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MODERN IMMUNOLOGICAL APPROACHES TO ASSESS MALARIA TRANSMISSION AND IMMUNITY AND TO DIAGNOSE PLASMODIAL INFECTION

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The present paper reviews our recent data concerning the use of immunological methods employing monoclonal antibodies and synthetic peptides to study malaria transmission and immunity and to diagnose plasmodial infection. As concerns malaria transmission, we studied the main vectors of human malaria and the plasmodial species transmitted in endemic areas of Rondônia state, Brazil. The natural infection of anopheline was evaluated by immunoradiometric assay (IRMA) using monoclonal antibodies to an immunodominant sporozoite surface antigen (CS protein) demonstrated to be species specific. Our results showed that among six species of Anopheles *found infected,* An. darlingi *was the main vector transmitting* Plasmodium falciparum *and* P. vivax *malaria in the immediate vicinity of houses. In order to assess the level of anti-CS antibodies we studied, by IRMA using the synthetic peptide corresponding to the repetitive epitope of the sporozoite CS protein, sera of individuals living in the same areas where the entomological survey has been performed. In this assay the prevalence of anti-CS antibodies was very low and did not reflect the malaria transmission rate in the studied areas. In relation to malaria diagnosis, a monoclonal antibody specific to an epitope of a 50 kDa exoantigen, the major component of supernatant collected at the time of schizont rupture, was used as a probe for the detection of* P. falciparum *antigens. This assay seemed to be more sensitive than parasitological examination for malaria diagnosis since it was able to detect plasmodial antigens in both symptomatic and asymptomatic individuals with negative thick blood smear at different intervals after a last parasitologically confirmed attack of malaria.*

Key words: malaria – diagnosis – *Anopheles* – plasmodial infection

The development of new immunological methods using monoclonal antibodies and synthetic peptides in the past decade contributed for a better knowledge on malaria transmission and immunity as well as for the diagnosis of plasmodial infection. This report review data obtained applying these methods as tools to evaluate the malaria situation in the state of Rondônia. The results presented here, have been originally published elsewhere (Oliveira-Ferreira et al., 1990, 1991; Ferreira-da-Cruz et al., 1991).

Concerning malaria transmission, studies on the sporozoite stage led to the identification of an immunodominant sporozoite surface antigen (CS protein) demonstrated to be species specific. The development of an Immunoradiometric Assay – IRMA (Zavala et al., 1982) using anti-CS monoclonal antibodies allowed the identification of the infecting *Plasmodium* species in the invertebrate host, an event that entomologists were so far unable to do. Using this assay we studied the main vectors of human malaria and the plasmodial species transmitted, by examining 12336 *Anopheles* for the presence of sporozoite in endemic areas of Rondônia State.

The nucleotide sequence of the sporozoite CS protein showed intramolecular tandem repeats of few aminoacids (3 repeats of 4 aminoacids – NANP, in the case of *P. falciparum* for instance) that constitute the main epitope of CS protein. Such repetitive epitopes, which appear to be the target of antibody recognition, have been produced both by chemical synthesis and recombinant DNA technology and are now being tested as potential malaria vaccines. Using a synthetic peptide, an IRMA was developed to assess the level of

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anti-sporozoite antibody (Zavala et al., 1986). This assay allowed us to test anti-*P. falciparum* CS protein antibodies in 1636 individuals living in endemic areas of Rondônia state northwestern Brazil.

As concerns malaria diagnosis it is well known that in endemic regions malaria infection is diagnosed by parasitological methods. Besides the intensive labor and the need of a skilled microscopist to perform the examination of blood smears, low parasitaemia may lead to false negative results. A monoclonal antibody specific to an epitope of a 50 kDa exoantigen (Delplace et al., 1985), which is a breakdown product of a 126 kDa schizont protein, the major component of supernatant collected at the time of schizont rupture, was used as a probe for the detection of *P. falciparum* antigens. This assay seemed to be more sensitive than parasitological examination for malaria diagnosis since it was able to detect plasmodial antigens in both symptomatic and asymptomatic individuals with negative thich blood smear at different intervals after a last parasitologically confirmed attack of malaria.

MALARIA SITUATION IN BRAZIL

Malaria is the most prevalent disease in the world and the number of cases increases each year in diverse geographical areas including Brazil where the number of registered cases jumped from 52,000 in 1970 to 577,590 in 1990. However 97% of the cases are from the Amazon region or come from this area that occupies half of the national territory but holds only 12% of the country population.

The increase in the malaria frequency in this area in the last 30 years is due to the migration of human populations mostly from non-endemic areas attracted by prospecting mining and settlement projects sponsored by the government. Consequently marked environmental changes have occurred in this region allowing a great contact of man and vectors leading to the rise in malaria transmission. This can be exemplified by the analysis of the case of the state of Rondônia, where 238,270 cases were registered in 1989 almost 50% of the total cases of the country and a 200% population increase from 1980 to 1987.

These data show that it was necessary to perform epidemiological studies to determine vectors of human malaria in the present situation in the state of Rondônia after the important environmental alteration due to human migration and colonization programs in this area.

For this reason we studied the vectors of human malaria and their role in the process of malaria transmission in endemic areas of Rondônia state (northwestern – Brazil).

POPULATION STUDIED

In Rondônia four endemic areas were studied: the town of Ariquemes (rural area – Linha), two recent settlements, Machadinho and Cujubim, both started in 1985, and Itapoã do Oeste. According to the Ministry of Health, malaria is endemic in all four localities without a marked seasonal variation during the studied period (unpublished annual report of the Ministry of Health). In some of the localities (Machadinho and Cujubim) the number of registered cases of malaria was higher than the population (Table I).

TABLE I

Number of registered malaria cases in the studied localities

Localities	POP/NC 1986	POP/NC 1987	POP/NC 1988
Ariquemes	96.793/43.515	112.439/ 75.969	130.659/81.010
Machadinho	9.060/17.711	13.000/ 23.972	21.220/ 5.445
Cujubim	1.941/ 2.259	2.051/ 2.501	[a]2.461/ 8.431
Itapoã	3.156/ 2.537	4.713/ 4.284	[a]7.038/ 3.143
Total	110.950/66.022	132.203/106.726	161.371/98.029

POP: population, NC: number of cases; *a* estimative.

MALARIA TRANSMISSION BY ANOPHELINE

Concerning malaria transmission, in order to incriminate a species of *Anopheles* as a malaria vector it is necessary to detect sporozoite in their salivary glands. In this study, the natural infection of anopheline in four localities in the municipality of Ariquemes was evaluated by an immunoradiometric assay – IRMA (Zavala et al., 1982) using monoclonal antibodies species (*P. falciparum* and *P. vivax*) specific (Oliveira-Ferreira et al., 1990). In addition it is indispensable to show their epidemiological importance in malaria transmission in the region. This has been done by studying the anopheline behavior through different types of captures on human bait indoors, outdoors in the close vicinity of houses (1-2m) and far from houses (40-60m), and in forest. Animal bait were also used in open terrain (Lourenço-de-Oliveira et al., 1989).

A total of 12336 *Anopheles* of 12 species have been collected, 61 mosquitos belonging to 6 species were found to be positive by IRMA: 47 *An. darlingi*, 5 *An. triannulatus*, 4 *An. albitarsis*, 2 *An. brasiliensis*, 2 *An. strodei*, and 1 *An. oswaldoi*. As concerns the *Plasmodium* species 41 *Anopheles* harboured *P. falciparum* and 20 were infected with *P. vivax*. These results show clearly that *An. darlingi* was the most frequent species found infected and in some localities the only one. All these species had been previously found infected by direct examination of salivary glands (Classical method) or by Immunological tests (IRMA or ELISA) (Arruda et al., 1986; Tadei et al., 1988). Our data add to these finds the plasmodial species in *An. triannulatus*, only previously found infected with *P. vivax* in which we also detected *P. falciparum*. In *An. brasiliensis* only found infected by classical method (Deane et al., 1946), we have identified the infecting plasmodial species; *P. falciparum* and *P. vivax*. Immunological methods have a great advantage in relation to classical method because the former can identify the *Plasmodium* species in the *Anopheles* and dead mosquitoes can be used. However, recent studies have shown that monoclonal antibodies can also react with oocyst sporozoite (Lombardi et al., 1987; Posthuma et al., 1988). Therefore, as most of the studies done in the Amazon region including ours used the whole mosquitoes instead of head and thorax separately, the detection of a positive specimen does not necessarily mean the identification of a malaria vector. Indeed not all species of *Anopheles* can support the parasite development up to sporozoite stage in the salivary glands (Rosenberg, 1985). Therefore the incrimination of a malaria vector in the Rondonia state could only be done after extensive studies by IRMA/ELISA and/or by studying the *Anopheles* behavior.

In relation to the epidemiological importance of the species *An. darlingi* was by far the most abundant species (80.1%) followed by *An. triannulatus* (7.5%), *An. evansae* (3.0%), *An. albitarsis* (2.8%), *An. oswaldoi* (2.3%) and all remaining species being scarce. Comparing the incidence of the species biting man, again *An. darlingi* was more frequent, mostly in the immediate vicinity of and inside the houses (70%), less frequent far from houses and very rare in the deep forest (Table II).

Another important datum in our studies concerns the comparative captures performed in man and animal simultaneously. *An. darlingi* clearly showed feeding preferences for man. In the same way, 77% of infected *An. darlingi* was found biting man what did not occur with other species that were more frequent biting animals. These results, added to the high frequency of this species in the vicinity of houses and low frequency far from houses, suggest a high degree of antropophily of *An. darlingi* in the studied area.

We have also analysed the incidence of *Anopheles* species in malarious and non-malarious areas and we observed a close asssociation between the presence of *An. darlingi* and the existence of malaria. This species was more frequent in malarious (77.8%) than in non-malarious (22.2%) areas.

In the past, extensive studies performed in the Amazon region and other states (Galvão et al., 1942; Deane, 1947; Deane et al., 1948; Causey et al., 1946) showed that *An. darlingi* transmitted malaria inside houses. In our study a different behavior was observed. In our opinion the shifting from endophylic to exophylic might be the result of an acquired resistance of this vector to contact with insecticides sprayed all year around in the region.

In conclusion, our results on the malaria transmission showed that among 6 species of *Anopheles* found infected, *An. darlingi* was the main vector transmitting *P. falciparum* and *P.*

TABLE II

Species of collected *Anopheles* in different types of captures

Species	Human Bait				Animal bait	Total
	Inside houses No. (%)	Close to houses No. (%)	Far from houses No. (%)	Forest No. (%)	No. (%)	No.
An. darlingi	1572 (97.6)	7752 (92.8)	140 (42.3)	1 (0.7)	420 (22.3)	9885
An. triannulatus	11 (0.7)	239 (2.9)	81 (24.5)	93 (62.0)	507 (26.7)	931
An. evansae	2 (0.1)	89 (1.1)	14 (4.2)	– –	271 (14.3)	376
An. albitarsis	21 (1.3)	125 (1.5)	2 (0.6)	– –	198 (10.5)	346
An. oswaldoi	– –	50 (0.6)	63 (19.0)	50 (33.3)	125 (6.6)	288
An. strodei	1 (0.1)	20 (0.2)	4 (1.2)	– –	184 (9.7)	209
An. braziliensis	– –	24 (0.3)	5 (1.5)	– –	113 (6.0)	142
An. galvoi	4 (0.2)	11 (0.1)	15 (4.5)	– –	20 (1.1)	50
An. rangeli	– –	14 (0.2)	– –	1 (0.7)	32 (1.7)	47
An. nuneztovari	– –	22 (0.3)	– –	2 (1.3)	14 (0.7)	38
An. argyritarsis	– –	2 (0.0)	3 (1.0)	– –	10 (0.5)	15
An. mediopunctatus	– –	1 (0.0)	4 (1.2)	3 (2.0)	1 (0.1)	9
Total	1611 100	8349 100	331 100	150 100	1895 100	12336

vivax malaria in the immediate vicinity of houses. Therefore vector control measures used in the area based on insecticide spray inside houses are not sufficient. In relation to other species found infected, they could be considered as secondary vectors with little epidemiological importance.

ANTI-SPOROZOITE IMMUNITY

In view of the perspective of the availability of a malaria vaccine in a not too remote future, we have also studied, in parallel with the entomological survey the patterns of naturally occurring anti-sporozoite response in these areas and their relationship with the malaria transmission. This study was performed by IRMA using a synthetic peptide corresponding to the immunodominant epitope of CS protein.

Epidemiological studies carried out in several endemic areas, using synthetic peptides or recombinant proteins, reproducing the repeat region of the *P. falciparum* CS protein, or even whole sporozoite from salivary glands, showed that the frequency of anti-sporozoite antibodies increases with age in autochthonous individuals from African and Asian endemic areas of malaria and can reach up to 70% to 100% in subjects with more than 40 years of age (Nardin et al., 1979; Del Giudice et al., 1987; Tapchaisri et al., 1983). In Brazil, few data are available on the prevalence of anti-sporozoite antibodies in endemic areas of malaria (Tosta et al., 1986, Arruda et al., 1989).

Using the data from the entomological studies we have estimated the number of infective bites per man per night and our data show a high rate of transmission in the studied areas, an overall of 0.26 infective bites per man per night or 7.8 per month (Oliveira-Ferreira et al., 1991), figures that are comparable to some observed in other malaria endemic areas of Africa (Esposito et al., 1988; Druilhe et al., 1986).

In the same way the prevalence of anti-blood stage IgG antibodies shows that 50.1% of the population had contact with *Plasmodium*. In spite of these data, in the present study the frequency of individuals with antibodies to the CS repeat of *P. falciparum* was very low (Table III). In fact the prevalence of 6.0% for anti-(NANP)4 IgG antibodies differs from those observed in Gambia (70%), in Thailand (79%), and in Burkine Faso (88%) (Tapchaisri et al., 1983; Esposito et al., 1986; Beier et al., 1987). These differences could be explained by the fact that most of the studies performed in Africa and Asia concerned native populations of hyperendemic areas whereas in our studies we dealt with migrant populations mostly from malaria free areas of Brazil, and living in Rondônia state for two to four years. Low frequencies of anti-$(NANP)_4$ anti-

bodies were also observed in nonimmune temporary residents of Africa (Philpott et al., 1990; Miller et al., 1988). Therefore, it is possible that individuals concerned by this report have not been exposed enough to malaria infection to develop detectable levels of anti-$(NANP)_4$ antibodies or sporozoites in doses injected by the mosquitoes in the studied areas are poorly immunogenic. One other possibility could involve an immunosuppressive effect by the blood stage malaria parasites on the anti-$(NANP)_4$ antibodies response, as shown in mice and humans (Orjih & Nussenzweig, 1979; Marsh & Greenwood, 1986). Nevertheless it must be said that in the population studied only 11.8% of the population had detectable parasites in the thick blood smears and among these individuals 20% had anti-$(NANP)_4$ antibodies against 10% of individuals with negative thick blood smears.

TABLE III

Frequency of anti-NANP4 antibodies in the studied localities

Localities	IRMA Neg. No. (%)	IRMA Pos. No. (%)	Total No.
Ariquemes	433 (96.7)	15 (3.3)	448
Linha	506 (92.2)	43 (7.8)	549
Cujubim	291 (94.8)	16 (5.2)	307
Machadinho	291 (93.0)	96 (6.0)	313
Total	1521 (94.0)	96 (6.0)	1617

IRMA: immunoradiometric assay.

In responding individuals the antibody production was influenced by the previous malaria experience since subjects with more than 9 attacks of malaria in the past presented the higher frequency of anti-$(NANP)_4$ IgG antibodies (Table IV) suggesting that infective bites, in cumulative numbers, should occur before anti-$(NANP)_4$ antibodies develop at detectable levels. A higher frequency of IgG antibodies was also observed in individuals over 30 years of age, as in studies performed in Africa and Asia, and in those ranging from 31 to 40 years of age for IgM antibodies. These results can not be explained by the time of exposure of individuals to the risk of malaria infection since in our populations age does not necessarily reflect time of exposure as in natives of Africa and Asia and other factors including social and professional activities could be involved.

Finally, in view of the fact that in the studied population the prevalence of antibodies to $(NANP)_4$ does not reflect the malaria transmission rate and that the development of stage specific antibodies seems to depend on the exposure of cumulative number of sporozoites one could conclude that the population concerned by this report have not been exposed long enough to sporozoite antigens to develop such antibodies. One may also wonder if individuals could have been exposed and have had the evaluation of CS antibodies response at a time distant from the actual sporozoite exposure suggesting a short-lived antibody response. One non exclusive explanation for the present finding is that these anti-$(NANP)_4$ antibodies rise as a consequence of stimulation by blood stage cross-reacting antigens that would not be represented in the strains present in the localities concerned by our studies. Therefore follow-up studies of the immune response to the CS protein in these areas should be performed before definitive conclusions are drawn.

MALARIA DIAGNOSIS THROUGH ANTIGEN DETECTION

The transmission of malaria infection represents a risk for transfusion medicine in urban areas of endemic regions. This is also true in countries in which the disease is not prevalent since travel facilities from and to endemic countries, have increased the chances of including infected individuals among asymptomatic donors. The criterion of deferring any blood donor for 3 years after cessation of therapy for a confirmed case of malaria (WHO) can not be applied in malaria endemic areas where most of the individuals have several infections during the year. Donor screening by direct parasitological examination is labor intensive and not sensitive enough for use in large scale (Mackey et al., 1980). Conversely, malarial antibody detection is of limited value in blood banks located in areas where malaria is endemic since the presence of anti-plasmodial antibody, very frequent in these regions, does not necessarily reflect active infection.

The detection of *P. falciparum* circulating antigens has been proposed by several authors as a strategy to diagnose malaria infection and recently it was demonstrated that this methodology could be more sensitive than microscopic

TABLE IV

Frequency of anti-NANP4 antibodies according to the number of past attacks of malaria (NPA) in the studied localities

	IRMA									
	Ariquemes		Linha		Cujubim		Machadinho		Total	
NPA	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.
0	92	1	67	3	28	1	23	–	210	5
(%)	(99)	(1)	(96)	(4)	(97)	(3)	(100)	–	(98)	(2)
1-3	169	4	118	7	83	3	63	6	433	20
(%)	(98)	(2)	(94)	(6)	(97)	(3)	(91)	(9)	(96)	(4)
4-9	87	3	118	9	72	7	88	3	365	22
(%)	(97)	(3)	(93)	(7)	(91)	(9)	(97)	(3)	(94)	(6)
>9	75	7	202	24	108	5	115	12	500	48
(%)	(91)	(9)	(89)	(11)	(96)	(4)	(91)	(9)	(91)	(9)
Total	423	15	505	43	291	16	289	21	1508	95
	(97)	(3)	(92)	(8)	(95)	(5)	(93)	(7)	(94)	(6)

IRMA: immunoradiometric assay.

examination (Khusmith et al., 1988). Therefore data provided by the use of such a sensitive test could be used as indicators of present or recent infection for blood screening in malarial endemic areas.

In the availability of an appropriate monoclonal antibody (Delplace et al., 1985), we have standardized an ELISA for the search of malarial circulating antigens (Ferreira-da-Cruz et al., 1991). The original procedure (Fortier et al., 1987) was modified and became 10 times more sensitive. This increase in sensitivity enabled us (a) to confirm malaria diagnosis in *P. falciparum* and *P. vivax* parasitized individuals, (b) to detect malaria infeciton in symptomatic individuals with negative thick blood smear and (c) to detect plasmodial antigens in asymptomatic subjects with negative thick blood smear and recent history of malaria.

Sera were obtained from subjects living in an endemic region (Ariquemes). They corresponded to 175 polyinfected individuals and 17 prime-infected subjects. These individuals were distributed in three groups: group A consisted of 70 malarious patients with positive thick blood smear (TBS) (34 with *P. falciparum*, 33 with *P. vivax* and 3 with mixed infections); group B included 25 symptomatic individuals with negative TBS and studied at different intervals after a last parasitologically confirmed attack of malaria (due to *P. falciparum* in 16 cases, to *P. vivax* in 8 and to mixed infections in 1 case) and group C comprised 96 asymptomatic individuals studied at different intervals after a last positive TBS (61 with *P. falciparum*, 33 with *P. vivax* and 2 with mixed infections).

The sensitivity of four different protocols in detecting *P. falciparum* antigens was studied. The monoclonal for coating and a polyclonal hyperimmune human gamma globulin as the second antibody, was shown to be the most sensitive and consequently this system was selected for the detection of soluble malarial antigens in human sera.

Among patients with active disease (Group A) 100% of the cases of *P. falciparum* or mixed infections and 91.7% of those with *P. vivax* malaria were positive for the presence of plasmodial antigens. The lower parasitaemia detected was 0.0003% for *P. falciparum* and 0.001% for *P. vivax* malaria. The test was also capable to detect plasmodial antigens in 13(52%) of the symptomatic individuals studied. The increase in the frequency of antigen positive individuals with time after a confirmed attack of malaria, in our opinion, may reflect the increased risk of reinfection in these individuals that remained living in endemic areas. In relation to individuals with positive TBS, no direct correlation was observed between parasitaemia and ELISA ratio values.

As concerns symptomless individuals, tested at the same interval periods, after a confirmed attack of malaria, positive results were found

in 18.8% of individuals studied ≤ 30 days, 0% of those examined >30 <180 days and in 16.1% of those tested ≥ 180 ≤ 360 days. These data may also represent reinfection of the individuals.

Comparing the detection of antigens by ELISA and of antibodies by Immunofluorescence antibody test (IFAT), in prime and polyinfected patients with parasitologically confirmed malaria, we have observed that for both groups of individuals, antigen screening was more sensitive than the antibody detection. The sensitivity for antigen detection seems to be greater for prime than for polyinfected individuals but actually all prime-infected individuals studied have had *P. falciparum* malaria, and the negative results obtained were those due to *P. vivax* infections.

According to these preliminary data one could conclude that this assay was shown to be very sensitive since it was capable of detecting circulating plasmodial antigens in 100% of patients with parasitologically confirmed *P. falciparum* malaria and also in 33.3% of symptomatic individuals with negative TBS and even in 38.7% of asymptomatic subjects examined up to 30 days after a last positive TBS. Comparison of antigen detection by ELISA and antibody screening by IFAT in parasitized individuals, points to a greater sensitivity of the former, suggesting that the presence of circulating antigens can be a better indicator of disease activity than the existence of serum antibodies.

As between 30 and 180 days after a last positive TBS subjects concerned by the present study are free of circulating antigens, further studies are necessary to allow the conclusion of whether or not the donation of Ag free blood in this period is safe.

Finally, this assay, by virtue of its high sensitivity and the facilities in processing a large number of specimens, can prove to be useful in endemic areas for the recognition of asymptomatic malaria and consequently for the screening of blood donors.

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