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Immunogenicity of a non-repetitive sequence of *Plasmodium falciparum* circumsporozoite protein in man and mice

G. DEL GIUDICE,* Q. CHENG,* D. MAZIER,† N. BERBIGUIER,† J. A. COOPER,* H. D. ENGERS,* C. CHIZZOLINI,‡ A. S. VERDINI,§ F. BONELLI,§ A. PESSI§ & P.-H. LAMBERT* **WHO-Immunology Research and Training Centre, Department of Pathology, University of Geneva, Geneva, Switzerland, †Department of Parasitology, INSERM U 313, Groupe Hospitalier Pitié-Salpétrière, Paris, Cédex, France, ‡Centre International de Recherches Médicales de Franceville, Franceville, Gabon, West Africa and §Polypeptide Synthesis Department, Eniricerche, Monterotondo, Italy*

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SUMMARY

In the present work, the hypothesis that individuals naturally exposed to *Plasmodium falciparum* malaria infection in endemic areas produce antibodies directed against non-repetitive epitopes of the circumsporozoite protein was investigated. Using a synthetic peptide reproducing the non-repetitive group-conserved region I sequence, we have shown that specific anti-region I antibodies are detectable in sera from endemic countries. Of these sera, 87% also had antibodies against the immunodominant repetitive epitope (Asn-Ala-Asn-Pro, NANP) of *P. falciparum*. In order to study the immunogenicity of this non-repetitive epitope, a synthetic peptide consisting of both region I and three (NANP) repeats [RI-$(NANP)_3$] was used to immunize inbred strains of mice. H-2^b mice produced antibodies against both the repetitive and the non-repetitive epitope. These antibodies were specific for each epitope, recognized *P. falciparum* sporozoites in immunofluorescence, and inhibited sporozoite penetration into human liver cells *in vitro*. Non-H-2^b mice were completely unresponsive. Lymph node cells from H-2^b mice immunized with RI-$(NANP)_3$ peptide proliferated in the presence of RI-$(NANP)_3$ and of $(NANP)_4$ peptide, but never in the presence of RI peptide alone. These findings demonstrate that in the configuration used (i) the non-repetitive epitope region I does not carry T-helper epitopes; (ii) the (NANP) repetitive epitope may act as a carrier for the immune response to region I in mice; and (iii) therefore, immune response to region I in man probably depends on the recognition of T-cell epitopes similar to those involved in the anti-NANP response: i.e. such a T epitope may be NANP itself in responding individuals or another, not yet recognized, sporozoite T-cell epitope.

INTRODUCTION

The immunogenicity of some plasmodial antigens, as well as the protection they confer to infected hosts and the elucidation of their molecular structure, suggests that the development of malaria vaccines consisting of selected amino acid sequences either synthesized chemically or genetically engineered (Miller *et al.*, 1986) is possible. One of the candidate epitopes is the tandemly repeated amino acid sequence NANP (Asn-Ala-Asn-Pro) of the *Plasmodium falciparum* circumsporozoite (CS) protein (Young *et al.*, 1985; Zavala *et al.*, 1985). Recent work carried out in our (Del Giudice *et al.*, 1986; Togna *et al.*, 1986) and in other (Good *et al.*, 1986) laboratories has shown that the immune response to this repeated sequence is genetically controlled in mice, and is linked to the presence of the *b* allele in the I-A subregion of the murine MHC (H-2) complex. Non-H-2^b mice responded to $(NANP)_n$ only when the peptides were conjugated to heterologous carrier proteins.

Non-repeated sequences flanking the $(NANP)_n$ epitope, such as the so-called regions I and II (RI and RII) (Dame *et al.*, 1984), have been found to be highly conserved in *P. knowlesi* (Ozaki *et al.*, 1983), *P. vivax* (McCutchan *et al.*, 1985), *P. berghei* (Eichinger *et al.*, 1986) and *P. cynomolgi* (Galinski *et al.*, 1987) CS proteins. Synthetic peptides from these regions, coupled to carrier proteins, are immunogenic in animals, and the antibodies raised were shown, by immunofluorescence, to recognize sporozoites of different *Plasmodium* species (Ballou *et al.*, 1985; Vergara *et al.*, 1985b). Although no evidence has been presented about the immunogenicity of these CS regions in naturally infected individuals, it has been demonstrated that rabbits (Vergara *et al.*, 1985a) and monkeys (Sharma *et al.*, 1986), repeatedly immunized with *P. knowlesi* sporozoites, produce antibodies against non-repetitive epitopes of the CS protein.

In the present study we tested the hypothesis that individuals naturally exposed to malaria infection in endemic countries would produce antibodies to a non-repetitive epitope, i.e. region I,

Correspondence: Dr G. Del Giudice, WHO-Immunology Research and Training Centre, Dept. of Pathology, University of Geneva, CMU, 1 rue Michel Servet, 1211 Geneva 14, Switzerland.

of *P. falciparum* CS protein. Moreover, we investigated the possibility to raise anti-region I antibodies in a murine model by using synthetic peptides consisting of the region I sequence or of region I plus three (NANP) repeats [(RI-(NANP)$_3$].

MATERIALS AND METHODS

Peptides

Synthetic peptides reproducing RI [Lys-Pro-Lys-His-Lys-Lys-Leu-Lys-Gln-Pro-Gly-Asp-Gly-Asn-Pro, according to Dame *et al.* (1984)], RI plus three (Asn-Ala-Asn-Pro) repeats [RI-(NANP)$_3$], and 40 and four (Asn-Ala-Asn-Pro) repeats [(NANP)$_{40}$ and (NANP)$_4$] of the *P. falciparum* CS protein, were produced at the Polypeptide Synthesis Department, Eniricerche, Monterotondo, Italy. The original synthesis of these peptides has been described in Italian Patent Applications No. 21144, July 16, 1986, and No. 21718, June 25, 1985, respectively, and will be the subject of a forthcoming publication. The lyophilized material was resuspended in sterile distilled water at 1 or 10 mg/ml, divided into aliquots, and stored at −70° until use.

Enzyme-linked immunosorbent assay (ELISA) for the detection of anti-(NANP) and anti-RI antibodies

Anti-(NANP)$_{40}$ antibodies were detected in human and mouse sera by ELISA, as described previously (Del Giudice *et al.*, 1986, 1987a, 1987b). The ELISA for the detection of anti-RI antibodies was carried out in the same way, except that plates were coated with RI peptide at 10 μg/ml. Individual sera were tested at a dilution of 1:200.

In some experiments, mouse and human sera, diluted 1:200, were preincubated for 1 hr at 37° with different concentrations of RI or (NANP)$_{40}$ peptides. Then, 100 μl of these mixtures were tested by ELISA in microwells coated with RI or (NANP)$_{40}$.

Human sera

Randomly selected sera were obtained from 93 adults (age range: 20–70 years) living in Okondja, Gabon, West Africa, where *P. falciparum* malaria is endemic and accounts for 99% of all the cases of malaria. Sera were also obtained from 102 healthy blood donors at the Blood Transfusion Centre, University Cantonal Hospital, Geneva, Switzerland.

Mice

Eight to 12-week-old mice of either sex were used throughout the study. C57BL/6 mice were produced at the animal facilities of ISREC, Lausanne, Switzerland. C57BL/10, B10.A(5R), BALB/c, and CBA/Ca mice were produced at the animal facilities of CMU, Geneva, Switzerland. The original breeding pairs for these mice originated from the Jackson Laboratories, Bar Harbor, ME.

Immunizations

Twenty micrograms of RI and RI-(NANP)$_3$ without carrier were emulsified 1:1 in complete Freund's adjuvant (Difco Laboratories, Detroit, MI) and 50 μl of the suspension were injected at the base of the tail. Fifteen days later, mice were boosted in the same way with peptides emulsified in incomplete Freund's adjuvant (Difco Laboratories). Fifteen days later, they were boosted again with 20 μg of peptides diluted in saline (500 μl/mouse, i.p.). Mice were bled several times by retro-orbital plexus puncture until 6 days after the last injection. Single sera were tested by ELISA; pools of mouse sera were tested by IFA and by inhibition of sporozoite invasion of human liver cells (see below).

Peptide-induced proliferation assay

Eight to 10 days after the second immunization with RI-(NANP)$_3$, inguinal and periaortic lymph nodes were removed. Cells were resuspended in DMEM containing L-glutamine (2 mM), HEPES buffer (25 mM), 2-mercaptoethanol (5×10^{-5} M) and FBS (5%) and were seeded at 2×10^5 cells per 200 μl cultures in round-bottomed microtitre plates (Greiner, Nürtingen, FRG) in the presence of peptides at the desired concentrations. The cultures were pulsed with 1 μCi of [^{3}H]thymidine on Day 4, the cells harvested 18 hr later, and thymidine incorporation was measured by liquid scintillation counting.

Immunofluorescence assay (IFA)

Antibodies reacting with *P. falciparum* sporozoite-surface antigens were measured by IFA using wet preparations of sporozoites attached to poly-L-treated glass slides, as described elsewhere (Druilhe *et al.*, 1986). In some experiments wet preparations of *P. yoelii* (17XL strain) and air-dried preparations of *P. yoelii*, *P. vivax* (Sal 1 strain) and *P. gallinaceum* sporozoites were employed.

Inhibition of sporozoite penetration into human liver cells

The inhibition assay was carried out as described previously (Mazier *et al.*, 1986). Briefly, human hepatocytes obtained from a liver biopsy were seeded in culture at a concentration of 10^5 cells per chamber in eight-chamber plastic Lab-Tek slides (Mile Laboratories Inc., Naperville, IL) and cultured for 24–48 hr before sporozoite inoculation. Sporozoites of *P. falciparum* were obtained from *Anopheles stephensi* after feeding through an artifical membrane on gametocytes from cultures of the NF54 strain. Salivary glands, aseptically dissected, were homogenized with culture medium in a tissue homogenizer and 25 μl containing 50,000 sporozoites were added to each chamber containing 25 μl of a 1/5 dilution of the test sample. The number of sporozoites that invaded hepatocytes and developed into trophozoites was determined after 2 and 6 days of culture with a fluorescent assay using a mouse monoclonal antibody directed against the *P. falciparum* CS protein.

RESULTS

Immunogenicity of RI in individuals naturally exposed to malaria infection

In order to investigate the possibility that anti-RI antibodies could be induced by sporozoites in individuals naturally exposed to malaria infection in endemic countries, an ELISA was developed employing a 15 amino acid synthetic peptide reproducing the RI sequence of *P. falciparum* CS protein. Randomly selected sera from 93 adults living in a rural community in Gabon (Del Giudice *et al.*, 1987b) were tested for the presence of anti-RI and anti-(NANP)$_{40}$ antibodies, and the results were compared with those obtained with 102 sera from healthy blood donors living in Geneva. As shown in Fig. 1, 39 sera were positive for anti-RI antibodies and 87% of these were also positive for anti-(NANP)$_{40}$ antibodies. However, of 61 sera

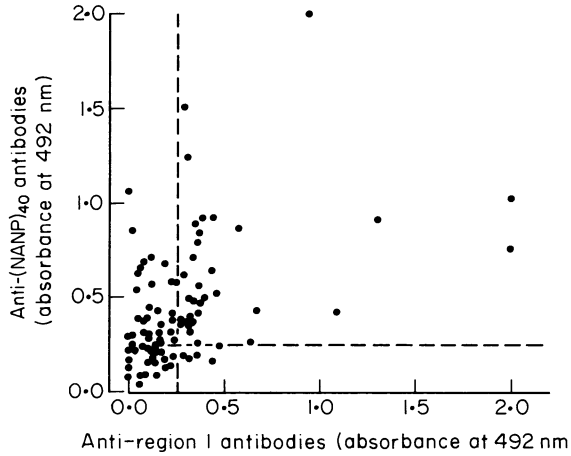

Figure 1. Anti-$(NANP)_{40}$ and anti-RI antibodies in sera from adults living in Okondja, Gabon. Sera diluted 1:200 were tested individually by ELISA in wells coated with $(NANP)_{40}$, 1 μg/ml, or with RI peptide, 10 μg/ml. Dotted lines represented the upper limit of normal value (98th percentile: 0·25 OD), obtained by testing 102 sera from normal blood donors from Geneva.

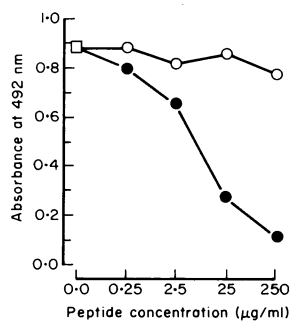

Figure 2. Specificity of human anti-RI antibodies. Serum from an adult living in Gabon, was diluted 1:200 and mixed with different concentrations of $(NANP)_{40}$ (○) or RI (●) peptide (1 hr at 37°) before being tested by ELISA for anti-RI antibodies.

positive for $(NANP)_{40}$ antibodies, only 34 (55·7%) had anti-RI antibodies; whereas, among sera negative for anti-$(NANP)_{40}$ antibodies, five (15·6%) were positive for anti-RI antibodies. There was a statistically significant correlation between anti-$(NANP)_{40}$ and anti-RI antibodies in these sera (Spearman rank correlation: $r = 0{\cdot}43$; $P < 0{\cdot}001$).

In competitive experiments, anti-RI antibodies appeared not to cross-react with the repetitive (NANP) sequence. In fact, their binding to solid-phase RI peptide was specifically inhibited by preincubation with RI, but not by preincubation with $(NANP)_{40}$ peptide (Fig. 2).

Immunogenicity of RI and RI-$(NANP)_3$ peptides in mice

In order to investigate the immunogenicity of the non-repetitive epitope RI in mice, groups of inbred strains of mice were immunized with RI peptide, 20 μg in Freund's adjuvant at the base of the tail. After three immunizations with carrier-free RI, no mice produced antibodies against this peptide (data not shown).

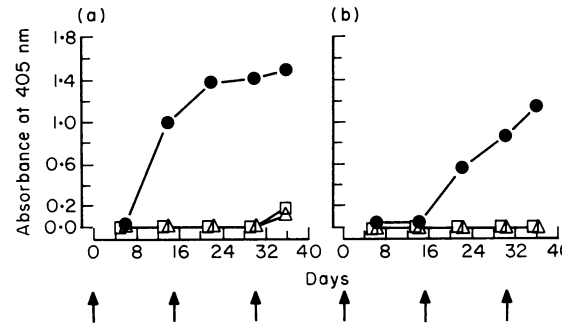

Figure 3. Antibody response to RI-$(NANP)_3$ peptide in mice. Five to nine C57BL/6 (●), BALB/c (□) and CBA/Ca (△) mice were immunized three times, 2 weeks apart, with RI-$(NANP)_3$ peptide, 20 μg in Freund's adjuvant at the base of the tail and were bled on different days. Sera diluted 1:200 were tested individually by ELISA in wells coated with $(NANP)_{40}$ 1 μg/ml (a), or with RI peptide, 10 μg/ml (b). Arrows indicate the days of immunization.

However, when C57BL/6 mice were immunized with the RI-$(NANP)_3$ peptide, anti-(NANP) antibodies were detectable 14 days after the first injection and the antibody levels increased after the second and third injections (Fig. 3a). Concomitantly, these mice also produced anti-RI antibodies, as detected by ELISA employing RI peptide for the coating of the plates. Anti-RI antibodies appeared after the second immunization with RI-$(NANP)_3$ and increased after the third injection (Fig. 3b). Non-H-2^d mice [BALB/c (H-2^d) and CBA/Ca (H-2^k)] immunized with RI-$(NANP)_3$ produced neither anti-$(NANP)_n$ nor anti-RI antibodies, even after the third injection.

In order to investigate the specificity of the anti-RI antibodies, sera from C57BL/6 mice immunized with $(NANP)_{40}$ and with RI-$(NANP)_3$ were preincubated with $(NANP)_{40}$ or RI peptides and then tested by ELISA in microwells coated with $(NANP)_{40}$ or with RI. As shown in Fig. 4, only the homologous peptide inhibited the binding of antibodies to the relevant peptide-coated plates.

A pool of sera from C57/BL6 mice immunized with RI-$(NANP)_3$ peptide recognized wet preparations of *P. falciparum* sporozoites by IFA at titres similar to those observed with sera from C57BL/6 mice immunized with carrier-free $(NANP)_{40}$ (1:500,000). A pool of sera from BALB/c mice gave negative results. In spite of the expected homology of the RI sequences among different *Plasmodium* species, such sera failed to recognize wet preparations of *P. yoelii* sporozoites by IFA. However, when air-dried sporozoites were employed for IFA, a positive fluorescence was observed on *P. yoelii* (1:50) and *P. vivax* (1:800), but not *P. gallinaceum* sporozoites.

Furthermore, pools of anti-RI-$(NANP)_3$ sera from C57BL/6 mice were inhibiting sporozoite penetration into cultured human liver cells, with a high degree of activity (86%). A pool of sera from immunized BALB/c mice did not show any inhibition.

Peptide-induced cell proliferation

Experiments were then conducted in order to investigate the peptide-driven cell proliferation *in vitro* in C57BL/6, C57BL/10, B10.A(5R) (all of them I-A^b) and BALB/c (I-A^d) mice 8–10 days after the second immunization with the RI-$(NANP)_3$ peptide. Lymph node cells from C57BL/6 mice proliferated well in the

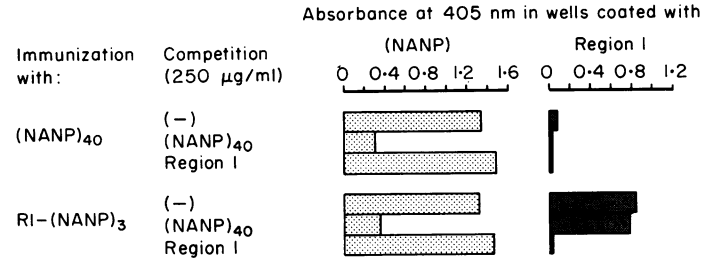

Figure 4. Specificity of anti-$(NANP)_n$ and anti-RI antibodies in groups of five to six C57BL/6 mice immunized with either $(NANP)_{40}$ or RI-$(NANP)_3$ peptide. Sera taken 6 days after the last immunization and diluted 1:200 were mixed with $(NANP)_{40}$ or RI peptides (250 μg/ml) (hr at 37°) before being tested by ELISA for anti-$(NANP)_{40}$ or anti-RI antibodies.

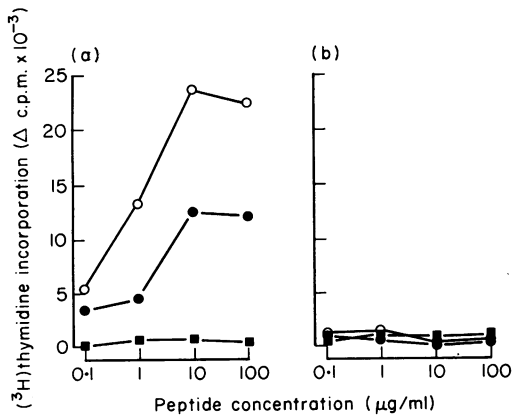

Figure 5. Peptide-driven cell proliferation. Lymph node cells were taken from C57BL/6 (a) and BALB/c (b) mice 8–10 days after the second immunization with RI-$(NANP)_3$ peptide (see legend to Fig. 3). Cells, 2×10^5, were seeded in triplicate in round-bottomed microwells in the presence of different concentrations of RI-$(NANP)_3$ (○), $(NANP)_4$ (●) and RI (■) peptides. Cell proliferation was evaluated by adding 1 μCi of [^{3}H]thymidine per culture on Day 4, and harvesting cells 18 hr later. Results are expressed as the difference in c.p.m. obtained in wells with peptides and in wells with DMEM. Background values ranged from 1,400 to 2,500 c.p.m.

presence of RI-$(NANP)_3$, as well as in the presence of $(NANP)_4$ peptide (10 μg/ml). However, RI peptide alone was ineffective, at any concentration tested, in stimulating proliferation (Fig. 5a). Similar proliferative patterns were observed in assays performed using lymph node cells from C57BL/10 and B10.A(5R) mice. On the other hand, cells from BALB/c mice did not proliferate in the presence of any peptide (Fig. 5b).

DISCUSSION

Previous work by other investigators has shown that non-repeated, group-conserved CS RI and RII are able to induce an immune response in animals when these epitopes are injected as synthetic peptides conjugated to heterologous carrier proteins (Ballou *et al.*, 1985; Vergara *et al.*, 1985b). It was concluded that such regions were not immunogenic under natural exposure to whole sporozoites because (i) no anti-RI or RII monoclonal antibody was ever obtained after fusion of myeloma cells with spleen cells from mice immunized with sporozoites; and (ii) all anti-sporozoites antibodies produced by individuals living in malaria endemic countries appeared to be directed against the CS repetitive epitope. However, the immunogenicity of non-repetitive epitopes of the *P. knowlesi* CS protein was shown recently in monkeys and rabbits immunized with sporozoites in which antibodies specifically recognizing repetitive and non-repetitive synthetic epitopes were detectable (Vergara *et al.*, 1985a; Sharma *et al.*, 1986).

This work demonstrates that antibodies specifically directed against one non-repeated CS sequence (RI) are produced by individuals exposed to natural malaria infection in endemic areas. The great majority (87%) of sera containing anti-RI antibodies also contained anti-$(NANP)_{40}$ antibodies. This would suggest that the repetitive (NANP) epitope may act as a carrier protein for the immune response against RI in a natural human *P. falciparum* malaria infection. However, other, not yet recognized, T-helper epitopes could also be present on the sequence of *P. falciparum* CS protein, helping the production of both anti-$(NANP)_{40}$ and anti-RI antibodies *in vivo*. In fact, Good *et al.* (1987) have shown that a non-repetitive sequence from the *P. falciparum* CS protein sequence could function as a T-helper epitope for the immune response against the repetitive $(NANP)_n$ sequence in I-A^k mice. The role (if any) of these anti-RI antibodies is unknown. Previous studies have shown that anti-RI antibodies from immunized animals do not inhibit sporozoite penetration into human hepatoma (Ballou *et al.*, 1985) or hepatic (Mazier *et al.*, 1986) cells in culture. This finding contrasts with recent data showing that a synthetic amino acid sequence belonging to *P. falciparum* and *P. knowlesi* CS RI can bind to human hepatoma cell line HepG2-A16 and that a specific rabbit IgG can inhibit this binding (Aley *et al.*, 1986). In the present work we observed that sera from C57BL/6 mice immunized with RI-$(NANP)_3$ efficiently inhibited sporozoite penetration into human hepatic cells, but at a degree similar to that observed with sera from C57BL/6 mice immunized with $(NANP)_{40}$.

In a murine model, RI peptide was not immunogenic, whereas RI-$(NANP)_3$ peptide was as immunogenic as $(NANP)_{40}$ in inducing anti-(NANP) antibodies in H-2^b mice. These mice also produced anti-RI antibodies that did not recognize the repetitive epitope. However, non-H-2^b mice produced neither anti-(NANP) nor anti-RI antibodies. The occurrence of anti-RI antibodies alone in those mice capable of producing anti-(NANP) antibodies, and the absence of specific antibodies in mice immunized with RI peptide alone, suggests that the RI sequence does not contain any T-helper epitope, confirming the results recently reported by Good *et al.* (1987). The results obtained in cell proliferation assays demonstrated that $(NANP)_3$ functioned as an immunogenic carrier for the immune response to the contiguous RI epitope. Indeed, lymph node cells

from immune I-A^b mice positive for both anti-(NANP) and anti-RI antibodies proliferated *in vitro* only in the presence of RI-$(NANP)_3$ or $(NANP)_4$ peptides, but never in the presence of RI peptide alone. It should be noted that the RI-$(NANP)_3$ peptide used in this study lacks two amino acids (asparagine and proline) which, in the native CS protein, separate the RI from the repetitive sequence (Dame *et al.*, 1984; Lockyer & Schwarz, 1987). This could create a particular folding of the peptide with the generation of a new T-helper epitope. However, the persistence of the genetic restriction in the immune response to this peptide, the pattern of the antibody responses and the results of lymphocyte proliferation do not suggest a major role of such a putative new T site. Thus, in mice immunized with the RI-$(NANP)_3$ peptide the $(NANP)_3$ sequence probably represents the T-helper epitope for the immune response to RI.

Finally, as expected from the homology existing among the region I sequences so far known, serum antibodies from C57BL/6 mice immunized with the RI-$(NANP)_3$ peptide recognized *P. yoelii* and *P. vivax*, in addition to *P. falciparum*, but not *P. gallinaceum* sporozoites, thus confirming the results reported previously by other investigators (Ballou *et al.*, 1985; Vergara *et al.*, 1985b). However, such a cross-reactivity was observed only when air-dried sporozoites were employed for IFA, not on wet preparations of sporozoites. Therefore, one could hypothesize that the RI sequence is not easily accessible on the intact sporozoite surface for antibody recognition The drying procedure could have exposed this epitope through disruption of the sporozoite surface integrity.

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