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Synthesis and evaluation of monoclonal antibody against *Plasmodium falciparum* merozoite surface antigen 2

Afra Khosravi[1], Eghbaleh Asadollahy[2*], Sobhan Ghafourian[3], Nourkhoda Sadeghifard[3], Reza Mohebi[3]

[1]*Immunology Department, Faculty of Medicine, Ilam University of Medical Sciences, Ilam, Iran*
[2]*Food and Drug Department, Food and Drug Deputy, Ilam University of Medical Sciences, Ilam, Iran*
[3]*Clinical Microbiology research Centre, Ilam University of Medical Sciences, Ilam, Iran*

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ABSTRACT

Objective: To assess the quality of expressed MSP-2 and also to confirm the immune response against different domains of these proteins. **Methods:** Mice were immunized with a schizont extract to stimulate the immune system to make antibodies against different antigens of the late stage parasite including production of antibodies against different domains of *Plasmodium falciparum* (*P. falciparum*) MSP-2. B lymphocytes of immunized mice were extracted from the spleen and the fusion was performed using NS-1 myeloma cells and the hybridoma cells were assayed by ELISA either with a schizont extract or different domains of MSP-2 and/or by IFAT with whole schizont preparation. Fusion of NS-1 and spleen cells was performed. The positive hybrids were cloned and ELISA was applied against different dilutions. The positive clones were transferred to a small tissue culture flask and after developing they were assayed against schizont extract and the different MSP-2 domains. The positive clones were expanded to large (75 cm^2) flask and cultured under the same conditions, checking them using both ELISA and IFAT and the positive cells were frozen as soon as possible. **Results:** A total number of 7 fusions including 26 plates (2496 wells) were performed, of which 1336 hybrids were produced and the overall efficiency (1336/2496 × 100) was about 53%. ELISA was performed to detect the positive hybrids against crude schizont extract by which the highest frequency to crude schizont extract was found for the supernatant of the hybrids produced in fusion number 3 (66 out of 315 hybrids). The supernatant of both B5 and F1 hybridoma cells were more positive against domain 2 of the MSP-2 recombinant protein in Western blotting test. Western blotting results also showed that different domains of the MSP-2 recombinant protein and also the MSP-2 of the *P. falciparum* parasite were recognized by some of the positive clones and also immune sera. **Conclusions:** Bringing together all the results of this study it has been confirmed that some clones have recognized both schizont extract and different domains of the MSP-2 recombinant protein and therefore confirming the quality of the MSP-2 domains.

1. Introduction

Several methods are available for the characterization of GST fusion proteins, the choice largely depends on the experimental situation[1]. For example, SDS-PAGE analysis, although frequently used for monitoring results during expression and purification, may not be the method of choice for routine monitoring of samples from high-throughput screening[2]. Functional assays based on the properties of the protein of interest (as compared to the GST tag) are useful, but must be developed for each specific protein. Expression and purification of GST fusion proteins can be monitored by Western blot analysis to enhance the sensitivity of detection system. This requires the availability of a specific antibody against the protein of interest and monoclonal antibodies are widely used for this purpose. As monoclonal antibodies are extremely specific, they can be used in association with Western blotting for confirming the expression of MSP-2 proteins of different domains and also to assess their quality and purity[4]. Hence the production of monoclonal antibodies to different regions of MSP-2 has been performed in order to assess the quality of expressed MSP-2 and also to confirm the immune response against different domains of these proteins. Although the technique of monoclonal antibody is relatively straightforward[1], the successful production of hybrid cell lines producing the

*Corresponding author: Eghbaleh Asadollaphy, Food and drug Department, Food and Drug Deputy, Ilam University of Medical Sciences, Ilam Iran.
E-mail: eghbaleh@yahoo.co.uk

monoclonal antibody of desired specificity requires a lot of time, effective efforts and probably luck. After isolating a positive hybrid line several stages have to be done in order to effectively clone a positive hybridoma cell and prepare it for long term storage, while ensuring that it has kept its potential to produce the antibody of interest.

2. Materials and methods

Mice were immunized with a schizont extract, to stimulate the immune system to make antibodies against different antigens of the late stage parasite, including the production of antibodies against different domains of *Plasmodium falciparum* (*P. falciparum*) MSP-2. B lymphocytes of immunized mice were extracted from the spleen and the fusion was performed using NS-1 myeloma cells and the hybridoma cells were assayed by ELISA either with a schizont extract or different domains of MSP-2[5-7] and/or by IFAT with whole schizont preparation[6]. Fusion of NS-1 and spleen cells was performed.

2.1. Retrieval and cultivation of myeloma (NS-1) cells

One Eppendorf tube of NS-1 cell lines (Professor Marcel Hommel, LSTM kindly provided the NS-1 cells) were taken out from liquid nitrogen and put in a 37 ℃ water bath for 2-3 minutes to thaw the frozen cells quickly. The tube was wiped with a 70% ethanol-wet tissue and was taken to a laminar flow hood. The NS-1 cell lines were transferred to a sterile 15 mL conical tube and washed with 10 mL of Iscove's plus 20% FCS (Foetal Calf Serum) pipetting up and down to mix the cells suspension and centrifuge at 1 000 rpm for 5 minutes to remove the DMSO (dimethylsulfoxide) cryoprotectant. 5 mL complete medium (Iscove's plus 10% FCS, 3 mL L-glutamine, 300 μL gentamicine and 1 mL 8-azaguanine per 100 mL of medium) was added before counting the cells using the trypan blue dye exclusion method and a haemocytometer. Dilution was made to a density of 3×10^5 cells/mL and maintained in 25 cm^2 tissue culture flask (or 15-20 mL in 75 cm^2 flask). Cell cultures were incubated at 37 ℃ incubator with a humid atmosphere of 8% CO_2 in air and they were checked every 3-4 days using an inverted microscope, and if healthy and confluent, were diluted to a count of 2×10^4 cells per mL in complete medium. On the day before fusion the viability of cells was determined, as a high viability (3×10^5/mL) is required.

2.2. Cell fusion

All required media including Iscove's without serum, Iscove's 15% FCS and Iscove's with OPI and HAT were prepared before fusion. The immunized mouse was sacrificed according to the Home Office (UK) guidelines, under terminal anesthesia in the animal section of School of Tropical Medicine, by a qualified and authorized technician. Serum was obtained from the mouse by tail snip bleeding[6-8] or by cardiac exsanguinations[9]. Blood samples were incubated at room temperature for a few hours; the clot removed using sterile tweezers and the serum clarified by centrifugation at 4 ℃ for 10 minutes. The sera were aliquoted in 0.5 mL tubes and stored at -20 ℃. A polystyrene white board has been prepared and the mouse was pinned out on it and sprayed with 70% ethanol. A vertical incision was made in the abdomen and the spleen was removed aseptically using sterile dissecting instruments while care was taken avoiding any injury to intestines or spleen. Three Petri dish containing 8 mL of Iscove's without serum were prepared already in order to wash the spleen. The spleen was placed in the first Petri dish and transferred to a laminar flow hood. The attached tissues and fats were removed under the laminar hood and the spleen was washed firstly by swirling the Petri dish gently and then by transferring the spleen into another Petri dish. Two sterile needles, one bent at an angle of 90 ℃ to hold the spleen down and the other straight to tease the spleen cells out, were used. The cell suspension was then pipetted into a 15 mL sterile conical tube using a 10 mL pipette. The suspension was left under the hood for few minutes to allow large fragments to settle and then the cell suspension was transferred to a new 15 mL sterile conical tube centrifuging at 1 000 rpm for 5 minutes at room temperature. Supernatant was removed and the cells were re-suspended in 2.5 mL of sterile 85% NH_4OH (ammonium chloride), pipetting up and down to mix the suspension and leaving at room temperature for 2 minutes to lyse the red blood cells. 10 mL of Iscove's plus 15% FCS was added and the cells were centrifuged at 1 000 rpm for 5 minutes in order to wash the NH_4OH. The supernatant was removed and 10 mL of Iscove's plus 15% FCS was added and gently mixed. An aliquot of spleen cells (e.g. 0.1 mL) was placed in an Eppendorf tube and an equal volume of 4% aqueous trypan blue was added and cells were counted. It's usually expected that 10^8 spleen cells to be obtained from the spleen of one mouse. An aliquot of NS-1 cells was counted and cells were combined at a ratio of 10:1 of spleen cells to NS-1 cells in a sterile 50 mL conical tube centrifuging at 1 000 rpm for 5 minutes. The supernatant was removed and the cells were re-suspended in 10 mL of Iscove's without serum in order to remove traces of serum. The cells were centrifuged again at 1 000 rpm for 5 minutes at room temperature, removing the supernatant and mixing the cells by tapping the base of the tube. 0.3 mL of warm PEG (Poly Ethylene Glycan) 1450 was added over the period of 60 seconds with constant mixing. In order to increase the number of hybrids PEG/ 10% DMSO solution (Sigma) was used[8]. 15 mL of Iscove's without serum was added slowly over a period of 90 seconds. The cells were incubated at room temperature for 10 minutes followed by centrifugation at 1 000 rpm for 5 minutes. The supernatant was removed and the cells were re-suspended in 30 mL of OPI+HAT medium for 10^8 spleen cells. The cells were distributed into a 96-well flat-bottomed plate, using a 10 mL pipette and adding one drop per well. The plates were labeled and incubated in humidified 8% CO_2 incubator at 37 ℃. The cells were re-fed with one drop of OPI+HAT

medium after 24 hours. After 6 days the plates were checked for hybrid clones and re-feed with OPI+HAT medium. The supernatant of hybridoma positive wells were assayed using ELISA and IFAT after day 10th to 14th of incubation.

2.3. Cloning

The positive hybrids were removed by pipetting the contents of wells up and down to re-suspend the cells and transfer them into a new plate in 3 different dilutions of neat, 1:10 and 1:100. A few days later a second dilution was performed and again ELISA was applied against different dilutions. The positive clones were transferred to a small tissue culture flask and after developing they were assayed against schizont extract and the different MSP-2 domains. The positive clones were expanded to large (75 cm^2) flask and cultured under the same conditions. Checking them using both ELISA and IFAT and the positive cells were frozen as soon as possible.

2.4. Fusion efficiency

The accuracy of fusion protocol in producing monoclonal antibody was evaluated by calculation of fusion efficiency as an indication of produced hybridoma using this procedure. In other words the higher fusion efficency, the more chance of producing monoclonal antibody to the desired antigen. The fusion efficiency, which is the number of hybridoma-containing wells to the total number of wells, was calculated for each fusion separately. It has been demonstrated that the dilution of cells during OPI-HAT selection is an important factor in the growth of single hybrid so that the greater dilution of the cells produces fewer cells with multiple clones and a higher percentage of wells with single clones[10]. More than 25 percent fusion efficiency was obtained when the spleen cells and NS-1 were combined at a ratio of approximately 1:1 instead of 1:10 respectively. Usually from day 10-14 a scoring sheet was used to record the result of each fusion, identifying the hybrids for which the detection test should have been done.

2.5. Synthesis and purification of MSP-2 protein

MSP-2 recombinant protein was synthesized as previously described[5,11-13]. Briefly, the corresponding primers for domains 1, 2, 3 and 4 of the 3D7 isolate were PCR-amplified for directional cloning into BamHI- NotI-linearised pGEX-4-T-1. After cloning into TOPO-TA vector, the DNAs were ligated into the expression vector (pGEX 4T-1) and transformed into DH5 followed by a mini-preparation of plasmid DNA. Transformation of DE3 competent cells was performed through culturing the transformant cells on LB agar plates. Purification of MSP-2 fusion proteins was performed according to the Amersham instruction handbook and aliquots of each domain was kept at -20 ℃ until it was used in further steps. As conserved C and N terminal domains of MSP-2 are reportedly not as immunogenic as the repeated ones and due to the problematic expression of this domain, the analyzed results of domain 1 was not achieved at this stage[5].

2.6. Characterization of clones

In order to characterize each hybrid, several tests were performed at different times during each stage of cloning and sub-cloning. Only the ELISA positive hybrids against schizont extract were tested by IFAT and the positives hybrids were cloned. The clones were grown and tested against crude schizont extract and MSP-2 different domains to find the positive clones. The next step was sub-cloning in the tissue culture flasks.

2.7. Enzyme linked immunosorbent assay (ELISA)

ELISA was carried out as described by Voller[6]. ELISA plates were coated with 100 μL per well of the schizont extract or with MSP-2 recombinant protein of different domains in carbonate coating buffer pH 9.6 and incubated at 4 ℃ overnight. Unbound antigen was removed by three times washing with TST (Tris, sodium chloride and Tween 20) washing buffer, after which plates were pre-blocked with 5 % (w/v) skimmed milk in TST and stored at 4 ℃ overnight. Monoclonal antibodies were diluted at a ratio of 1:100 with blocking buffer, of which 100 μL was added per well comparing to one positive control (100 μL of hyper immune γ-globulin) and one negative control (100 μL of human normal serum) at the same dilution in order to determine the reaction of each single serum to different domains of MSP-2. A horseradish peroxidase (hrp)-conjugate anti human IgG was added at a dilution of 1: 1 000 incubating at room temperature for two hours. The fresh ABTS (2, 2′-azino-di-3-ethylbenzthiaoline sulfonic acid) substrate was added to develop the reaction. After developing, the reaction was stopped by adding 100 μL of H_2SO_4 1M. The OD values were read at 405 nm using a Dynatech Microtiter Plate reader after 60 minutes. Positive samples were defined as those giving a specific OD above the normal range for the control European sera. The normal range was taken as the Mean ± Standard Deviation (SD) of 20 normal human sera with no history of malaria.

2.8. Preparation of IFAT (Indirect Fluorescent Antibody Test) slides

Infected RBCs with 5%-10% parasitaemia with the majority of late stage schizont mostly releasing the merozoites were used for IFAT. The culture was centrifuged at 2 000 rpm for 5 minutes removing the supernatant. The PRBCs were washed twice in PBS. 50 μL of these washed PRBCs was re-suspended in one ml of PBS. 25 μL of this suspension were dispensed on 12-spot teflon-coated multi-spot microscope slides (Hendley-Essex Multiple Microscopic Slide, UK) and left under the laminar hood to dry. Slides were labeled and kept at -20 ℃ in a plastic slide box until use.

2.9. Ethical committee

This study was approved by the ethical committee of Liverpool School of Tropical Medicine and as the Keneba sera collection was donated by Prof. McGregor to the Liverpool School of Tropical Medicine. The source of sera used was this collection.

3. Results

A total number of 7 fusions including 26 plates (2 496 wells) were performed, of which 1 336 hybrids were produced and the overall efficiency was about 53%, the fusion efficiency was acceptable amongst the first 2 fusions performed (plates 1-6, 54%). In the next batch of fusion (fusion number three) a total number of 315 hybrids were produced hence the fusion efficiency was 82%, which is the highest level of efficiency amongst all fusions (Table 1) The lowest level of fusion efficiency was in fusion number 6 (27% efficiency).

From all 7 fusions performed 243 clones were grown in 96 wells plates as they were detected positive against crude schizont extract but only 10 clones were finally detected positive against different domains of the MSP-2 recombinant protein after sub-cloning in tissues culture flasks before freezing. The number of the positive hybrids against schizont extract antigens for the four plates of the fusion number three was as follows: 15 positive hybrids for the plate number one, 17 positive for the plate number two, 21 for the plate number three and 13 for the plate number four. From these positive hybrids to schizont antigens only two clones (C6 and A3) remained positive, hence, they were expanded in tissue culture flask after sub-cloning and have been frozen down, while their supernatant was kept at -20 ℃ for analysis. These two positive clones reacted to domain 2 and 4, after second sub-cloning and cultivation in tissue culture flasks. The results of the final selected clones in different fusions are shown in Table 1.

ELISA was performed to detect the positive hybrids against crude schizont extract by which 53 out of 310 hybrids, produced in fusion 1 and 2, were detected as positive. Only 15 out of 179 hybrids produced in fusion number four detected positive against schizont extract using ELISA. For the fusion number four after sub-cloning to a new plate by limiting dilution the wells A6, A11, B5, G9, F1, F2 and H4 recorded as positive against both schizont extract and MSP-2 domains, hence, they have been grown in small tissue culture flasks. After expanding the hybrids into a 75 cm^2 tissue culture flask, ELISA was performed using their supernatant in order to monitor the reactivity of the positive clones to schizont extract and MSP-2 different domains before freezing the clones. The ELISA results showed that B5 and F1 clone were positive for MSP-2 domains 2 and 4. The highest frequency of the ELISA positive hybrids to crude schizont extract was found for the supernatant of the hybrids produced in fusion number 3 (66 out of 315 hybrids).

In order to confirm the positivity of the selected clones, Western blotting was carried out using different domains of the MSP-2 recombinant protein, GST, crude schizont extract and RBC. The supernatant of both B5 and F1 hybridoma cells were more positive against domain 2 of the MSP-2 recombinant protein in Western blotting test. In addition, the reaction of both the mice immune serum and the mice normal serum have been assessed against the *P. falciparum* infected RBC (late stage), GST and the MSP-2 different domains (Figures 1-5). The immune serum of both mice and human has reacted to the most parts of PRBC (schizont extract) and also to MSP-2 different domains while normal serum had no reaction to them. Western blotting results showed that, different domains of the MSP-2 recombinant protein and the MSP-2 of the *P. falciparum* parasite were recognized by some of the positive clones and immune sera.

Figure 1. C6 positive hybrid against MSP-2 different domains and GST.

Figure 2. Western blot results for mouse immune serum against MSP-2 different domains and RBC infected with *P. falciparum*. From left lanes 1 and 2 are domain 2, lane 3 is domain 3 and lane 4 is domain 4. Lanes 5 and 6 are infected RBC and the last lane is wide range marker. Mice immune serum reacted with different domains of MSP-2 and also with all antigens of crude schizont extract as this serum was immunized with crude schizont extract.

Table 1

The frequency and efficiency of the different fusions.

Fusions	Number of Wells	Number of hybrids	Fusion efficiency	Schizont positive clones	MSP-2 positive clones	Final selected clones
Fusion 1, 2	576	310	54%	53	1	A6
Fusion 3	384	315	82%	66	2	C6, A3
Fusion 4	384	179	48%	15	2	B5, F1
Fusion 5	384	192	50%	60	1	E6
Fusion 6	384	106	27%	16	2	B5, B4
Fusion 7	384	234	60%	33	2	G5, E5
Total	2496	1336	53%	243	10	-

Overall fusion efficiency was 53% with the higher efficiency for fusion 3. 10 clones had reaction against different domains of MSP-2.

Figure 3. Western blot results for mouse normal serum against MSP-2 domains and GST.

Lane 1 is GST, lane 2 is MSP-2 domain 3, lane 3 is domain 2, lane 4 is domain 4 and lane 5 is marker. There is no reaction of mouse normal serum against any antigen indicating that mouse immune serum in previous and next figures was effectively immunized to most antigens of late stage.

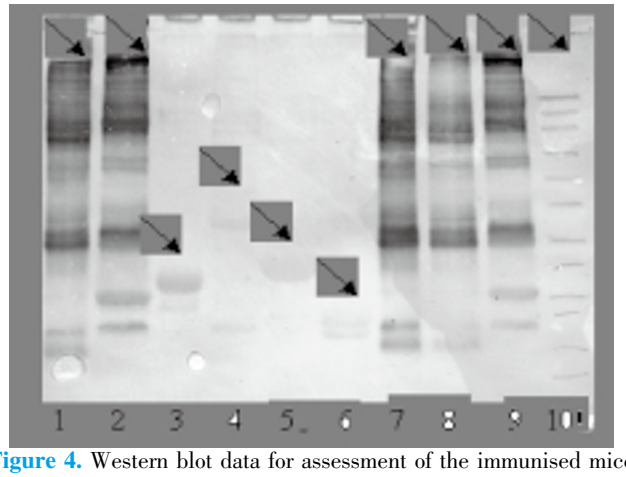

Figure 4. Western blot data for assessment of the immunised mice serum to schizont extract and MSP-2 different domains.

Lane1 and 2 are schizont extract Triton soluble and insoluble, lane 3 is MSP-2 domain1, lane 4 is MSP-2 domain3, lane 5 is MSP-2 domain 2, lane 6 is MSP-2 domain 4, lanes 7-9 are schizont extract and lane 10 is marker. Mice immune serum reacted with all MSP-2 domains and Triton soluble and insoluble extracts indicating that mice were immunized properly.

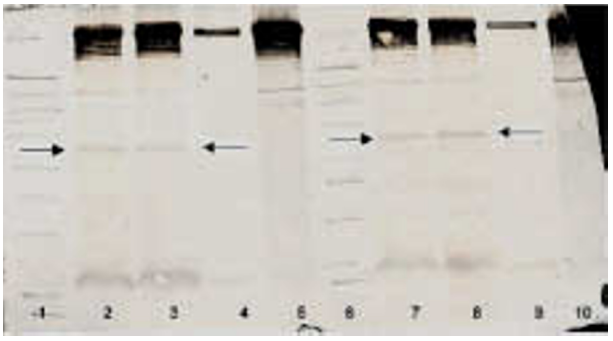

Figure 5. Western blot image of monoclonal antibody against Schizont extract antigen compared to RBC.

From left, the Lane 1 and 6 is marker, lanes 2, 3, 7 and 8 are late stage *P. falciparum* A4 isolate Triton soluble and insoluble, lanes 4 and 9 is RBC and lane 5 and 10 are PRBC. Monoclonal antibody has recognized MSP-2 intact molecule at about 50 kDa.

4. Discussion

The high reactivity of immune serum to schizont extract antigens as a control for the monoclonal antibody was expected as some researchers have already reported it[11]. MSP-2 has been detected by monoclonal antibody C6 at approximately 48 kDa on Western blo. Also this monoclonal antibody has detected the domains 2 and 4 of MSP-2 on Western blot strongly. These findings confirmed the quality of the MSP-2 recombinant proteins synthesized in the current study, which was important for my purpose in order to assess the age, related immune responses to MSP-2 different domains. The crude schizont extract was used to immunize the mice[14,15] and produce monoclonal antibody against different domains of MSP-2. Western blot was performed using different domains of MSP-2 and crude schizont extract in order to identify each monoclonal antibody. Once the specific monoclonal antibody was recognized, the quality of recombinant proteins were analysed in comparison to other evidences. As mice immune sera and monoclonal antibody C6 both recognized the full-length intact MSP-2 protein and also the MSP-2 different domains have been recognized by monoclonal antibody

and by malaria immune sera from Keneba collection, these data together with the data of analyses sequencing of MSP-2 different domains all confirmed the quality of MSP-2 recombinant proteins[15,16]. Western blotting test was more accurate than ELISA in detection of positive clones against different domains of the MSP-2 recombinant protein. A total number of 243 out of 1 336 (22%) of the grown hybrids in all 7 fusions were detected positive against schizont extract using ELISA. The results of the IFAT showed less frequency of positive hybrids against schizont extract antigens but mostly confirmed the ELISA positive hybrids e.g. 36 out of 53 of the schizont positive hybrids by ELISA were confirmed positive by IFAT. The supernatant of some wells without hybrids were tested against schizont extract as negative control in order to compare with ELISA positive hybrids and the results showed that there was no reaction against schizont extract and MSP-2 domains (results not shown).

Bringing together all the results of this study it has been confirmed that some clones have recognized both schizont extract and different domains of the MSP-2 recombinant protein and therefore confirming the quality of the synthesized recombinant MSP-2 domains.

Conflict of interest statement

We declare that we have no conflict of interest.

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