

A rhoptry antigen of *Plasmodium falciparum* is protective in *Saimiri* monkeys

R. G. RIDLEY¹, B. TAKACS², H. ETLINGER² and J. G. SCAIFE^{1*}

¹Department of Molecular Biology, University of Edinburgh, King's Buildings, Mayfield Road, Edinburgh EH9 3JR, Scotland

²Central Research Units, F Hoffman-La Roche Limited, CH-4002 Basel, Switzerland

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SUMMARY

A non-polymorphic antigen associated with the rhoptry organelles of *Plasmodium falciparum* has been purified by immuno-affinity chromatography. The antigen, RAP-1 (rhoptry associated protein-1), which is defined by monoclonal antibodies which inhibit parasite growth *in vitro*, is a multi-component antigen consisting of four major proteins of 80, 65, 42 and 40 kDa and two minor proteins of 77 and 70 kDa. These proteins were electro-eluted from preparative sodium dodecyl sulphate polyacrylamide gels and protected *Saimiri sciureus* monkeys from a lethal blood-stage infection of *P. falciparum* malaria. Sera from the protected animals recognized only proteins of the RAP-1 antigen when used to probe a Western blot of total parasite protein extract, confirming that RAP-1 is responsible for eliciting the protective immune response.

Key words: *Plasmodium falciparum*, *Saimiri sciureus* monkeys, rhoptry antigen, protection.

INTRODUCTION

The ability of some species of new world monkeys to support the erythrocytic stage of malarial infection has allowed vaccination studies to be undertaken in these primates and early experiments showed that vaccination with merozoites or schizonts from *Plasmodium falciparum* protected against a lethal blood-stage infection (Mitchell *et al.* 1977; Siddiqui, 1977). Many merozoite and schizont antigens have since been identified and their genes cloned and sequenced. However, only two well-defined antigens have so far been purified to homogeneity and successfully used to protect monkeys from infection; the major merozoite surface antigens derived from the protein PMMSA (Hall *et al.* 1984; Perrin *et al.* 1985a; Siddiqui *et al.* 1987) and a 41 kDa rhoptry antigen (Perrin *et al.* 1985b). In the case of one antigen, RESA, recombinant protein derived from a repeat region of the gene has offered some protection (Collins *et al.* 1986). Synthetic peptides have been used in some vaccination experiments. A synthetic peptide corresponding to the N-terminus of the PMMSA derived 83 kDa protein gave good protection (Cheung *et al.* 1986) and a mixture of three peptides corresponding to the N-termini of the PMMSA derived 83 kDa protein and two proteins of 55 kDa and 35 kDa, which have not been well characterized, gave almost full protection (Pattaroyo *et al.* 1987). These peptides also formed part of a complex of peptides from a variety of malarial antigens which showed some promise in a human vaccine trial (Pattaroyo *et al.* 1988).

To our knowledge, only PMMSA has been

repeatedly shown to offer a high degree of protection in this type of experiment (Hall *et al.* 1984; Perrin *et al.* 1985a; Siddiqui *et al.* 1987; our unpublished results). Because of the small number of candidate antigens for vaccine development which have been rigorously tested in model animal systems, we have sought other antigens which may have protective properties.

One source of such antigens may be the rhoptry organelles, which are located at the apex of the merozoite and are intimately involved in erythrocyte invasion (Aikawa, 1971; Bannister *et al.* 1986). In fact, early studies on the rodent malarial parasite *Plasmodium yoelii* revealed a rhoptry antigen which could partially protect mice against malaria (Holder & Freeman, 1981). We have previously reported two monoclonal antibodies 2.13 and 7.12, which define an antigen, RAP-1 (rhoptry associated protein-1) associated with the rhoptries (Hall *et al.* 1983). They both inhibit parasite growth *in vitro* (I. A. Hope and J. G. Scaife, unpublished observations) suggesting that their target antigen could induce protective immunity in an anti-malarial vaccine. The RAP-1 antigen, which also offers the advantage of being non-polymorphic (Clark *et al.* 1987) has recently been characterized and its gene cloned (Ridley *et al.* 1990). We report here the purification of this antigen and assess its ability to protect *Saimiri sciureus* monkeys from malarial infection.

MATERIALS AND METHODS

Standard techniques

Indirect immunofluorescence assays were performed as described previously (Hall *et al.* 1983). Sodium

* Reprint requests to Professor J. G. Scaife.

dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), using the method of Laemmli (1970), together with Western blotting analysis have also been previously described (Takacs & Staehli, 1987). *P. falciparum* *in vitro* culture was maintained according to the method of Trager & Jensen (1976), later modified by Zolg *et al.* (1982).

Affinity-purification of RAP-1 antigen

Purified mAb 2.13 (Hall *et al.* 1983) was coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia Ltd, Milton Keynes, UK) as recommended by the manufacturer. Cell pellets of bulk cultures of a *P. falciparum* isolate, K1 from Thailand (Thaithong & Beale, 1981) were harvested as previously described (Goman *et al.* 1982) and stored at -70°C until required. They were then extracted at 4°C for 1 h with an extraction buffer (2% (w/v) sodium deoxycholate, 1% (w/v) Nonidet P40, 50 mM Tris-HCl (pH 8.0), 5 mM ethylenediaminetetra-acetic acid (EDTA), 5 mM 1,2-di(2-aminoethoxy)ethane-*N,N,N',N'*-tetra-acetic acid (EGTA)) containing 5 mM-iodoacetamide, 0.2 mM phenylmethylsulphonyl fluoride (PMSF) and 10 $\mu\text{g}/\text{ml}$ each of the protease inhibitors pepstatin, chymostatin, antipain, leupeptin and soybean trypsin inhibitor (all from Sigma Chemical Co. Ltd, Poole, UK). Fresh PMSF was added to 0.2 mM and the extract was centrifuged at 100000 *g* for 3 h at 4°C . The supernatant fraction had fresh PMSF added to 0.2 mM and was passed through a 10 ml column of Sepharose 4B-bound mAb 2.13 equilibrated with the extraction buffer. The column was then washed extensively with wash buffer (0.5% (w/v) sodium deoxycholate, 1% (w/v) Nonidet P40, 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 5 mM EGTA) followed by at least 5 column volumes of the wash buffer containing 0.15 M NaCl and was finally re-equilibrated in salt-free wash buffer before eluting with 50 mM diethylamine in wash buffer at pH 11.5. The protein solution was neutralized with solid glycine and the cocktail of protease inhibitors at 10 $\mu\text{g}/\text{ml}$ was added prior to storage at -70°C .

Monkey immunization experiment

RAP-1 was purified from the K1 strain of *P. falciparum* by affinity chromatography followed by electro-elution of the protein bands from 15% preparative SDS-polyacrylamide gels. Four *Saimiri sciureus* monkeys each received, subcutaneously, 25 μg protein in complete Freund's adjuvant on day 1 and 25 μg protein in incomplete Freund's adjuvant on days 24 and 48. Control monkeys received the adjuvant in phosphate-buffered saline on the same days. Sera were taken on day 56. On day 60, monkeys received, intravenously, 3.5×10^7 *P. falciparum* (Uganda Palo Alto strain) infected erythro-

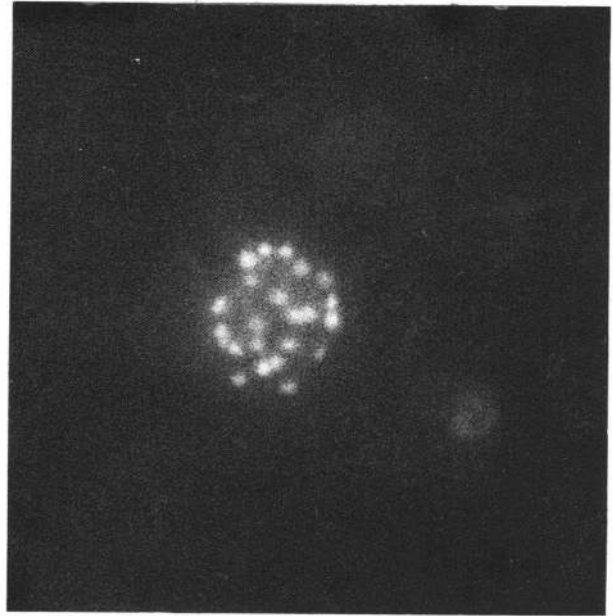


Fig. 1. Monoclonal antibody 2.13 punctate immunofluorescence pattern obtained with the schizont stage of a *Plasmodium falciparum*-infected erythrocyte.

cytes from a donor monkey. Parasitaemias were determined by light microscopy of blood smears stained with Giemsa.

RESULTS

Localization of the antigen RAP-1 to the rhoptry organelles of the merozoite is shown in Fig. 1. The punctate fluorescence pattern produced by mAb 2.13 defines rhoptry organelles of parasites at the late schizont stage of the erythrocytic cycle. Each parasite within the schizont contains a pair of rhoptry organelles.

The RAP-1 antigen was purified from extracts of cultured parasites by affinity chromatography using mAb 2.13. It consists of four major bands (p80, p65, p42 and p40) with $M_r = 80, 65, 42$ and 40 kDa and two minor bands, $M_r = 77$ and 70 kDa which varied in intensity depending on the preparation (Fig. 2). The high yield of p80 with respect to p70 was heavily dependent on the addition of protease inhibitors during extraction, purification and storage of the antigen, suggesting that p70 is a degradation product of p80.

The ability of human immune sera to recognize RAP-1 was tested (Fig. 3). The components p80, p77, p70, p65 and p40 were all well recognized by the sera; however, the signal for p42, though present, was very weak and is not visible in the figure. Interestingly, p80 showed much stronger immune recognition than any of the other bands, suggesting that it contains an immunodominant epitope lacking in the other RAP-1 components.

The protein bands observed by Coomassie staining in Fig. 2 were further purified by electro-elution

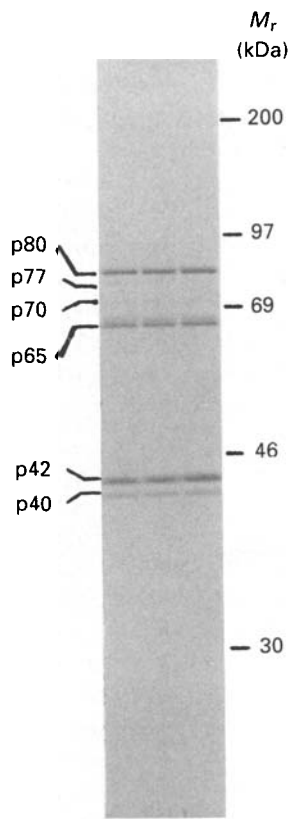


Fig. 2. Immuno-affinity purified rhoptry antigen RAP-1 was subjected to SDS-PAGE in the presence of 2-mercaptoethanol and stained with Coomassie Blue. The major fractions eluted from an immuno-affinity column are shown and the sizes of the major bands are indicated.

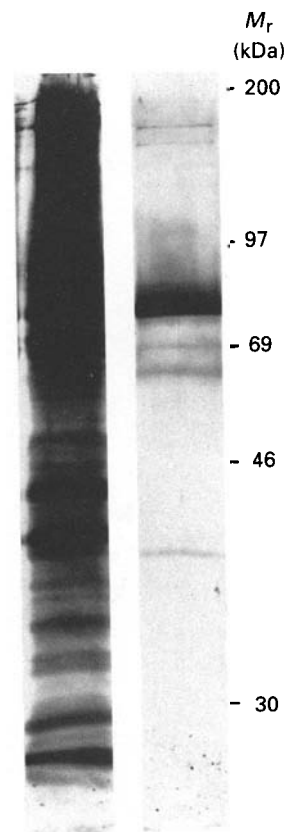


Fig. 3. Recognition of RAP-1 by human immune sera. Ten samples of Nigerian human immune sera were pooled and used to probe a Western blot of total *Plasmodium falciparum* protein (lane 1) and affinity-purified RAP-1 (lane 2).

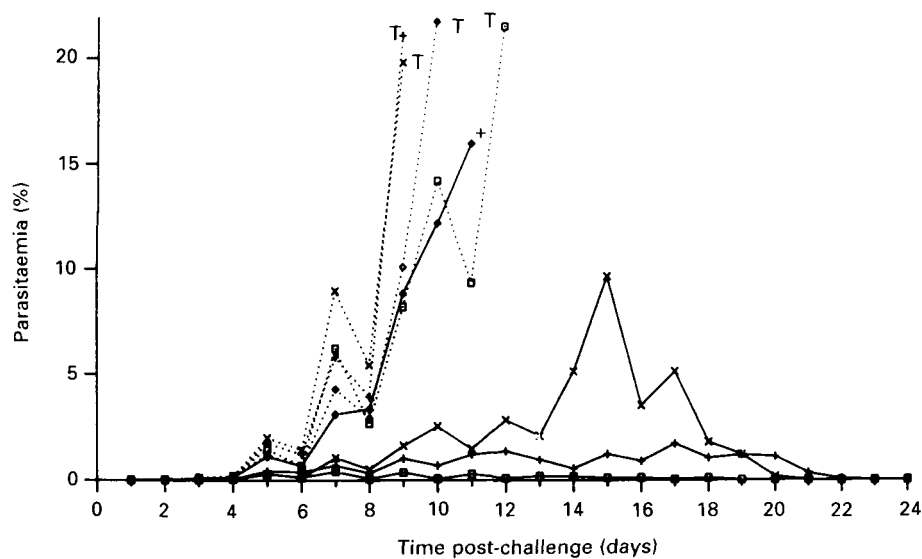


Fig. 4. Effect of inoculation with RAP-1 on parasitaemia in *Saimiri* monkeys. Control monkeys are represented by dotted lines and experimental animals by full lines. Positions are marked to indicate when drug treatment was administered (T) or death occurred (+).

from preparative gels and used to inoculate *Saimiri* monkeys. Four animals were treated with the antigen while four control animals were injected with saline (Fig. 4). All eight animals were challenged with blood-stage parasites (Fig. 4). One of the four

experimental monkeys died prematurely with a ruptured spleen at a parasitaemia significantly lower than is normally tolerated in these animals. This monkey produced the lowest titre of antibody by indirect immunofluorescence. The other three re-

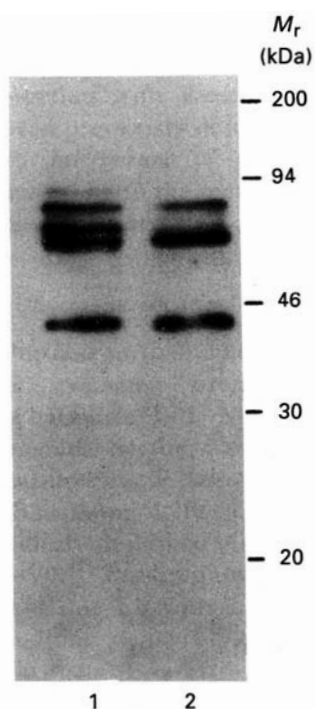


Fig. 5. Recognition of RAP-1 by monkey antisera. Sera taken from the experimental monkeys prior to parasite challenge were pooled and used to probe a Western blot of total *Plasmodium falciparum* protein (lane 1) and affinity-purified RAP-1 (lane 2).

covered completely after developing relatively low parasitaemias. By contrast, all of the control animals showed a rapid rise in parasitaemia to 20% by days 8–12 and were cured by chloroquine and Fansidar treatment. No correlation was observed between antibody titre and peak parasitaemia in the three protected animals.

Sera taken from the animals after immunization and four days prior to parasite challenge, were assayed for reactivity against RAP-1 and for reactivity against total parasite protein (Fig. 5). The sera, which stained rhoptries in indirect immunofluorescence (not shown), recognized purified RAP-1 in Western blots (lane 2) and detected only components of the RAP-1 antigen when used to probe a Western blot of total malarial protein extract (lane 1). The sera of the four control animals gave negative results in these tests.

The protein bands in the total protein extract recognized by the monkey sera in lane 1 correspond to the bands at 80, 70, 65 and 42 kDa observed in Fig. 2. We are satisfied that the lower band observed in Fig. 5 corresponds to p42 as co-migratory signals have been observed using rabbit antisera raised specifically against this protein. The protein detected above p80 at 83 kDa is frequently observed when blotting total malarial protein extract with both monoclonal and polyclonal antibodies against RAP-1 and most likely corresponds to a newly synthesized precursor protein prior to cleavage of its N-terminal signal sequence (Ridley *et al.* 1990). The 70 kDa

band is likewise frequently observed in total malarial protein extract (lane 1) even though it is of low abundance in affinity-purified RAP-1 as a result of using protease inhibitors during the extraction and purification of antigen (lane 2) (see also Fig. 2).

Importantly, no proteins were recognized by the monkey sera other than those associated with the RAP-1 antigen. This confirms the purity of the immunizing antigen and strongly suggests that there are no cross-reactive epitopes on other malarial antigens. We conclude that the RAP-1 antigen elicits an immune response in *Saimiri* monkeys which confers protection against *falciparum* malaria.

DISCUSSION

In this report, we show that the non-polymorphic rhoptry antigen RAP-1 (Clark *et al.* 1987) can be extracted from *P. falciparum* and can protect *Saimiri* monkeys against a lethal infection of *falciparum* malaria. It effectively inhibits early multiplication of the pathogen and permits complete recovery without further intervention. The sera from the immune animals recognized only RAP-1 antigen proteins when used to probe a Western blot of total parasite protein, confirming that the antigen is responsible for generating this protection. Furthermore, in keeping with the antigen's non-polymorphic nature, the response is not strain limited. The immunizing antigen, purified from a Thai isolate, K1, protected against an isolate of East African origin, Palo Alto Uganda.

It is not possible at this stage to specify how humoral and cellular mechanisms participate in the protection of the monkeys. However, some comments on the immunogenicity of this antigen are possible.

The RAP-1 antigen is obviously complex, consisting of up to six protein species. The recognition by monoclonal antibodies of all the higher molecular weight proteins (p80, p77, p70 and p65) and pulse-chase data (Clark *et al.* 1987) has led to the conclusion that these proteins are all derived from the same precursor protein (Ridley *et al.* 1990). In addition, evidence has also been presented that p42 and p40 may be derived from these higher molecular weight proteins (Ridley *et al.* 1990). In the light of these observations it is interesting to note that the 80 kDa protein is much more strongly recognized by human immune sera than the other proteins, suggesting that a strong immunogenic determinant is present on the 80 kDa protein which is lacking on the other proteins. In contrast, the 42 kDa component appears to be poorly immunogenic in man, though the denatured protein was immunogenic in monkeys.

It should be noted that sera from the experimental animals recognized a recombinant fusion peptide derived from the merozoite surface antigen, PMMSA (Gentz *et al.* 1988) (not shown). However,

the sera did *not* recognize parasite PMMSA or its products in a Western blot (Fig. 5), nor did they give the immunofluorescence pattern characteristic of this antigen (not shown). Perhaps the epitopes recognized in the fusion peptide (made in *Escherichia coli*) are special to this construct.

It needs to be stressed that RAP-1 is different from another antigen of similar size and subunit composition, defined by a mAb Hb31c13, previously used in monkey immunization experiments (Perrin *et al.* 1985*b*). Antibodies raised against the two antigens do not cross-react (R. Ridley and U. Certa, unpublished observations). In addition, the 80 kDa component of the antigen defined by mAb Hb31c13 has been identified as a serine protease (Braun-Breton, Rosenberry & Pereira da Silva, 1988). The gene sequence encoding RAP-1 has been cloned and sequenced and does not contain any of the motifs characteristic of serine proteases (Ridley *et al.* 1990).

In summary, a non-polymorphic rhoptry antigen, RAP-1, has been purified from *P. falciparum* malarial extracts using immuno-affinity chromatography and electro-elution from polyacrylamide gels. This antigen was successfully used to protect *Saimiri* monkeys from a lethal infection of *P. falciparum* malaria. Further assessment of the antigen's value as a vaccine requires monkey immunization experiments using recombinant protein material derived from the gene sequence of RAP-1. Attempts to express the gene in bacteria are currently in progress.

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