Mem. Inst. Oswaldo Cruz, Rio de Janeiro, Vol. *86* (2): 153-158 apr./jun. 1991

CELLULAR IMMUNE RESPONSE OF HUMANS TO THE CIRCUMSPOROZOITE PROTEIN OF *PLASMODIUM VIVAX*

MAURICIO M. RODRIGUES/*/ +; ARARIPE P. DUTRA** & NOBUKO YOSHIDA*

Department of Medical and Molecular Parasitology, New York University, New York, NY 10010
*Disciplina de Parasitologia, Escola Paulista de Medicina, Caixa Postal 20207, 04023, São Paulo, SP, Brasil
**Superintendência de Controle de Endemias (SUCEN), Rua Cardeal Arcoverde 2878, 05408, São Paulo, SP, Brasil

The cellular immune response to the circumsporozoite (CS) protein of Plasmodium vivax *of individuals from malaria-endemic areas of Brazil was studied. We examined the* in vitro *proliferative response of the peripheral blood mononuclear cells (PBMC) of 22 individuals when stimulated with a CS recombinant protein (rPvCS-2) and two other synthetic peptides based on the sequence of the* P. vivax *CS protein. Seven of the individuals from malaria-endemic area displayed an antigen-specific* in vitro *proliferative response to the recombinant protein PvCS-2 and one out of 6, proliferative response to the peptide 308-320. In contrast, none of the individuals displayed a proliferative response when stimulated with the* D/A *peptide which represent some of the repeated units present in this CS protein. Our study, therefore, provides evidence for the presence, within the major surface antigen of* P. vivax *sporozoites, of epitopes capable to induce proliferation of human PBMC.*

Key words: circumsporozoite protein – sporozoite immunity – malaria – T cell epitopes

Sporozoites are the infective stage of malaria parasites present in the salivary glands of *Anopheles* mosquitoes. Immunization with irradiated sporozoites is known to induce protective immunity against rodent, simian and human malaria. This immunity is species and stage specific (Cochrane et al., 1980). $CD8^+$ T lymphocytes, gamma-interferon and antibodies play a major role in the protective immunity induced in mice by irradiated sporozoites (Schofield et al., 1987; Nussenzweig & Nussenzweig, 1989). These findings indicate that a synthetic or recombinant vaccine, capable of inducing a long lasting and specific immunity against sporozoite infection, would be most effective if it contained parasite derived epitopes recognized by antibodies as well as by T helper and cytotoxic T lymphocytes.

The predominant surface antigen of mature and infective sporozoites is the circumsporozoite (CS) protein. They constitute a family of structurally related proteins, present in all plasmodial species and are believed to play an important role in sporozoite infectivity (Nussenzweig & Nussenzweig, 1985). One of the similarities among these proteins resides in the central region of the molecule which is composed of tandemly repeated amino acid sequences (Nussenzweig & Nussenzweig, 1985). These repeat sequences are recognized by most sporozoite-specific antibodies (Zavala et al., 1985). Immunization of mice with synthetic peptides, representing these tandemly repeated sequences of *Plasmodium berghei,* coupled to tetanus toxoid, induced high antibody levels and resulted in protection of 87% of animals against sporozoite challenge (Zavala et al., 1987).

The major tandemly repeated sequence (NANP) of *P. falciparum* appears to be poorly recognized by murine and human T cells (Good et al., 1986, 1988; Sinigaglia et al., 1988a, b). Efforts are therefore being made to define other epitopes recognized by CS primed T cells. Several epitopes of the *P. falciparum* CS protein which are recognized by proliferating human T cells from individuals living in malaria-endemic areas and by a sporozoite vaccinated volunteer, have been described (Good et al., 1988; Siniga-

This work was supported by WHO and Rockfeller Foundation.
+ Research Fellow CNPq.

Received 5 December 1990.
Accepted 10 April 1991.

glia et al., 1988a; Nardin et al., 1989). There is no consensus regarding the presence of an immunodominant helper T cell epitope in the CS protein. The synthetic peptide corresponding to one of the T cell epitopes was found to be a "promiscuous" epitope, since antigen presenting cells of different HLA class II haplotype were capable of presenting it to antigen-specific cloned human $CD4^+$ cells (Sinigaglia et al., 1988b).

The aim of the current study was to characterize whether peripheral blood mononuclear cells (PBMC) of individuals from malaria-endemic areas, in Brazil, displayed a proliferative response *in vitro* when stimulated with a yeast derived recombinant *P. vivax* CS protein and two synthetic peptides based on the sequence of this protein.

MATERIALS AND METHODS

Recombinant and synthetic peptides – The recombinant protein PvCS-2, which encompasses amino acids 77 to 340 of the *P. vivax* CS protein (Belém strain), was produced and purified as described (Barr et al., 1987). The *D/A* peptide (DGQAGDRAAGQPAGDRA), which represents two of the repeat units of the central region of the *P. vivax* CS protein, was synthesized as previously reported (Romero et al., 1987). Another synthetic peptide representing part of the amino acid sequence P308-320 of the C-terminal region of the *P. vivax* CS protein was also used (Rodrigues et al., 1991).

Blood samples – Blood samples were obtained from individuals coming from several *P. vivax*-endemic areas of the Amazon region, Brazil, during the year of 1988. Detailed description including age, sex, number of malarias and time after the last infection is summarized in Table I.

In vitro *proliferation assays* – The culture medium used was composed of RPMI 1640 (GIBCO, Grand Island, NY) supplemented with 2 mM L-glutamine, 5×10^{-5} M 2- mercaptoethanol, 1 mM sodium pyruvate, 1% (vol/vol) non-essential amino acid solution and 10% (vol/vol) normal human serum.

Peripheral blood mononuclear cells (PBMC) were purified using a Ficoll-Hipaque gradient (Pharmacia, Uppsala, Sweden). The cells harvested from the plasma-Ficoll interface were washed 3 times in phosphate buffered saline (PBS) and resuspended in culture medium. The concentration of the cells was adjusted to 10^6 viable cells per ml. The assay was performed in 96 well flat bottom Costar plates. 0.2 ml of the cell suspension was added to each well, together with 20 μl of the antigen, at the desired concentration. Each determination was performed in triplicate. The cultures were incubated at 37 °C in an atmosphere of 5% CO_2 for six days. During the last 18-24 h of culture, 1 μCi of tritiated thymidine (^{3}H-TdR; New England Nuclear, Boston, MA) was added to each well.

At the end of the incubation period, human lymphocytes were collected with the aid of a semi-automatic cell harvester and the amount of radioactive thymidine, which had been incorporated, was measured by liquid scintillation spectroscopy. The results are presented as the mean of triplicates.

Other reagents – Concanavalin A was purchased from Calbiochem (La Jolla, CA), and was used at a final concentration of 100 μg/ml.

Statistical analysis – The null hypothesis was that the two populations (individuals from malaria-endemic areas and individuals never exposed to malaria) are identical regarding the distribution of their individual stimulation indices (SI). The null hypothesis was tested by the non parametrical Kolmogorov-Smirnov test (Hollander & Wolfe, 1973), designed to detect all possible deviation from the hypothesis. The value of the SI of each individual, observed upon stimulation of the PBMC with the rPvCS-2 protein were used in this analysis.

RESULTS

The cellular immune response of PBMC of individuals from malaria-endemic areas was investigated using a recombinant CS protein (rPvCS-2) and two synthetic peptide, both based on the sequence of the *P. vivax* CS protein. The cells were tested for their capacity to produce a proliferative response upon *in vitro* stimulation with these antigen.

The corresponding data are tabulated in Tables I and II as the SI, determined by dividing the ^{3}H-TdR uptake of cultures with antigen by the uptake of those without antigen. Upon

TABLE I

Malaria endemic area individuals

#	Sex	(age)	No. of malarias	Days after the last	Parasitemia[a]	Species[b]	SI[c]
1	M	(32)	19	20	–	*P. f.*	1.68
2	M	(38)	04	25	–	*P. f.*	0.97
3	M	(56)	03	0	+	*P. v.*	1.02
4	M	(32)	22	126	–	*P. v.*	1.71
5	M	(29)	26	153	–	*P. v.*	1.45
6	M	(37)	10	44	–	*P. v.*	1.92
7	F	(12)	12	31	–	*P. v.*	1.54
8	M	(25)	10	36	–	*P. f.*	1.71
9	M	(39)	10	0	+	*P. v.*	0.88
10	M	(48)	31	0	+	*P. f.*	1.43
11	M	(60)	05	21	–	*P. v.*	1.21
12	M	(34)	09	0	+ *(P. v.)*	*(P. v./P. f.)*	1.43
13	M	(34)	15	0	+	*P. v.*	1.35
14	F	(28)	20	70	–	*P. v.*	1.35
16	F	(28)	04	25	–	*P. v.*	*3.38*
17	M	(52)	34	0	+ *(P. v.)*	*(P. v./P. f.)*	*4.56*
18	M	(33)	04	62	–	*P. v.*	*3.85*
19	M	(40)	10	56	–	*P. v.*	*2.42*
20	M	(20)	07	0	+	*P. v.*	*4.58*
21	F	(27)	06	90 *(P. v.)*	–	*(P. v./P. f.)*	*3.96*
22	M	(29)	70	0	+	*P. v.*	*3.81*
Normal controls							
1	M	(27)	–	–	–	–	1.26
2	F	(30)	–	–	–	–	1.31
3	M	(46)	–	–	–	–	1.14
4	M	(38)	–	–	–	–	1.05
5	M	(26)	–	–	–	–	0.98
6	F	(34)	–	–	–	–	0.97
7	M	(42)	–	–	–	–	1.50
8	M	(50)	–	–	–	–	*2.70*

a: presence of erythocytic stages detected by blood smears at the day the blood was collected.
b: species of *Plasmodium* detected in previous malaria infections.
c: SI of PBMC upon stimulation with rPvCS-2, for detail see Table II.

stimulation with the recombinant protein PvCS-2, the lymphocytes of seven out of 22 individuals displayed a proliferative response, with SI greater than 2.0. The proliferative response of the PBMC of these individuals failed to correlate with the age, number of malarial episodes (Table I), or with the time of residence in an endemic area (data not shown). When PBMC from healthy individuals (never exposed to malaria) were analyzed, one out of eight presented a SI higher than 2.0, upon stimulation with 30 μg of the recombinant protein (Table I and II).

The cells of six individuals from the malaria-endemic areas were also assayed upon incubation with a synthetic peptide corresponding to the sequence 308-320, within the C-terminal region of the *P. vivax* CS protein. One patient (#22) demonstrated a proliferative response to both the peptide P308-320 and also the rPvCS-2. This synthetic peptide contains the same amino acid sequence as the corresponding region of the recombinant CS protein, except that its two cysteines have been replaced by alanines. The SIs of the PBMC of patient #22 were 3.8 and 4.1 with the rPVCS-2 and this peptide, respectively (Table II). None of the six healthy individuals tested with this peptide had SIs higher than 2.0.

The synthetic peptide *D/A*, which represents two different repeat units, failed to produce a proliferative PBMC response, i.e., the SI was less than 2.0 in all individuals tested (data not shown).

TABLE II

Cellular immune response of individuals from malaria-endemic areas to rPVCS-2 and peptide P308-320

Donor #	rPvCS-2		P308-320[a]	
	6 μg/ml	30 μg/ml	5 μg/ml	25 μg/ml
#1-13	< 2[b]	< 2	NT[c]	NT
#14-15	< 2	< 2	< 2	< 2
#16	< 2	3.4	NT	NT
#17	3.9	4.5	NT	NT
#18	2.2	3.8	NT	NT
#19	2.3	2.4	< 2	< 2
#20	2.9	4.6	< 2	< 2
#21	< 2	4.0	< 2	< 2
#22	3.4	3.8	2.3	4.1
Total[d] 1/6	5/22 (23%)	7/22[e] (32%)	(16%)	1/6 (16%)
Controls				
#1-2	< 2	< 2	NT	NT
#3-7	NT	< 2	NT	< 2
#8	NT	2.7	NT	< 2
Total	0/2 (12.5%)	1/8	NT	0/6 (0%)

a: the PBMC of all donors and normal controls were also tested with the D/A peptide (30 μg/ml) and Con A. All of them displayed a proliferative response lower than 2 SIs when stimulated with the D/A peptide. The cpm values of the cultures without antigen ranged from 569 to 2140. The SIs obtained in response to Con A in both groups ranged between 15-247.
b: results are given as Stimulation Index (SI).
c: NT = not tested.
d: number of donors with SI higher than 2.0 per total number of individuals analyzed.
e: $0.05 < P < 0.1$; non parametrical Kolmogorov-Smimov Test.

TABLE III

High percentage of responders in individuals recently afflicted with *Plasmodium vivax* malaria

	Control healthy individuals	*P. vivax* individuals	Non-*P. vivax* individuals
Number of donors with SI higher than 2.0 per total	1/8	7/13	0/9
Percentage	12.5%	54%[a]	0%

a: $P < 0.05$.

The statistical analysis, compared the two populations, individuals from malaria endemic area and normal healthy individuals, in their capacity to respond to the rPVCS-2. The hypothesis that the two population were the same was rejected at the level of 10%, however accepted at the level of 5%, indicating a borderline level of significance. This borderline significance could be due to the fact that some of the low responder had *P. falciparum* malaria

but had no contact with *P. vivax* malaria. For that reason we divided the individuals from malaria endemic area in two groups, the ones who were recently afflicted with *P. vivax* malaria and the others which did not have this infection recently. These informations are quite precise and were obtained from records of SUCAM, carried by those individuals, and from SUCEN where malaria diagnosis was performed. As shown in Table III, although the number of subjects is limited per group, all the patients which had SI higher than 2.0, except one, are segregated in the group of individuals from malaria-endemic area recently afflicted with *P. vivax* malaria. The hypothesis that this group and the controls are the same population is now rejected at the level of 5%.

DISCUSSION

This first investigation of the cellular immune response to the major surface antigen of *P. vivax* sporozoites of individuals living in malaria endemic areas revealed that the PBMC of 32% of these individuals produced a proliferative response upon stimulation by the CS antigen. This finding, based on a single determination, is likely to underestimate greatly the percentage of individuals sensitized and potentially capable of responding to the sporozoite antigen. Another possibility relates to the fact that the recombinant *P. vivax* CS protein we used for the *in vitro* stimulation of the PBMC contains only approximately 75% of the sequence of this antigen. The most N-terminal amino acids (aa 1 to 76) and the most C-terminal portion of this sequence (aa 341 to 378) are not contained in this recombinant construct. In this regard, we found that lymphocytes of mice, immunized with synthetic peptides selected according to the T cell epitope prediction of Rothbard & Taylor (1988), did recognize as T cell epitopes, two sequences in the C-terminal region (Rodrigues et al.,1991), which are not contained in rPvCS-2. Whether these T cell epitopes of the CS protein of *P. vivax* are also recognized by human immune cells, remains to be determined. A third reason which possibily reduced the percentage of responder is that some of the patients may not have had contact with *P. vivax* sporozoites. In fact by dividing them in two groups, the one that had contact with *P. vivax* sporozoites the percentage of responder increased to 54% (Table III).

A third T cell epitope (P308-320), recognized by B10 (H-2^b) mice (Rodrigues et al., 1991), also produced *in vitro* proliferation of the PBMC of 1 out of 6 individuals we assayed (Table II). However, none of the PBMC from the individuals from malaria endemic areas, which proliferated in response to the rPvCS-2 protein, recognized the repeat peptides *D/A* as a T cell epitope.

The PBMC of one out of eight subjects never exposed to malaria displayed a SI higher than 2.0 when stimulated *in vitro* with the protein rPvCS-2. Previous studies described that PBMC of some subjects never exposed to malaria demonstrated an *in vitro* proliferative response to a synthetic peptide based on the sequence of the *P. falciparum* CS protein. The generation of T cell clones from PBMC of these subjects confirmed the presence of peptide-specific $CD4^+$ T cells (Sinigaglia et al., 1988a, b).

Our present data indicate the presence of epitopes, within the CS protein of *P. vivax*, capable to induce proliferative response of PBMC of patients from malaria-endemic areas of Brazil. Ongoing studies of the cellular response of individuals living in *P. vivax* endemic areas and of sporozoite-immunized chimpanzees will clarify whether these and/or additional epitopes, are recognized by their immune T cells. Such information will be relevant not only for monitoring the cellular response to this antigen, but also for future vaccine design.

ACKNOWLEDGEMENTS

To Dr R. S. Nussenzweig, for all the support received during the development of this project, also for her kindness in reviewing and discussing the manuscript. To Dr Laurent Excossier and Mrs Rejane Lima from the Center for Theoretical and Applied Genetics at Rutgers University, NJ, for their valuable suggestions for the statistical analysis. To Dr P. Barr from Chiron Corporation for the recombinant PvCS-2, Dr Elizabeth Nardin for providing the D/A peptide, and Mrs Terry J. Huntley for typing the manuscript.

REFERENCES

BARR, P. J.; GIBSON, H. L.; ENEA, V.; ARNOT, D. E.; HOLLINGDALE, M. R. & NUSSENZWEIG, V., 1987. Expression in yeast of a *Plasmodium vivax* antigen of potential use in a human malaria vaccine. *J. Exp. Med., 165:* 1160-1171.

COCHRANE, A. H.; NUSSENZWEIG, R. S. & NARDIN, E. H., 1980. Immunization against sporozoites, p. 163 In J. P. Kreier *Malaria*, Vol. 3. Academic Press, New York.

GOOD, M. F.; BERZOFSKY, J. A.; MALOY, W. L.; HAYASHI, Y.; FUJII, N.; HOCKMEYER, W. T.; & MILLER, L. H., 1986. Genetic control of the immune response in mice to a *Plasmodium falciparum* sporozoite vaccine. Widespread nonresponsiveness to single malaria epitope in highly repetitive vaccine. *J. Exp. Med., 164:* 655-660.

GOOD, M. R.; POMBO, D.; QUAKYI, I. A.; RILEY, E. M.; HOUGHTEN, R. A.; MENOM, A.; AILING, D. W.; BERZOFSKY, J. A. & MILLER, L. H., 1988. Human T-cell recognition of the circumsporozoite protein of *Plasmodium falciparum:* Immunodominant T-cell domains map to the polymorphic regions of the molecule. *Proc. Nat. Academy of Science USA, 85:* 1199-1203.

HOLLANDER, M. & WOLFE, D. A., 1973. *Nonparametric statistical methods.* John Wiley and Sons, New York.

NARDIN, E. H.; HERRINGTON, D. A.; DAVIS, J.; LEVINE, M.; STUBER, D.; TAKACS, B.; CASPERS, P.; BARR, P.; ALTSZULER, R.; CLAVIJO, P. & NUSSENZWEIG, R. S. 1989. Conserved repetitive epitope recognized by $CD4^+$ clones from a malaria-immunized volunteer. *Science, 246:* 1603-1606.

NUSSENZWEIG, V. & NUSSENZWEIG, R. S., 1985. Circumsporozoite proteins of malaria parasites. *Cell (Cambridge, Mass.), 42:* 401-402.

NUSSENZWEIG, V. & NUSSENZWEIG, R. S., 1989. Rationale for the development of an engineered sporozoite malaria vaccine. *Adv. Immunol., 45:* 283-334.

RODRIGUES, M. M.; PAIVA, A.; DUTRA, A.; YOSHIDA, N. & NAKAIE, C. 1991. Multiple epitopes within the circunsporozoite protein of *Plasmodium vivax* recognized by murine $CD4^+$ lymphocytes. *Exp. Parasitol., 72:* 271-277.

ROMERO, P.; HEIMER, E. P.; HERRERA, S.; FELIX, A. M.; NUSSENZWEIG, R. S. & ZAVALA, F., 1987. Antigenic analysis of the repeat domain of the circumsporozoite protein of *Plasmodium vivax. J. Immunol., 139:* 1679-1682.

ROTHBARD, J. B. & TAYLOR, W. R., 1988. A sequence pattern common to T cell epitopes. *EMBO J., 7:* 93-100.

SCHOFIELD, L.; VILLAQUIRAN, J.; FERREIRA, A.; SCHELLEKENS, H.; NUSSENZWEIG, R. & NUSSENZWEIG, V., 1987. Gamma interferon, $CD8^+$ T cells and antibodies required for immunity to malaria sporozoites. *Nature (London), 330:* 664-666.

SINIGAGLIA, F.; GUTTINGER, M.; GILLENSEN, D.; DORAN, D. M.; TAKACS, B.; MATILE, H.; TRZECIAK, A. & PINK, J. R. L., 1988a. Epitopes recognized by human T-lymphocytes on Malaria circumsporozoite protein. *Eur. J. Immunol., 18:* 633-638.

SINIGAGLIA, F.; GUTTINGER, M.; KILGUS, J.; DORAN, D. M.; MATILE, H.; ETLINGER, H.; TRZECIAK, A.; GILLESSEN, D. & PINK, J. R. L., 1988b. A malaria T cell epitope recognized in association with most mouse and human MHC class II molecules. *Nature (London), 336:* 778-780.

ZAVALA, F.; TAM, J. P.; BARR, P. J.; ROMERO, P. J.; LEY, V.; NUSSENZWEIG, R. S. & NUSSENZWEIG, V., 1987. Synthetic peptide vaccine confers protection against murine malaria. *J. Exp. Med., 166:* 1591-1596.

ZAVALA, F.; TAM, J. P.; HOLLINGDALE, M. R.; COCHRANE, A. H.; QUAKYI, I.; NUSSENZWEIG, R. S. & NUSSENZWEIG, V., 1985. Rationale for development of a synthetic vaccine against *Plasmodium falciparum* malaria. *Science, 228:* 1436-1438.