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SHARING OF ANTIGENS BETWEEN *Plasmodium falciparum* AND *Anopheles albimanus*

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SUMMARY

The presence of common antigens between *Plasmodium falciparum* and *Anopheles albimanus* was demonstrated. Different groups of rabbits were immunized with: crude extract from female *An. albimanus* (EAaF), red blood cells infected with *Plasmodium falciparum* (EPfs), and the SPf66 synthetic malaria vaccine. The rabbit´s polyclonal antibodies were evaluated by ELISA, Multiple Antigen Blot Assay (MABA), and immunoblotting. All extracts were immunogenic in rabbits according to these three techniques, when they were evaluated against the homologous antigens. Ten molecules were identified in female mosquitoes and also in *P. falciparum* antigens by the autologous sera. The electrophoretic pattern by SDS-PAGE was different for the three antigens evaluated. Cross-reactions between *An. albimanus* and *P. falciparum* were found by ELISA, MABA, and immunoblotting. Anti-*P. falciparum* and anti-SPf66 antibodies recognized ten and five components in the EAaF crude extract, respectively. Likewise, immune sera against female *An. albimanus* identified four molecules in the *P. falciparum* extract antigen. As far as we know, this is the first work that demonstrates shared antigens between anophelines and malaria parasites. This finding could be useful for diagnosis, vaccines, and the study of physiology of the immune response to malaria.

KEYWORDS: *Anopheles albimanus*; *Plasmodium falciparum*; Shared antigens; Cross-reaction; Multiple Antigen Blot Assay (MABA).

INTRODUCTION

Several studies have reported that, during the process of co-evolution, parasites have shared and expressed equivalent molecules with their hosts. These cross-reactions may probably result from a process of adaptation of these invertebrates to counteract protective immunological mechanisms[3,4,24].

Specifically, these shared antigens between parasites and their hosts have been demonstrated among different species of trematodes and their intermediate hosts[8,9,10,16,17,29,45]. One of them is tropomyosin, a protein shared by *S. mansoni* and its intermediate host *Biomphalaria glabrata*[20] .

It has been proposed that the presence of common antigens between some parasites and their hosts would facilitate the search for candidate vaccines and diagnostic targets, when observing the abundance of antigenic material that can be obtained from those hosts, as demonstrated in schistosomiasis. The existence of shared antigens between *Schistosoma* and *Biomphalaria* has been shown by the presence of anti-*Biomphalaria* antibodies in experimental animals infected with the parasite. The opposite has also been demonstrated in animals immunized with uninfected snails, which have shown anti-*Schistosoma* antibodies. Additionally, anti-*Biomphalaria* antibodies have been found in infected humans with *Schistosoma mansoni*[1,18,19,22,25,27,33].

Malaria is a vector-borne disease transmitted through the bite of anopheline mosquitoes, the only genus that transmits human malaria[26,47]. In America, the most important species of *Anopheles* are: *An. darlingi*, *An. albimanus*, *An. aquasalis,* and *An. pseudopunctipennis*[23]. However, so far, no study has been carried out to determine shared antigens between *Plasmodium* and their vectors.

For this reason, the purpose of this research is to identify shared antigens between *An. albimanus* and *P. falciparum* that could have potential relevance in immunodiagnosis and immunoprophylaxis of malaria. It is also useful in explaining false positive results observed against malaria antigens in individuals who were exposed to multiple anopheline bites. In preliminary studies carried out with humans who did not have any previous malaric episodes and who come from a non endemic area in Venezuela, a percentage of seropositivity against molecules from *An. albimanus* and *P. falciparum* crude extracts was found (unpublished results). The identification of common antigens between the parasite and its vector could be of value in a multi-target vaccine (anti-parasite and anti-vector), once the feasibility of anti-arthropod vaccines is demonstrated[11,44].

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MATERIALS AND METHODS

Source of adult An. albimanus mosquitoes: A mosquito colony from *An. albimanus* was maintained in the laboratory according to the methodology described by ZERPA *et al*.[46].

Preparation of An. albimanus crude extract antigen: A crude anopheline preparation was obtained from female mosquitoes (EAaF) five to six days old. Female newborn mosquitoes were fed only with sugar solution until the moment of the extract preparation. None of the mosquitoes were exposed to a blood meal. These were immobilized by exposure to a temperature below 0 °C, during 15 min in a freezer. Afterwards, legs and wings were removed without damaging the abdomen, thorax and head. Each lot of antigen was prepared with 25 mosquitoes and homogenized in 0.5 mL of saline solution 0.85% (w/v) with a homogenizer (Potter Eveljhan), through 30 cycles as described by ALGER & CABRERA[2] and SUTHERLAND & EWEN[38]. After the homogenization, the mosquito extract was sonicated in a Braun-Sonic 1510 (B. Braun Melsungen AG) at 100 watts, using six cycles of one min each at 5 ºC. The sonicated sample was centrifuged at 4,960 g for one h at 5 °C. Finally, one extract was obtained by mixing all the preparations from the same colony of mosquitoes, and then, only the supernatant was used as antigen.

Preparation of Plasmodium falciparum antigen: *P. falciparum* (FCB_2 strain) parasites were obtained from continuous *in vitro* culture with a parasitemia of 10% to 12%, according to the methodology of TRAGER & JENSEN[41]. Parasites were concentrated by a discontinuous gradient of Percoll: 75% (v/v), 60% (v/v), 40% (v/v) as described by KRAMER *et al*.[28] and RIVADENEIRA *et al*.[35]. The layer of cells enriched with parasitized red blood cells (PRBC) was centrifuged at 861 g for 15 min at 4 ºC. Three types of *P. falciparum* antigens were obtained: (a) *Crude extract (EPfd) obtained with* detergent: PRBC were treated with saponin solution at 0.2% (w/v) in a 7:1 ratio, strongly stirred during 20 s and centrifuged at 1,240 g for 10 min. After two washes in PBS at 1,240 g X 10 min, the pellet containing parasites and the ghost red blood cells were collected. The PRBC lysis was completed by treating with a buffer solution, containing SDS at 10% (v/v), EDTA 1M, PMSF 100 mM or 300 µL Protease Inhibitor Cocktails (Sigma, P 2714), in a 7:1 ratio, and thereafter by shaking with a vortex at maximum speed during five min. Finally, the homogenate was centrifuged at 15,800 g in a microcentrifuge for 20 min, discarding the pellet and keeping the supernatant according to SALCEDO *et al.*[37]. This extract was used for the assays. (b) *Crude extract obtained by sonication* (EPfs): The PRBC was resuspended in 1 mL of PBS-Dulbecco and sonicated in a Braun-Sonic 1510 (B. Braun Melsungen AG) at 100 watts by six cycles of one min each at 5 ºC. Thereafter, it was centrifuged at 12,000 g for 30 min, discarding the pellet and keeping the supernatant. Only one extract was prepared by this methodology and used as antigen. (c) *Preparation of culture Supernatant (Spt) antigen: In vitro* culture of *P. falciparum* was centrifuged at 1,240 g for 10 min at 4 ºC, and the supernatant obtained was used as the control antigen in MABA. Protein concentration of the different antigen preparations was quantified following the BRADFORD[5] method.

Immunization of rabbits: Three months old New Zealand rabbits were injected subcutaneously with 0.5 mg of SPf66 vaccine, 0.5 mg of EPfs, or 1 mg of EAaF extracts, emulsified in Complete Freund's Adjuvant (CFA) for the first dose and with Incomplete Freund's Adjuvant (IFA) for the subsequent two doses every 15 days (1:1 ratio vol/vol of antigen/adjuvant). EPfs (extract obtained by sonication) was used for immunization because it had no detergent, and it was the most enriched with parasite material. Rabbits immunized with SPf66 vaccine were boosted six times. Bleeding of rabbits, previously anesthetized with 10 mg/kg Ketamine Chlorhydrate, was carried out before the first immunization and 10 days after the third dose.

Preparation of red blood cells (RBC): RBC antigen was obtained with the same methodology for EPfd as described by SALCEDO *et al*.[37].

Immunogenicity and cross-reaction of An. albimanus and P. falciparum extracts: The detection of specific anti-mosquito and anti-*Plasmodium* rabbit antibodies was carried out by ELISA, as described by ENGVALL & PERLMANN[21] and by VOLLER *et al*.[42], with a protein concentration of 7 µg/well for the *An. albimanus* antigen (EAaF), 3 µg/well for the *P. falciparum* antigen (EPfs), and 1 µg/well for the SPf66 vaccine. Additionally, the multiple antigen blot assay (MABA), described by NOYA & ALARCÓN DE NOYA[30], was used, sensitizing the nitrocellulose membrane with protein (between five and 20 µg/mL). The immune sera were also evaluated by Immunoblotting[40] at a dilution of 1:50 for anti-SPf66 vaccine and 1:100 for anti-female mosquito (EAaF) and anti-*P. falciparum* (EPfs). The protein concentration of the different antigenic preparations (EPfd, EPfs, EAaF, and RBC) used for immunoblotting was 1.0 µg/µL. Anti-IgG rabbit conjugated peroxidase was used at a 1:4000 dilution. A chemiluminiscent substrate Luminol® was used (Amersham, ECL Detection System). Strips were then exposed to a film (Hyperfilm®, ECL Detection System, Amersham).

Statistical analysis: Statistical comparisons using median and standard deviation were made. Three experiments were performed by enzyme-linked immunosorbent assay (ELISA).

RESULTS

Electrophoretic pattern by SDS-PAGE: Silver stained *An. albimanus* mosquito crude extracts revealed sixteen protein bands for the female antigen (EAaF), ranging from 24 to 95 kDa. In the *P. falciparum* preparation (EPfd), there were eighteen molecules between 26 to 161 kDa (data not shown).

Immunogenicity of An. albimanus female mosquitoes, P. falciparum and SPf66 antigen preparations: Immune rabbit sera against EPfs, SPf66 vaccine, or EAaF, evaluated by MABA are shown in Fig. 1 and by ELISA in Fig. 2. Evaluations done by immunoblotting are shown in Fig. 3.

Ten antigenic components were revealed in EAaF extract (apparent molecular weight from 30 to 124 kDa) by anti-EAaF sera (Fig. 3). Likewise, anti-EPfs sera recognized ten antigenic molecules between 12 to 143 kDa in EPfd (Fig. 3).

Cross-reaction by MABA: Rabbits immunized with EPfs recognized the following antigens: excretory-secretory in the culture supernatant of *P. falciparum* (Spt), EAaF, SPf66 vaccine, and normal

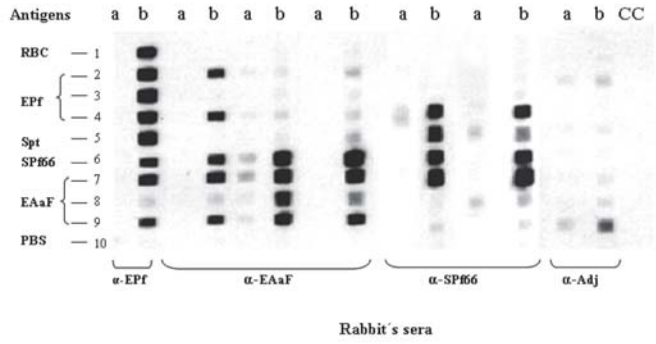

Fig. 1 - Immunogenicity and cross-reactivity of *Plasmodium falciparum* and *Anopheles albimanus* crude extracts, evaluated by MABA. RBC: Red Blood Cells (1), EPf: extract of RBC parasitized with *P. falciparum* treated with saponin detergent (2) and sonicated (3 and 4) with concentration of 5 µg/mL and 20 µg/mL respectively. Spt: Supernatant of culture *P. falciparum* (5). SPf66: Vaccine anti-*P. falciparum* (6). EAaF: Extract of *An. albimanus* female with concentration of: 20 µg/mL (7), 5 µg/mL (8) and 10 µg/mL (9), PBS (10). Preimmune serum (a), Immune sera (b). Adj: Adjuvant. CC: Control of conjugate.

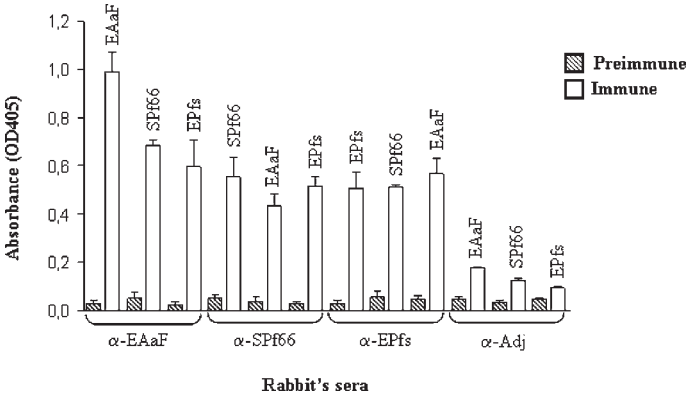

Fig. 2 - Immunogenicity (homologous sera) and antigen cross-reactivity (heterologous sera) between *P. falciparum* and *An. albimanus* by ELISA. Rabbit's sera immunized with: Extract of *An. albimanus* female (EAaF) or Extract of *P. falciparum* by sonication (EPfs) or Vaccine malaria (SPf66). Preimmune serum (a), Immune sera (b). Adj: Adjuvant. CC: Control of conjugate. OD405: optical density at 405 nm. T: Standard deviation.

RBC components (Fig. 1). Rabbits immunized with EAaF recognized *P. falciparum* and SPf66 vaccine preparations (Fig. 1). Finally, rabbits immunized with SPf66 vaccine recognized one or more components in the culture supernatant of *P. falciparum*, EAaF, EPfs, and EPfd (Fig. 1). Noteworthy, only rabbit sera immunized with EPfs recognized RBC. The rest of the sera groups did not react with this antigen preparation.

Cross-reaction of P. falciparum and An. albimanus by ELISA: A reciprocal recognition was observed among the three extracts in rabbits immunized with the heterologous antigens. The crude EAaF was recognized by rabbit sera immunized with EPfs or SPf66, and similarly, the crude EPfs was identified by anti-EAaF and anti-SPf66. Moreover, the SPf66 vaccine was recognized by anti- EAaF and anti-EPfs antigen immune sera (Fig. 2). Weak non-specific antibody responses were observed with control rabbits immunized only with adjuvant.

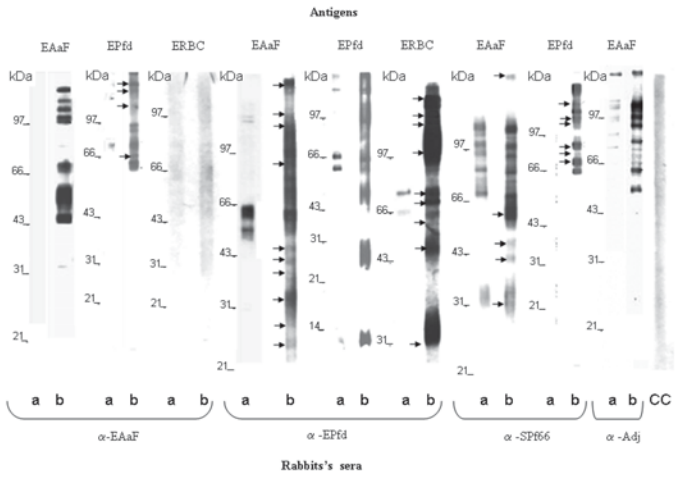

Fig. 3 - Pattern of antigenic recognition of *An. albimanus* female extract (EAaF), *P. falciparum* treated with saponin (EPfd) and Red Blod Cell extract by rabbit sera: anti-EAaF, anti-EPfs (*P. falciparum* treated by sonication) and anti-SPf66, by immunoblotting. Adj: Adjuvant, CC: Conjugate Control, a: Pre-immune sera, b: Immune sera.

Cross-reaction by immunoblotting: The anti-EAaF rabbit sera recognized ten protein bands in the EPfd from 63 to 159 kDa, specifically 68, 110, 136, and 159 kDa bands (Fig. 3). The anti-EPfs reacted with fourteen protein bands in the EAaF between 25 and 160 kDa, ten of them being specifics with molecular weights of 25, 27, 36, 42, 44, 49, 83, 110, 118, and 160 kDa (Fig. 3). The anti-EPfs recognized nine molecules: 32, 53, 65, 70, 74, 99, 118, 126, and 130 (duplet) kDa in the RBC fraction (Fig. 3). Anti-SPf66 vaccine reacted with thirteen protein bands in the EAaF ranging from 31 to 152 kDa, with only five specific molecules: 31, 42, 44, 61, and 152 kDa (Fig. 3). The anti-SPf66 rabbit recognized ten bands in EPfd, from 64 to 132 kDa. The most specific molecules were: 70, 77, 82, 106, 110, and 127 kDa (Fig. 3). Some protein bands with molecular weights around 65, 74, 99 kDa are also present in rabbits immunized only with CFA and IFA (data not shown). These bands were excluded from analysis of specificity.

DISCUSSION

The *Schistosoma-Biomphalaria* association was the first host-parasite model which demonstrated shared antigens[8,22,43] . Different molecules are cross-reactive and most of them have not been characterized yet. Tropomyosin is one of the few molecules characterized and it is shared by *S. mansoni* and *B. glabrata*[20].

Since then, there are different epidemiological studies in which the presence of cross-reactive antibodies were observed[1,10,36]. More recently, the presence of two antigenic components from *B. glabrata* were recognized by mice sera, immunized with a crude antigen from *S. mansoni* adult worm (AWA), by immunoblotting[13].

Other models in which antigenic sharing between parasites and their intermediate hosts (snails) or vectors has been described: *S. haematobium*, *S. japonicum*, and *Paragonimus westermani, Leishmania major* and *Phlebotomus duboscqi* [9,27,28,33,39,43].

The purpose of this study is to identify shared antigens in another model: *An. albimanus - P. falciparum.*

Rabbits immunized with EPfs reacted strongly with the EAaF. Furthermore, rabbits immunized with female *An. albimanus* extracts recognized molecular components in only one of the two sonicated EPf. This was expected, however, the protein concentrations were different. The cross-recognition of possible common molecular components between these two organisms was also confirmed by ELISA and MABA assays. These anti-mosquito sera not only recognized molecular components of the *P. falciparum* antigen, but unexpectedly, also identified the SPf66 synthetic malaria vaccine, which contains short sequences of four distinct surface molecules: one from the sporozoite (NANP) and three from the asexual blood stages (83, 55, 33 kDa) of the parasite[31,32]. This findings deserves special attention and further experiments will be required to evaluate the possible anti-anopheline effect of this well-known protective vaccine[31,32] .

Also, it would be interesting to identify the topography of the antigens involved in the cross-reactivity in anophelines. A complementary support of the cross-reactivity observed in this study would be provided by cross-absorption of the antisera, with homologous and heterologous antigens, before testing them against both antigens.

It was expected to find a major recognition of the molecular components in the female mosquito extract because of the biochemical complexity of the digestive system. However, the low recognition of these molecules by the homologous or heterologous immune sera could be attributed to different reasons. Firstly, the enriched proteases content required for the digestion of the blood meal could cleave some proteins during the preparation of the extract and it could inhibit the antigenicity of them[12]. Although some extracts were prepared with protease inhibitors, the results obtained in both cases were similar. Secondly, it could be that proteases from the midgut and saliva of anophelines might induce immune suppression in the immunized rabbits. In fact, it has been demonstrated at the experimental level in the murine model, that the initial secretion of IL-2 or γ-interferon by TH1 cells can be suppressed by components present in the salivary gland extracts of *Aedes aegypti* and *Simulium vittatum* mosquitoes[14,15] .

Another possibility is that, the selection of an anti-IgG conjugate might have occluded the response to other important isotypes (IgM and IgE).

Moreover, the immunization with Complete and IFA adjuvants could induce a predominantly TH1 response, which is not the habitual immune reactivity to allergenic components of mosquito saliva. The presence of specific IgE and IgG_4 antibodies has been shown to be the main serological response induced in mice, rabbits, and humans against *Aedes aegypti*, *Ae. communis* and *An. stephensi* saliva[6,7,34]. Therefore, it will be necessary to evaluate the presence of specific anti-mosquito IgE and IgG_4 antibodies as well.

In summary, the study of the shared antigens of any parasite-host model should have three fundamental objectives: (i) In terms of vaccines, the possibility to induce simultaneous protection against the pathogenic agent and its vector; (ii) In terms of diagnosis, to eliminate those molecules responsible for inespecificity of any immunoassay, since exposure to the saliva of the vectors could be responsible for some false positive serology, as we have observed already in persons not exposed to malaria; (iii) In terms of parasite-host interactions, to elucidate the possible role of common molecules involved in immune evasion and immune modulation.

As far as we know, this is the first study to demonstrate sharing antigens between *Plasmodium* and anopheline vectors. The results presented herein not only could help to understand vector-parasite interactions, but could also contribute to elucidate and to interpret serological tests and immunoepidemiological data related to this parasitic disease.

RESUMEN

Antígenos compartidos entre *Plasmodium falciparum* y *Anopheles albimanus*

Epítopes de antígenos compartidos entre *Plasmodium falciparum* y *Anopheles albimanus* fueron identificados. Diferentes grupos de conejos fueron inmunizados con: extracto crudo de mosquito hembra de *An. albimanus* (EAaH), glóbulos rojos infectados con *P. falciparum* (EPfs) y la vacuna antimalárica sintética SPf66. Los anticuerpos policlonales producidos en conejos fueron evaluados por ELISA, inmunoensayo simultáneo de múltiples antígenos (MABA) e Immunoblotting. Todos los extractos resultaron inmunogénicos cuando se evaluaron por ELISA, MABA e Immunoblotting. Diez moléculas fueron identificadas en los mosquitos hembras y diez en los antígenos de *P. falciparum* por los sueros autólogos. El patrón electroforético por SDS-EGPA fue diferente para los tres antígenos evaluados. La reactividad cruzada de moléculas entre *An. albimanus* y *P. falciparum* fue demostrada por ELISA, MABA e Immunoblotting. Anticuerpos anti-*P. falciparum* y anti-SPf66 reconocieron diez y cinco componentes respectivamente en el extracto crudo de anofelinos (EAaH). Asimismo, sueros inmunes contra *An. albimanus* hembra identificaron cuatro moléculas en el extracto del antígeno de *P. falciparum*. Hasta el presente, este es el primer estudio en el que se demuestra la presencia de antígenos compartidos entre anofelinos y los parásitos de malaria. Este hallazgo podría ser de relevancia para el diagnóstico, vacunas e interpretación de la fisiopatología de la respuesta inmunitaria en malaria.

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