Immunogenicity and protective efficacy of *Plasmodium falciparum* liver-stage Ag-3 in *Aotus lemurinus griseimembra* monkeys

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Three recombinant proteins spanning the *Plasmodium falciparum* liver-stage Ag-3 (LSA-3) were used to immunize *Aotus* monkeys. The proteins were delivered subcutaneously without adjuvant, adsorbed onto polystyrene 0.5 μm particles at a concentration of 2 μg per immunization. Control animals received glutathione-S-transferase formulated similarly. Animals were challenged as late as 5 months after the last immunization, by intravenous inoculation of 100,000 *P. falciparum* sporozoites of a strain heterologous to the one from which the immunogens were derived. Sterile protection was achieved in three of the five immunized monkeys but in none of four controls. Antibodies were at low titer, but reacted with the native parasite protein and were boosted by parasite challenge. Ag-specific IFN-γ secretion was detectable in all LSA-3-immunized animals in response to the LSA-3-derived Ag. The protection was apparently associated with high levels of IFN-γ production in response to *in vitro* recall Ag. These results lend support to the vaccine potential of LSA-3 indicated by previous results obtained in chimpanzees, as well as the value of yet another Ag-delivery system. They also support the value of the *Aotus* model for the pre-clinical development of pre-erythrocytic-stage vaccines.

Received	11/7/02
Revised	2/2/03
Accepted	17/3/03

Key words: Immune response / Malaria pre-erythrocytic stage / *Aotus* monkey / Recombinant protein / Malaria vaccine

1 Introduction

Over the past 10 years, the study of anti-malaria immunity induced by irradiated sporozoites has provided substantial evidence that blockade of intra-hepatocytic parasite development is responsible for the protection [1–5]. In this context, our work has focused on the liver-stage antigenic repertoire [6, 7] and, for this purpose, liver-stage Ag-3 (LSA-3) has been selected as a potential vaccine candidate based on the differential immune responses observed between protected and non-protected volunteers immunized with irradiated sporozoites [8]. LSA-3 is a highly conserved, 200 kDa protein, expressed in both sporozoites and parasite liver-stages. It displays high antigenicity and immunogenicity, and has demonstrated protective efficacy in mice and chimpanzees using several distinct Ag-delivery systems [8–10].

Although these studies have provided valuable indications in favor of the protective capacity of LSA-3, they have the limitation of using either heterologous malaria species (*Plasmodium yoelii*) in mice or are limited by the small number of chimpanzees available. Taking advantage of the fact that *Aotus lemurinus griseimembra* monkeys are known to be highly susceptible to *P. falciparum* blood-stage infection and can produce circulating infective gametocytes, we have recently developed a model for *P. falciparum* sporozoite challenge by cyclical transmission through *Aotus* and mosquitoes [11]. We have now successfully used this model to assess the immunogenicity and efficacy of recombinant LSA-3 proteins, delivered as microparticles without adjuvant, against challenge by *P. falciparum* sporozoites.

[DOI 10.1002/eji.200323339]

Abbreviations: ELIspot: Enzyme-linked immunosorbent spot **GST:** Glutathione-S-transferase **IFAT:** Indirect immunofluorescent antibody test **LSA-3:** Liver-stage Ag-3 **MSP-3:** Merozoite surface protein-3 **RESA:** Ring erythrocyte surface antigen **SFC:** Spot-forming cells

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2 Results

2.1 Safety and protective efficacy

No local or generalized reactions were detected in *Aotus*, which are known to be fragile and sensitive animals, following subcutaneous immunizations with the LSA-3-coated 0.5 μm polystyrene microspheres. Hematological parameters remained within normal limits during the course of the immunization.

Upon challenge (Fig. 1), the three *Aotus* immunized with control Ag and one naïve animal (V97) developed patent *P. falciparum* parasitemia detectable after days 13–20. Among the LSA-3-immunized group, three monkeys (V84, M177 and M205) presented no parasitemia whatsoever at any time-point during the 55 days of the follow-up, whereas the remaining two (V83 and M211) developed patency from days 11 and 33, respectively. Parasitemia in the control group and in the two immunized but non-protected *Aotus* was moderate, ranging from 71 to 423 parasites/μl. All *Aotus* received drug treatment at day 60.

2.2 Immunogenicity

For practical reasons, not all *Aotus* could be studied by enzyme-linked immunosorbent spot (ELIspot) assays; however we were able to collect enough blood from three of the immunized monkeys to perform this assay. The frequency of specific IFN-γ-producing cells in the *ex-vivo* ELIspot assay performed with cells from these monkeys — M177, M205 and M211 — was higher in the two monkeys that were protected upon challenge (M177 and M205) as compared with the non-protected monkey (M211), and was specifically induced by either recombinant proteins (729, NN or PC) or peptides (NR2, NR2 or RE) (Fig. 2A). For monkeys V83 and V84, whose IFN-γ secretion was determined in PBMC supernatants, responses were observed to all peptides, but again the response of the protected *Aotus*, V84, was higher than that of the non-protected *Aotus*,V83, particularly in response to peptide RE (Fig. 2A). The specificity of IFN-γ production was demonstrated by negative results obtained from preimmunization samples (not shown), from postimmunization samples from the three control monkeys (Fig. 2A), and by glutathione S-transferase (GST) control wells (*i.e.* M211 is considered negative).

Ab responses determined by ELISA showed low but specific Ab (titers ranged from 100 to 900). As shown in Fig. 2B, four of the five monkeys from the test group produced Ab mainly against the 729 and NN recombinant proteins whereas no response was observed against the PC region of the protein. Although present at low levels, Ab were persistent and could be detected for up to five months after the last injection against the 729 recombinant protein in *Aotus* V83, V84 and M177, and against the NN protein in *Aotus* M177 and M205 (not shown). Although two of the three protected animals had the highest Ab titers, no clear relationship with protection

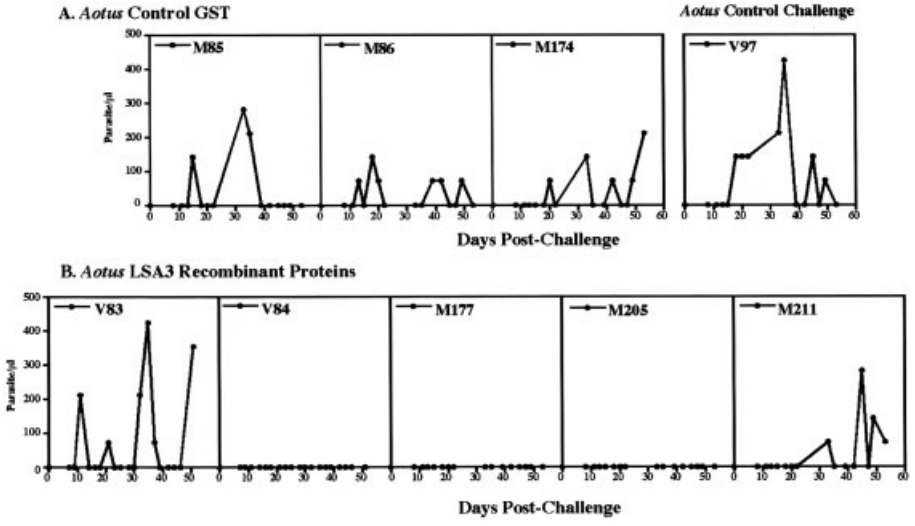
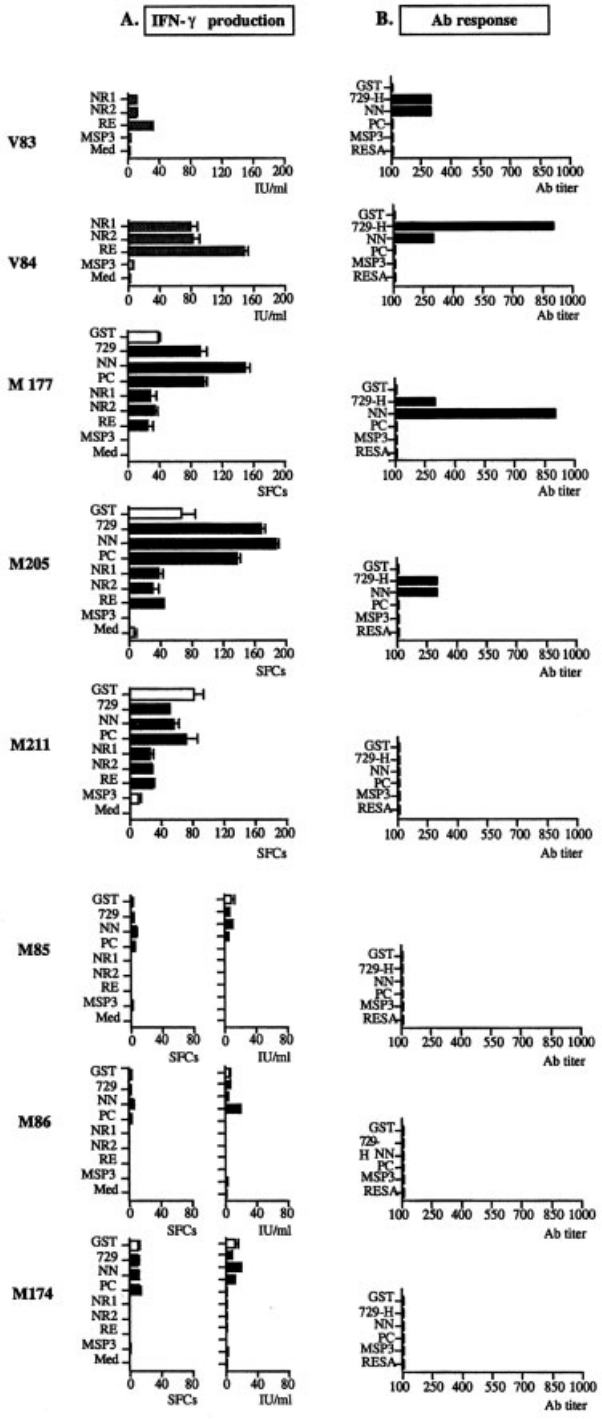


Fig. 1. Parasitemia follow-up after challenge of animals with *P. falciparum* sporozoites. Parasitemia in *Aotus* monkeys immunized with the three LSA-3 recombinant proteins (B) or GST alone (A) and challenged with 10^5 *P. falciparum* sporozoites from the Santa Lucia strain. Monkeys were followed-up for 55 days and parasitemia was evaluated using thick blood-smears. The number of parasite/μl was calculated based on the number of *Aotus* leukocytes/μl.



◀ *Fig. 2.* IFN-γ production and Ab responses in *Aotus* monkeys. *Aotus* V83, V84, M177, M205, and M211 were immunized with LSA-3 recombinant proteins. *Aotus* M85, M86, and M174 are control animals immunized with GST alone. (A) The IFN-γ production was evaluated by ELISA in *Aotus* V83 and V84 and is expressed in IU/ml, or was evaluated by ELIspot in *Aotus* M177, M205, and M211, and the results are expressed as the number of IFN-γ-SFC per 10^6 PBMC; controls were assessed by both methods. (B) Ab titers to LSA-3 and control Ag were determined by ELISA in sera collected 15 days after the last immunization, starting at a 1/100 dilution. Results obtained 5 months later are similar and are not shown.

was seen. The specificity of this response was ascertained by negative results in the control group, as well as by the lack of recognition of GST and of irrelevant peptides such as ring erythrocyte surface antigen (RESA) and merozoite surface protein-3 (MSP-3) by sera of LSA-3-immunized animals (Fig. 2B).

2.3 Reactivity with the native protein

Sera collected 140 days after the last immunization and sera collected 15 days after challenge were assessed for their reactivity with the native protein on the surface of sporozoites by the indirect immunofluorescent antibody test (IFAT). Although sera obtained after the last immunization did not recognize the protein expressed on sporozoites, specific anti-sporozoite Ab responses were induced in all LSA-3-immunized animals by the challenge. Since this was not seen in the challenged controls (GST-immunized and naïve), it indicates that the anti-LSA-3 response had been primed by immunization and boosted by sporozoite challenge (Fig. 3). The specificity of the Ab produced was also demonstrated by an absence of reactivity of all of the *Aotus* sera tested with *P. vivax* sporozoites (not shown).

3 Discussion

This study was aimed at evaluating the immunogenicity and protective efficacy of the *P. falciparum* LSA-3 protein in *A. l. griseimembra*. We recently reported that *Aotus* monkeys can be successfully infected with sporozoites derived from the *P. falciparum* Santa Lucia strain in a reproducible manner [11], *i.e.* similar to that obtained in chimpanzees [8] and in humans [6]. The results obtained in the four control monkeys support this observation, and those obtained in immunized animals are in agreement with the protective efficacy of LSA-3 previously demonstrated using other Ag-delivery methods in another primate species, the chimpanzee [8]. Despite the limited number of animals that can be studied, they confirm that LSA-3 can induce long-lasting immune responses that afford protection against a challenge with a strain heterologous to that used for designing the vaccine.

The LSA-3 recombinant proteins, injected at very low doses without adjuvant, induced high T cell IFN-γ

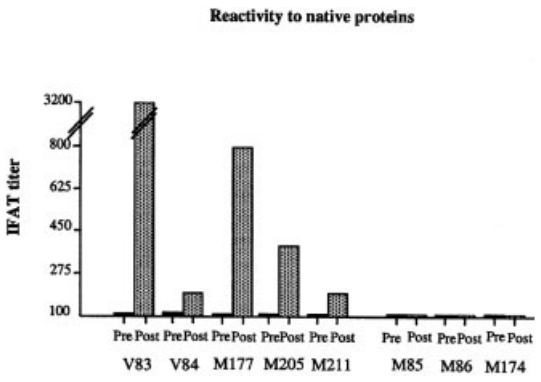


Fig. 3. Immune responses to the native protein. Ab titers determined by IFAT on *P. falciparum* sporozoites in pre- and post-challenge samples expressed as the reciprocal of the end-point titer showing specific fluorescence of the sporozoite surface (of serial two-fold dilutions starting at 1/100).

responses to each of the three recombinant proteins and the LSA-3-729-derived peptides, together with medium-to-low Ab responses. This was expected since this mode of immunization has been found to preferentially induce T cell responses associated with protection in mice (J.-P. Sauzet et al., manuscript in preparation). Our study confirm previous evidence that LSA-3 is a strong inducer of this cytokine [10, 12, 13]. *Aotus* previously immunized using LSA-3-729 lipopeptide formulations also developed high IFN-γ and significant Ab responses [12].

Although the formulation tested here induced a low antibody response before challenge, it was nevertheless enough to prime Th and B cell precursors to further respond strongly to native proteins upon challenge. This reflects an anamnestic Th cell response, in agreement with the high IFN-γ levels, which persisted for a long time. This also suggests a proper processing of both natural and "artificial" epitopes and confirms results previously described in mice and in chimpanzees [9, 10].

Three of the five immunized *Aotus* were fully protected and another one presented a delay in patency as compared with the four controls and to the other immunized but non-protected animal. These results are preliminary since the size of the cohort studied does not allow for statistical analysis. They nevertheless represent the first report of a malaria pre-erythrocytic subunit vaccine candidate capable of inducing protection in *Aotus* monkeys against heterologous *P. falciparum* sporozoite challenge. This conclusion is supported by: (i) the reproducibility of the challenges in *Aotus* by sporozoites of the Santa Lucia strain, established previously in more than 11 infections [11]; (ii) previous trials conducted in *Aotus* with pre-erythrocytic Ag, which have so far failed to induce protection after challenge with *P. falciparum* sporozoites [14]; (iii) the protection previously obtained with the same vaccine candidate, LSA-3, in chimpanzee using two other Ag-delivery methods [8]; and (iv) the fact that in all studies protection seemed to be associated with the ability of primed T cells to secrete IFN-γ.

The production of IFN-γ, which was recorded with all of the LSA-3 vaccine formulations employed so far, is of particular interest since this cytokine is known to be particularly efficient against *P. falciparum* liver forms, even at low cytokine concentrations. Its role in protection has been described *in vitro* for *P. falciparum* [15–17] as well as *in vivo* for rodent malaria upon immunization with irradiated sporozoites [18–21] and in chimpanzees immunized either with irradiated sporozoites or with LSA-3-derived antigens [6, 9]. It is unfortunate that all monkeys could not be studied by the same technique; however it was those *Aotus* showing full protection (V84, M177 and M205) that also displayed the highest *in vitro* IFN-γ production. In this and in previous studies, there seems to be an association between protection and the ability of mice, chimpanzees and *Aotus* to produce high IFN-γ responses, and to provide help to Ab production upon challenge [8, 9], *i.e.* the induction of Th cells is an important component of defense or at least appears as the best available surrogate marker of protection.

Protection was long-lasting since challenge was performed as late as 5 months after the last immunization. Protection extended to the Santa Lucia challenge strain, which is heterologous to that, K1, of the immunizing sequence. This is an important point in view of the limitations related to polymorphism in other malaria vaccine candidates such as CS and TRAP [22–24], and is in agreement with the high degree of LSA-3 sequence conservation [8]. These findings are in support of the vaccine potential of LSA-3, previously indicated using lipopeptide formulations without adjuvant, adjuvanted recombinant proteins, or genetic immunization [8, 13]. The present results add to this list another successful Ag-presentation means, that of microparticles without adjuvant. Finally, they contribute to support the *Aotus* monkey as a valuable model for the development of *P. falciparum* sporozoite and liver-stage vaccines.

4 Materials and methods

4.1 Immunization

A. l. griseimembra born at the Primate Center at Universidad del Valle (FUCEP, Cali, Colombia) were used. Animals were naïve adults, and males and females were used. They were matched according to their weight, size and age.

For the present study, three proteins covering the major part of the LSA-3 antigen and derived from the *P. falciparum* K1 strain sequence were cloned in a pGEX plasmid: LSA-3-729 from the N-terminal region, LSA-3-NN from the repeat region, and LSA-3-PC corresponding to the non-repeated C-terminal region [8]. All three GST-fused recombinant proteins were prepared according to the facturer's recommendations (Invitrogen, Carlsbad, CA, USA) and checked for the absence of LPS.

Immunization was performed using microparticles by injecting 2 μg of immunogen. The LSA-3 recombinant proteins or GST alone were passively adsorbed onto 0.5 μm polystyrene beads (Polysciences, Inc., Washington, USA), according to the manufacturer's recommendations. The efficacy of coating was controlled by an agglutination assay using an African human serum containing high anti-729, -NN and -PC antibody titers. Measurement of the protein concentration using optical density (280 nm) before and after bead-coating revealed that 70–80% of the protein was adsorbed on microparticles.

Previous studies in mice had shown that low doses (2–10 μg) of Ag adsorbed onto polystyrene beads and injected without adjuvant were more effective than high doses of Ag (50–100 μg). An experimental group of five *Aotus* (V83, V84, M177, M205 and M211) was immunized subcutaneously in the abdomen with 3.6×10^{10} beads coated with a mix of the three LSA-3 recombinants (2 μg each), in a final volume of 500 μl. Each monkey received three doses at intervals of 20 days. A group of three control monkeys (M85, M86 and M174) was immunized in the same manner with 2 μg of GST-coated polystyrene beads.

4.2 Challenge and parasitemia follow-up

The *P. falciparum* Santa Lucia strain (a gift from W. Collins, CDC Atlanta, GA) was passed through non-splenectomized monkeys in order to produce mature gametocytes for mosquito infection. Laboratory-reared *Anopheles albimanus* mosquitoes were fed with infected monkey blood on an artificial membrane, and mature infective sporozoites were collected in RPMI medium supplemented with 20% of normal *Aotus* serum and used for monkey challenge as described previously [14, 25, 26].

Immunized and control groups were challenged 156 days after the third immunization by intravenous inoculation of 10^5 sporozoites from a single sporozoite preparation. Blood was then sampled every 2 days during the next 55 days to monitor parasitemia. Giemsa-stained thick and thin smears were read blind in triplicate by three independent microscopists for 30 min per slide and parasite density expressed as parasites/μl [11]. At the end of the follow-up, the animals were treated with a combination of Sulfadoxine-Pyrimethamine [27, 28].

4.3 Cellular responses

B and T cell responses in immunized *Aotus* were assessed using two sets of proteins/peptides. First, LSA-3-NN and LSA-3-PC recombinant proteins, expressed as GST-fused recombinants, and LSA-3-729, expressed as a histidine-tailed fusion protein and purified on Ni-columns as recommended by the manufacturer (BioWhittaker). Secondly, the LSA-3-NR1, LSA-3-NR2 and LSA-3-RE synthetic peptides covering the sequence of the LSA3-729 fragment [8, 29].

T cell assays were performed using *Aotus* PBMC isolated on Ficoll Paque (Pharmacia Biotech) from the maximum ethically acceptable amount of blood from these small primates (<1 kg), *i.e.* 3 ml of blood taken by femoral venipuncture on day 0 (pre-immunization) and 15 days after the third immunization.

IFN-γ was studied here for two reasons: first, because it is a more reliable index of T cell activation than proliferation assays; and second, because it is established as a major mediator of protection against malaria liver stages [6, 30]. We have observed that culture of *Aotus* T cells offers more technical problems than human ones. Therefore, we chose to evaluate the number of IFN-γ-producing PBMC using a commercial kit for human IFN-γ ELIspot (MABTECH, Stockholm, Sweden). Microtiter plate wells (Millipore, MAHA S45, Bedford, MA, USA) were coated with 5 μg/ml of anti-human IFN-γ mAb (1-D1K, MABTECH) overnight at 4°C. After blocking with RPMI medium plus 10% FCS for 2 h at room temperature, a suspension of 5×10^5 PBMC/well was mixed with either recombinant proteins or synthetic peptides at 20 μg/ml. Plates were incubated for 40 h at 37°C in a 5% CO_2 **:** 95% air atmosphere. After washing with PBS plus 0.05% Tween-20, a biotinylated anti-IFN-γ mAb (7-B6-1, MABTECH) at 0.3 μg/ml was added and incubated overnight at 4°C. Streptavidine–alkaline-phosphatase (Boehringer Mannheim), diluted 1/1000, was added and the reaction revealed with the substrate 5-bromo-2-chloro-3-indolyl phosphatase / nitroblue tetrazolium (Sigma, St Louis, MO) leading to the appearance of dark-blue spots. The number of spots were determined using a stereomicroscope (×40) by two independent readers. Results are expressed as the mean number of IFN-γ spot-forming cells (SFC) per 10^6 PBMC.

However, in two of the investigated monkeys (V83 and V84) the number of lymphocytes recovered did not allow for the proposed study and we therefore decided to perform a more-classical determination of IFN-γ in stimulated lymphocyte culture supernatants that requires three-times less cells. IFN-γ concentrations in supernatants collected from triplicate wells on day 5 were assessed by a two-site capture ELISA as described previously [10] using another combination of anti-human-IFN-γ mAb identified as able to react with *Aotus* IFN-γ.

Negative and positive controls (unstimulated cells, cells stimulated by GST and cells stimulated by PHA) were included in each assay. The IFN-γ concentration (IU/ml) was calculated from a standard curve included in each plate and made from known amounts of recombinant human IFN-γ (Pharmingen International, 19751G). The specificity was determined by comparing the concentration in the test and control supernatants.

4.4 Antibody responses

Serum samples collected from immunized and control monkeys before and after immunization were kept at –20°C until use for specific Ab determination. Ab to the recombinant proteins and synthetic peptides were evaluated by an ELISA method using two-fold serial dilutions starting at 1/100, as described elsewhere [31], except that a rabbit anti-*Aotus* IgG at 1/2000 was used and was developed using peroxidase-conjugated anti-rabbit-IgG (Biosys, Compiègne, France) at a dilution of 1/4000. To define the cut-off value, each Ag was tested separately using sera from 10 control *Aotus* monkeys with no previous history of malaria exposure. The Ab titers were established as the last test sera dilution giving absorbances greater than the mean of *Aotus* normal control sera, plus 2 SD (using the optical density at 450 nm).

The presence of Ab to the native LSA-3 protein was determined by IFAT on the sporozoite surface as previously described [32]. *P. falciparum* sporozoites fixed with 0.001% glutaraldehyde were incubated with two-fold serial dilutions, starting at 1/100, of *Aotus* sera, and rabbit anti-*Aotus*-IgG diluted at 1/200 was employed as a second Ab.

Acknowledgements: This work was supported by The European Commission INCO-DC contracts 94–016 and 98–0387, Instituto Colombiano para la Ciencia y la Tecnología (Colciencias) contract CT 283–95 and World Health Organization contract 99–1006. B.-L. P. was supported by Colciencias and by the Société de Secours des Amis des Sciences-Paris, France. Peptides were synthesized and kindly provided by Dr Helene Gras-Masse. Thanks to Dr Jean Louis Perignon for critical reading of the manuscript. We thank C. Sokhna and all the team from IRD Dakar for their contribution to the microscopic examination of the slides. Thanks also to M. F. Yasnot and N. Puchot for technical assistance, and V. M. Salazar and L. A. Ruiz for care and handling the *Aotus* monkeys clony.

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