Project no. LSHP-CT-2005-012199

MALINV

Differential Expression of Malaria Parasite Invasion-Associated Proteins in the Sporozoite. Novel Vaccination Strategy

SPECIFIC TARGETED RESEARCH PROJECT

FP6-2003-LIFESCIHEALTH-3

Final activity report

Period covered: from 01/06/2005 to 30/11/2007

Start date of project: 01/06/2005 Duration: 30 months

Project coordinator: Rénia
Project coordinator organization: INSERM Revision [draft 1]

SUMMARY

Differential Expression of Malaria Parasite Invasion-Associated Proteins in the Sporozoite. Novel Vaccination Strategy
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This project was designed to establish whether a newly described set of host cell invasion-associated proteins can serve as novel targets of inducible protective immune responses against pre-erythrocytic *Plasmodium* parasites. In the last few years it was discovered that *Plasmodium* sporozoites express protein members of the EBL and the RH family thought till then to be exclusive to the erythrocytic stages of the parasite. Additional work has shown that sporozoite penetration and/or development can be inhibited by antibodies specific to some of these proteins. This confirms that members of the EBL and RH families may play a functional role during the pre-erythrocytic stages of the malaria infection. We have made use of different model systems, together with the human parasite to study the expression of these sporozoite invasion-associated (SIA) genes and investigate their role in host immunity. The different skills of the groups (whole animal studies through to molecular biology and bio-informatics) has allowed the production of reagents, expression of proteins and the development of laboratory techniques, which are generally applicable and available to all partners. This co-operative work has determined RNA transcription profiles and protein location in sporozoite and/or liver stage of many of these SIA genes and approached their role in sporozoite invasion and development. This information was used to perform preliminary investigations on their potential as a vaccine.

I Project execution

1. Project objectives

Efforts to develop vaccines against malaria still represent a substantial focus of current research activities. Antigens present in the erythrocytic pathogenic stages of the infection account for the majority of targets investigated for potential vaccines. Experimental vaccines targeting the pre-erythrocytic stages [the sporozoite injected by the mosquito, and the hepatic stage parasite) have encompassed a lesser diversity of parasite antigens]. This is the case despite the fact that experimentally induced sterile long-lasting immunity in humans has so far only been achieved through exposure to irradiated sporozoites. The acquisition and maintenance of this immunity depends on the use of invasive sporozoites and on the presence of developmentally arrested hepatic stage parasites.

The full repertoire of pre-erythrocytic antigens that underlie the sterile protection induced by irradiated sporozoites is not actually known. The three pre-erythrocytic antigens investigated to date (circumsporozoite protein, TRAP and LSA-3) may not be responsible for induction of optimal protective responses. This would account for the difficulties encountered in reproducing this sterile long-lasting immunity by current subunit vaccines. It would clearly be desirable to investigate other pre-erythrocytic antigens. Such efforts to expand targets of pre-erythrocytic stage immunity are justified because the infection is at its most vulnerable between the time sporozoites are injected and when hepatic merozoites emerge into the blood stream. During this period, two distinct stages (sporozoite and infected hepatocyte), whose numbers rarely exceed 100 in the human body, can be attacked. The protective responses could comprise not only humoral but also cellular effector mechanisms, since this is the only time during the infection, when the parasite can be found for 5 to 14 days inside a MHC-expressing cell. Even if suboptimal, the appropriate immune response could fully abrogate the infection, thus preventing both pathology and transmission to another host.

The global objective of this proposal was to establish whether a newly described set of host cell invasion-associated proteins can serve as novel targets of inducible protective immune responses against pre-erythrocytic *Plasmodium* parasites.

The measurable objectives of this project were:

1) To monitor expression of *ebl* and *rh* genes in the pre-erythrocytic stages of *P. falciparum*

2) To characterize of the role of the *P. falciparum* sporozoite-expressed EBL and RH proteins in human hepatocyte invasion using transgenic parasites lines with disrupted genes and in invasion inhibition assays using antibodies to selected EBL.

3) To identify of the orthologous sporozoite-expressed genes in two rodent malaria parasites

Objectives 1 to 3 were designed to demonstrate that invasion associated proteins expressed in the sporozoites of *Plasmodium* parasites have an important functional role in the invasion and development of sporozoites

4) To perform vaccination studies with selected genes in rodents

2. Participant list

List of Participants

Partic. Role*	Partic. no.	Participant name	Participant short name	Institution, Country	Date enter project **	Date exit project**
CO	1A	Rénia	Laurent	INSERM, France	Month 1	Month 30
CR	1B	Mazier	Dominique	INSERM, France	Month 1	Month 30
CR	2	Sauerwein	Robert	Univ. Nijmegen, Holland	Month 1	Month 30
CR	3	Mota	Maria	IMM, Portugal	Month 1	Month 30
CR	4	Cowman	Alan	WEHI, Australia	Month 1	Month 30

*CO = Coordinator; CR = Contractor
** Normally insert “month 1 (start of project)” and “month n (end of project)”

3. Achievements

Objective 1

1) Monitor expression of SIA (*ebl* and *rh)* genes in the pre-erythrocytic stages of *P. falciparum*

mRNA expression
Two different approaches were used. First specific primers for the different known *rh* and *ebl* genes were designed and validated using parasite DNA from the NF54 strain of *P. falciparum*. Primers we were tested them by RT-PCR with RNA purified from midguts and freshly dissected salivary glands sporozoites maintained at 4°C. Second, although not originally proposed in our proposal, we also performed a microarray analysis on salivary gland NF54 sporozoites freshly dissected from salivary glands and maintained at 4C, and on NF54 sporozoites incubated at 37°C and incubated with human hepatocytes. The latter condition was shown recently by partner 1b to induce induction of sporozoite proteins necessary for *P. falciparum* sporozoites invasion and development in human hepatocytes (Siau *et al*., submitted).
The results are summarized in the Table I. We confirmed that EBA-175, MAEBL transcripts can be detected on salivary glands of NF54 sporozoites. Rh1 transcripts were detected by microarrays but not by RT-PCR on salivary gland sporozoites. However, it has to be stressed that the set of specific primers that we used were not very sensitive in a control assay using *P. falciparum* genomic DNA. This may explain our inability to detect Rh1 in salivary gland sporozoites if the amount of transcripts is low. When our data were compared with micro-

array data obtained by Le Roch *et al* (2003, Science) using a mixture of midgut and salivary gland sporozoites of the 3D7 strain of *P. falciparum* (a clone of the NF54 strain), a good correlation was observed. The major difference was that they observed no Rh1 and EBA-175 expression. This discrepancy might due to strain differences. When our data were compared with micro-array obtained by Le Roch *et al* (2003, Science) using a mixture of midgut and salivary gland sporozoites of the 3D7 strain of *P. falciparum* (a clone of the NF54 strain), a good correlation was observed. The major difference was that they observed no Rh1 and EBA-175 expression. This discrepancy might due to strain differences or to the limit of sensibility of the different techniques.

Table 1

		MALINV					Litterature			
Gene reference number	Gene name	Microarray (partner 1B)		RT-PCR (partner 1A)		IFAT (partner 1A)	Affymetrix microarray (Le Roch et al, 2003 Science)	Mass spetrometry (Florens et al 2002, Nature)	IFAT	
		Sg SPZ	Sg Spz at 37°C + hepatocytes	Mg SPZ	Sg SPZ	Sg SPZ	Sg+ Mg SPZ	Sg SPZ	Sg SPZ	References
PFD0110w	RH1	yes	yes**	yes	no#	yes (r)	no	yes*		
PF13_0198	RH2a	no	no	no	no	yes (h)	no	yes		
MAL13P1.17	RH2b	no	no	no	no		no	yes*		
PFL2520w	RH3	no	no	no	no	yes (r)	no	yes		
PFD1150c	RH4	no	no	not done	not done	no (r)	no	yes		
PFD1145c	RH5	no	no	not done	not done	not done	no	yes		
PF07_0128	EBA175	no	yes*	not done	yes	yes (h+r)	no	no	yes	Gruner et al, 2001, J Infect Dis
PFA0125c	EBA181	no	no	yes	no	yes (h+r)	yes	yes		
PFD1155w	EBA165	no	no	not done	not done	yes (h)	no	no		
MAL13P1.60	EBA140	no	no	yes	no	yes (h+r)	yes	no		
PFD1145c	EBL1	no	no	no	no	no (r)	no	yes*		
PF11_0486	MAEBL	yes	yesp**	yes	yes	yes (r)	yes	yes	yes	Preiser et al, 2004, Infect Immun; Ghai etal, 2002, Mol Biochem Parasitol

* just above background
** increased expression
#: poor sensitivity of different primer sets
Sg SPZ: salivary glands spororozoites
Mg SPZ : midgut sporozoites
h: immunopurified human antibodies from sera from immune individuals
r rabbit polyclonal serum raised against recombinant protein

Protein expressions

We made use of reagents such as recombinant proteins and rabbit and mouse polyclonal monoclonal antibodies to characterize SIA proteins. These reagents were principally generated and validated by partner 4. We also acquired other reagents through collaboration external to the consortium. *P. falciparum* or *P. yoelii* DNA plasmids encoding SIA proteins or *E. coli* recombinant SIA proteins have been produced and were used to immunized mice or rabbits. Serum containing antibodies were collected and used for further studies. We also purified human antibodies from hyperimmune serums using recombinant proteins provided by partner 4.

As shown in Table 1, rabbit antibodies specific to Rh1, Rh3, MAEBL (Fig. 2), EBA175, EBA140, EBA181 and Rh3 but not specific to Rh2a, Rh2b, RH4, EBL-1, and EBA-165, showed reactivity on methanol-fixed dried *P. falciparum* sporozoites. When protein-purified antibodies were tested, we confirmed some and observed additional reactivity with *P. falciparum* sporozoites. Human antibodies purified on Rh2a, Rh2b, EBA140, and EBA165 stained positively sporozoites. However, we cannot exclude that cross-reactivity to other antigens might have occur since the different Rh protein have high degree of homologies between them and since antibodies were purified on whole recombinant proteins. This is also true for the EBL proteins. We have planned to purify new batches of human antibodies for hyperimmune serums and perform western blot experiments to test the specificity of the different purified human antibody preparation.

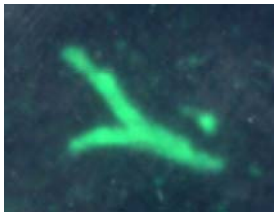

Figure 1. Immunofluorescence microscopy of *P. falciparum* salivary gland sporozoites stained with an anti rabbit anti-PfMAEBL antibodies (green).

In conclusion, expression during pre-erythrocytic stages of only a limited number of SIA genes could be demonstrated with certainty. Our results show that using only one technique to study gene expression might lead to wrong conclusions.

It is interesting to note that the different Rh and EBL molecules have been shown to be involved in different pathways of invasion of red blood cells by *P. falciparum* merozoites (Cowman and Crabb, 2006, Cell). We propose that these proteins might also have the same role in sporozoite invasion. This clearly deserves further studies.

This objective involved the coordinator and partner 1B, 2 and 4 and the subcontractor to the coordinator.

2) Characterize of the role of the *P. falciparum* sporozoite-expressed EBL and RH proteins in human hepatocyte invasion in invasion inhibition assays and using transgenic parasites lines with disrupted genes

Knock-out parasites. Constructs for targeting whole or fragments of the RH and EBL proteins were produced. They were transfected in the gametocyte producing 3D7 cloned line (NF54 strain). Specific disruptants (Rh1, Rh4, EBA-175, MAEBL, Rh2a, Rh2b, and Rh3) have been generated. We decided to study further 2 of these (EBA-175 and Rh3). Although MAEBL was an interesting candidate, previous experiments in *P. berghei* showed that MAEBL KO (Kariu *et al*, 2002, J Exp Med) are unable to develop further than the oocyst stage, thus preventing the production of sporozoites for in vitro studies.
Partner 2 conducted experiments with these parasites. The Rh3 KO clone was successfully grown *in vitro*, and gametocyte feeding experiments performed on 4 separate occasions gave the following results show a reduction in oocysts numbers. Further experiments are planned to measure sporozoite production and infectivity to human hepatocytes. For the EBA-175 KO clone, partner 2 did not succeed to grow parasites from a first shipment, thus a second shipment has been programmed to perform this experiment.

Antibody-mediated inhibition assay. We assessed the ability of rabbit to antibody against different recombinant EBL (EBL1, EBL140, PfMAEBL) and Rh (Rh2a+Rh2b, and Rh3) proteins to inhibit sporozoite invasion and development in primary culture of human hepatocytes. As shown in Figure 2, a significant inhibition was obtained only with rabbit serum to Pf MAEL (encoding the NM2 region). Experiments are planned to assess the inhibitory capacity of human antibodies from other batch of hyperimmune serum purified on already tested or to be tested recombinant proteins.

In conclusion, we have confirmed the role of MAEBL in invasion and development of sporozoite in human hepatocytes. And our data show that this molecule can consider as a candidate vaccine since antibodies to its region NM2 have potent inhibitory activity. The role of the other proteins (Rh1, RH3, and EBA140) remains to be determined.

This objective involved the coordinator and partner 1B, 2 and 4 and the subcontractor to the coordinator.

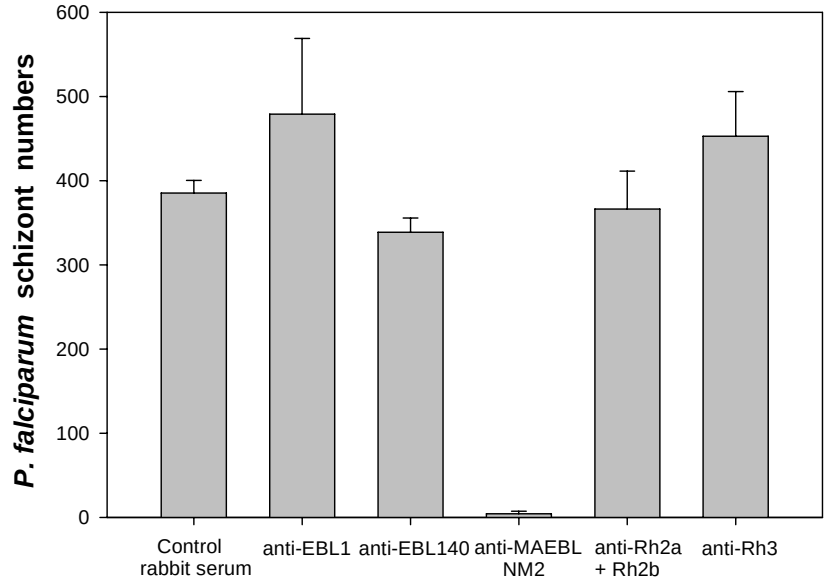

Figure 2. Inhibition assay of invasion and development of sporozoites in human hepatocytes by rabbit polyclonal antibodies to different EBL and Rh proteins. Antibodies to MAEL were produced against the NM2 fragment of the molecule. Human hepatocytes were incubated with *P. falciparum* NF54 sporozoites in the presence of serum from rabbit immunized with the different recombinant protein. The results are expressed as number of liver schizonts in triplicate wells (mean ± SD).

3) To identify of the orthologous sporozoite-expressed genes in two rodent malaria parasites

PyMAEBL and PbMAEBL were the first homologue of the PfEBL protein family to be identified (Kappe et al. 1998, PNAS), they were further shown to be expressed during the pre-erythrocytic stage (Preiser *et al*, 2004, Infect Immun). We have identified another orthologue of PfEBL, PyEBL-1, in the genome of *P. yoelii*. A similar bioinformatic mining search was performed for the *P. berghei* using the recently published genome (Hall *et al*, 2005, Science). Only one orthologue of the PfEBL genes, PbEBL-1, was identified. When we analyzed the pattern of expression of *Pyebl1* By RT-PCR using specific primers, EBL1 transcripts were detected only in very late liver stage parasites and in asexual blood stage parasites. Through collaboration with an external member of this consortium, Osamu Kaneko (Ehime University, Ehime, Japan), we gained access to monoclonal and polyclonal antibodies against the PyEBL1 protein. Using these reagents, we showed that the EBL1 was expressed only in the late liver schizonts (Figure 2) by not in sporozoites or early liver stage parasites, paralleling the *Pyebl1* transcript expression

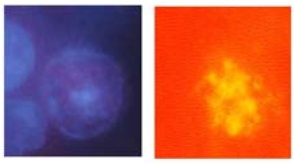

Figure 2. Immunofluorescence microscopy of methanol fixed late *P. yoelii* liver stage parasites with mouse polyclonal anti-PyEBL1 antibodies (yellow, right panel); nuclear staining with DAPI (left panel).

Next partner 3 attempted to disrupt *Pbebl1* in *P. berghei* parasites. No KO parasites could be generated. Similar results were obtained with *P. yoelii* (in collaboration with Osamu Kaneko, Nagasaki University, Japan) indicating that this molecule is essential for blood stage parasite development. We did not attempt to disrupt MAEBL in rodent malaria parasite since previous work showed that in *P. berghei* PbMAEBL KO (Kariu *et al*, 2002, J Exp Med) are unable to develop further than the oocyst stage.

In the contrary to the *ebl* genes, multiple homologues of the *rh* genes exist in the genome of *P. yoelii* (Py235 gene family) and *P. berghei* (Pb235 gene family) (Preiser *et al*, 2001, Science; Hall *et al*, 2005, Science). A restricted repertoire of *py235* was shown by us previously to be expressed during the pre-erythrocytic stage of *P. yoelii* and to be the target of inhibitory antibodies (Preiser *et al*, 2001, Science). There are around 10-15 *p235* genes described in the genome of rodent malaria parasites (Hall *et al*, 2005, Science). However, since these molecules are highly homologous the exact number of these genes. We have started to define the exact repertoire of *py235* genes and have already evidence that the number of *py235* genes is around 20 and not 15. This will help us to design new tools to identify precisely which *py235* member is expressed by the sporozoites.

In conclusion, we confirmed that rodent malaria parasites possess only a limited number of py235 and EBL proteins. For the EBL family, MAEBL is the only protein expressed in sporozoites and also in liver stage parasites.

This objective involved the coordinator and partner 3 and 4.

4) <u>To perform vaccination studies with selected genes in rodents</u>

As mentioned above, MAEBL is expressed by sporozoites and liver stage parasites and that antibody to recombinant MAEBL proteins can inhibit *P. yoelii* sporozoite development in hepatocytes (Preiser *et al*, 2004, Infect Immun). Moreover, we reported above that antibodies to PfMAEBL inhibited strongly *P. falciparum* sporozoite invasion and development in hepatocytes. Thus we produced different recombinant proteins. Since the MAEBL protein is a large protein, we attempted to produce different domains of the PyMAEBL protein (Kappe e*t al*, 1998, PNAS). We only succeeded to produce the NM2 domain as a recombinant protein of *E. coli*. All our attempts to produce other recombinant domains of PyMAEBL were unsuccessful.

In one experiment, we used the NM2 protein formulations in protection experiments. Mice were immunized with 4 injections of NM2 proteins together with Freund's adjuvant subcutaneously. Immunized mice developed low level antibodies against the parasites. Mice

were further challenged with sporozoites. Immunized were not protected since they developed parasitemia like control mice. These results indicate that better immunogens are needed.

We have also identified and characterized H2d-restricted T cell epitopes in the PyMAEBL proteins in order to analyze T cell response in mice immunized with the different MAEBL constructs. To validate the use of the T cell epitopes, we tested them using spleen cells from mice immunized with whole sporozoites (irradiated sporozoites or sporozoites under chloroquine prophylaxis). These protocols have been shown to induce full sterile protection (Nussenzweig *et al*, 1967, Nature; Belnoue *et al*, 2004, J immunol). We could show that these immunization protocols induce PyMAEBL specific T cells.

In a recent experiment, using transgenic parasites expressing circumsporozoite protein from different malaria species, we demonstrated this major surface sporozoite antigen is not involved in protection induced by irradiated sporozoites or sporozoite under chloroquine prophylaxis (Gruner *et al*, 2007, PLoS One). Using spleen cells from protected and not protected mice, we showed that PyMAEBL epitopes are recognized solely by T cells from protected mice suggesting that PyMAEBL specific-T cells may be responsible for protection. This experiment is being repeated. If confirmed, this would indicate that MAEBL constructs able to induce T cell responses should be designed.

One recombinant protein coding for a fragment of a Py235 expressed in sporozoite was produced (the original plasmid was obtained through collaboration with Peter Preiser, Nanyang Technological University, Singapore) and was used to immunize mice. Immunized mice developed antibodies against the parasites. However, after challenge with sporozoites, no protection was observed.

In conclusion, different vaccine formulations have been developed and tested. None of the formulation so far has induced good level of immune responses and protection. In order to study the immune responses induced by

We have initiated the production of new vaccine formulations (DNA vaccines and recombinant proteins) for further protection experiments.

This objective involved the coordinator and the subcontractor to the coordinator.

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II. Final plan for dissemination and use

The final plan for dissemination of scientific results of this proposal has and will follow standard practice. We have already published 3 articles and submitted 1 in peer-reviewed journals. Members of the consortium have presented some of their results at 2 conferences and have given or been involved in 5 seminars. Particular attention will be paid to protect intellectual rights via patents, prior to any public dissemination.

The creation of the MALINV consortium has been instrumental in the study of two families of malaria proteins. At present, no exploitable results can be reported as a result of the MALINV project. However, we have clearly obtained evidence that some members of the *ebl* family, and in particular MAEBL, and the *rh* genes family may be useful as vaccine targets for immunization against malaria.

Published results

Gruner AC, Mauduit M, Tewari R, Romero JF, Depinay N, Kayibanda M, Lallemand E, Chavatte JM, Crisanti A, Sinnis P, Mazier D, Corradin G, Snounou G and Renia L (2007) Sterile Protection against Malaria Is Independent of Immune Responses to the Circumsporozoite Protein. PLoS ONE 2: e1371-1376.

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