 incubator.

After seven to 10 days, the wells were inspected for hybrids. Supernatants from the culture of growing hybrids were removed and tested for anti-malarial antibody by IFA test. Cell populations positive for malaria antibody were then expanded by transferring into tissue culture bottles (Nunclon, Copenhagen) and further incubated. They were then cloned by limiting dilutions followed by further propagation, and the clone product analysed by the IFA test. Positive clones were cryopreserved in liquid nitrogen.

RESULTS

Of 600 wells plated, 326 hybrids were visible seven to 10 days after fusion, of which 125 secreted antibodies against *P. falciparum*. These hybrids were then cloned and their products tested. Based on their immunofluorescent reactivities, MABs produced were directed against different stages of parasite maturation and could be classified into 5 groups using the criteria similar to that of Hall *et al.*,[13] and the results are shown in Table 1. The IFA staining patterns are shown in Figure 1. In group I, there was a

Table 1 Groups of monoclonal antibodies, their IFA staining patterns and the numbers of monoclones produced.

Group	Pattern of IFA staining	No. of monoclones
I	Bright generalised staining of all blood stages including gametocytes	39
II	Strong reaction with merozoites, weak reaction with other stages	33
III	All asexual stages, with intense staining around the perimeter of individual merozoites and their reactions with the intra-schizont merozoites take the form of a cluster of grapes	33
IV	All asexual stages, but the reaction is more intense with the schizont producing an appearance of a cluster of small balls	15
V	Mottling surface appearance of schizont and trophozoite infected red blood cells	5
	Total	125

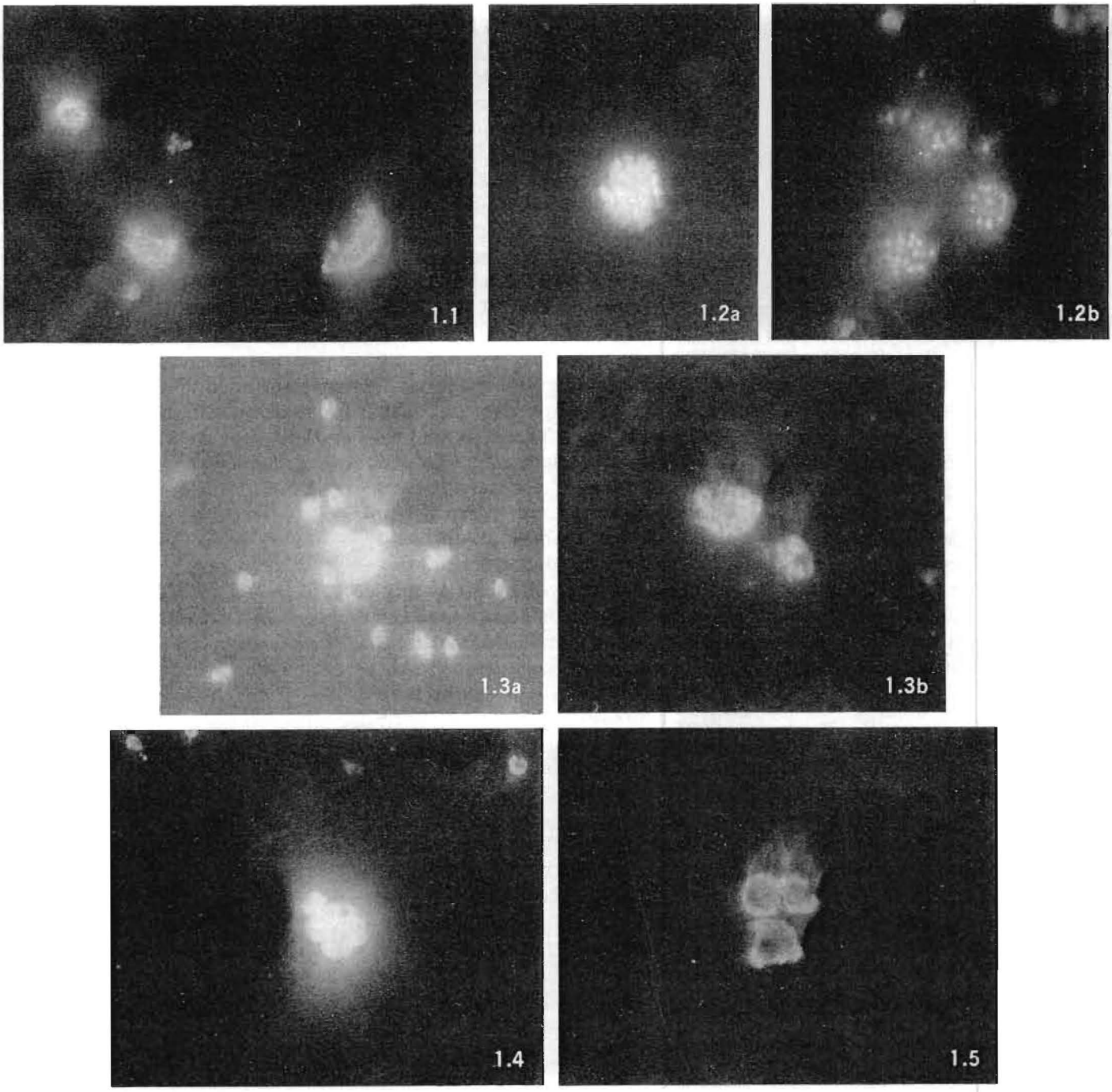

Fig. 1 Patterns of indirect immunofluorescent antibody (IFA) staining produced by monoclonal antibodies on acetone-fixed blood films of *P. falciparum*.

1.1 Staining with group I antibodies. Bright generalised staining of schizonts, rings and a gametocyte is shown.

1.2 Staining with group II antibodies showing reaction with whole intra-schizont merozoites (fig. 1.2a) and free merozoites as well as organelles (probably rhoptries) of intra-schizont merozoites (fig. 1.2b).

1.3 Staining with group III antibodies showing reaction with the perimeters of free merozoites releasing from a disrupted schizont (fig. 1.3a) and the reaction with intra-schizont merozoites making an appearance of a cluster of grapes (fig. 1.3b).

1.4 Staining with group IV antibodies showing an appearance of a cluster of small balls within a schizont.

1.5 Reaction with group V antibodies showing irregular (mottling) appearance on the surface of the schizonts or trophozoites.

uniformly bright generalised staining of all blood stages including gamethocytes (Fig. 1.1). Group II MABs reacted strongly with free merozoites and those within schizonts, and weakly with the earlier asexual forms. Of 33 group II MABs, 10 reacted with the whole merozoites (Fig. 1.2a) whereas the other 23 MABs reacted with an organelle or a group of an organelles probably be rhoptries (Fig. 1.2b). Group III MABs reacted with all asexual stages, but their reactions with the merozoites were confined only on the perimeter (Fig. 1.3a) and their staining of the intra-schizont merozoites took the form of a cluster of grapes (Fig. 1.3b). Our group IV and V MABs were different from those of Hall *et al.*[13] Our group IV MABs reacted with all asexual stages, with more intense staining in the schizont producing an appearance of a cluster of small balls (Fig. 1.4). Group V MABs reacted with the surface of a schizont or a late trophozoite showing a mottling appearance (Fig. 1.5).

DISCUSSION

In our hand the success rate of obtaining hybrids was 54.3 per cent, which was lower than that of Perrin,[14] who in 9 fusions obtained an average recovery rate of 69.6± 24.8 (S.D.) per cent. Among 326 hybrid clones tested, 125 clones (38.3%) were found to secrete antibodies against *P. falciparum*. Recovery rate of antibody specific clones was thus higher than that of Perrin[14] who showed that only 8.5 ±3.7 (S.D.) per cent of hybrids secreted anti-*P. falciparum* antibodies. The reason for a higher successful rate for antibody specific clones was probably attributable to the use of percoll enriched population of schizonts used in our study for immunisation.

Analysis of MAB reactivities showed that the immunofluorescent staining patterns of our MABs were in general similar to those reported by Hall *et al,*[13] except for group IV and group V MABs. The reaction of our group IV MABs showed a cluster of small balls appearance, the exact nature of which was not known. Our group V MABs showed a mottling surface staining of the schizont or the trophozoite infected cells. This irregular surface staining could be due to reactivity against certain antigen not uniformly distributed on the surface of parasitised cells.

The success in producing MABs against blood stages of *P. falciparum* will allow us to develop techniques for detection of low grade parasitaemia using the techniques of Mackey *et al,*[15] or Avraham et al.[16] These techniques will be useful for screening of blood donors as well as for detection of asymptomatic cases living in malaria endemic areas. MABs could also be used to detect circulating malaria antigens which could represent the malaria load more than the commonly used parasitological examination by blood smear. MABs with 'protective' functions by inhibiting merozoites re-invasion, by inhibiting intracellular growth of the parasites or by causing reversal of binding of infected cells to melanoma cells would make possible characterisation of candidate antigen(s) for use in the preparation of the future malaria vaccine. MABs could also be used for typing of strains of *P. falciparum* to see if there are any differences between strains causing acute uncomplicated malaria and those causing severe malaria.

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