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Anti-malaria antibody-producing B cell frequencies in adults after a *Plasmodium falciparum* outbreak in Madagascar

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SUMMARY

The central highlands of Madagascar offer a unique opportunity to explore the malaria immune memory, as the last murderous epidemic in the study area occurred 8 years ago. Quantification of the circulating memory B lymphocytes reacting to *Plasmodium falciparum* was assessed among 14 Madagascans by using a limiting dilution assay, applied to the EL4 culture system, which leads to activation, proliferation and differentiation into antibody-secreting cells (ASC) of most peripheral B cells. This system allowed us to observe, without any malaria-specific restimulation, a geometric mean frequency of one anti-*P. falciparum* ASC among 2992 circulating B cells, except for one Madagascan who did not have any detectable ASC. A geometric mean frequency of one anti-*P. falciparum* ASC among 1403 was obtained for six malaria hyperimmune Cameroonians, but conversely, no anti-malaria ASC was detected in the blood of six malaria non-immune French control subjects. Anti-*P. falciparum* ASC frequencies and serum specific antibodies were strongly related. Our results indicate that anti-malaria ASC are still present in peripheral blood of Madagascan subjects, who have not been exposed to *P. falciparum* for several years. These responder B cells reflect the malaria B cell memory acquired during the last epidemic.

Keywords antibody-secreting cells Madagascar memory *Plasmodium falciparum*

INTRODUCTION

Immunity against *Plasmodium falciparum* malaria is of a premunition type that requires regular contact with the parasite to persist. Adults living in endemic areas are protected against malaria, via cellular and humoral effector mechanisms. It is usually considered that protection decreases rapidly in the absence of antigenic stimulation [1]. Recently, we provided evidence that an anti-disease immunity may be of long duration, despite short-lived control of parasitaemia [2]. Moreover, it has been shown that humoral and cellular immune responses to several antigens from *P. falciparum* may persist in West African migrants after they have spent up to 13 years out of an endemic area [3]. In the present study, we investigated the persistence of B cell immunity against *P. falciparum* in individuals exposed to a falciparum outbreak 8 years ago.

Malaria transmission is unstable in the central highlands of Madagascar, where an epidemic of falciparum malaria occurred in the mid 1980s, in an area where it had been absent for almost 30 years [2,4]. Thereafter, malaria transmission decreased dramatically and has now nearly ceased [5]. Since January 1988, we have been following the immune responses to *P. falciparum* in a population from this area [6,7]. In a cohort study, cellular responses to several *P. falciparum* antigens decreased in parallel to the antigenic stimulus between 1988 and 1991 [8]. Since 1992, no *in vitro* cellular response to *P. falciparum* antigens has been detectable. Conversely, in 1991, humoral responses, assayed by quantification of serum antibodies to intraerythrocytic asexual stages of *P. falciparum*, were similar to those assayed during the malaria transmission season of 1988 [8], but have since slowly decreased.

The epidemiological status of malaria in this area thus allows a study of the decrease in specific immune responses against *P. falciparum* in the absence of antigenic stimulation, and offers a unique opportunity to explore further the specific immune memory. As serum antibodies persist over years, we dissected in 1994 the B lymphocyte functions in several individuals from this cohort, by using a limiting dilution assay, in order to quantify the circulating memory B lymphocytes reacting to *P. falciparum*. The results have been compared with those from both a group of Cameroonians living in the area of Yaounde, where *P. falciparum* malaria is hyperendemic, and a group of non-immune French subjects. The data should allow a better understanding of the mechanisms sustaining the natural maintenance of a specific human immunity.

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SUBJECTS AND METHODS

Subjects

Twenty-six adults were divided into three groups according to their exposure to *P. falciparum* parasites. The first group comprised 14 Madagascans (M1 to M14, mean age 37·6 years; range 19–60 years) belonging to the cohort previously described and from whom blood samples have been obtained annually since 1988. All Madagascan donors had been malaria-primed, as assessed by anti-*P. falciparum* serum antibodies at enrolment in 1988 [7,8]. The second group comprised six malaria-immune Cameroonians (C1 to C6, mean age 31·3 years; range 20–50 years) from the malaria hyperendemic area of Yaounde. A third group included six malaria-non-immune French subjects (F1 to F6, mean age 44·7 years; range 29–57 years).

Blood sample collection

Venous blood (30 ml) was drawn in 1994 into Vacutainer (Becton Dickinson, Oxnard, CA) heparinized tubes. Samples from Madagascar and Cameroon were shipped at room temperature to Paris, where they were processed within 36 h after bleeding. Malaria parasites were searched for on Giemsa-stained thick blood smears. When thick smears were positive, parasite densities were determined on Giemsa-stained thin blood smears per 20 000 erythrocytes, and parasitaemia was calculated on the basis of an erythrocyte count of $5 \times 10^6/\mu l$.

B cell isolation

Peripheral blood mononuclear cells (PBMC) were obtained by centrifugation on Ficoll–Paque (Pharmacia, St-Quentin-en-Yvelines, France) gradients. Most adherent cells were removed by incubation of PBMC for 1 h at 37°C in Petri dishes containing RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS; Polylabo Seromed, Strasbourg, France). Among the recovered non-adherent cell fraction, $CD19^+$ B cells were positively selected using Dynabeads M-450 Pan-B (CD19) magnetizable polystyrene beads coated with an anti-CD19 IgM MoAb (Biosys, Compiègne, France). Rosetted cells were incubated overnight at 37°C in RPMI–10% FCS, and pure, viable and bead-less B cells were then collected. Cell viability was checked at each step of the B cell isolation, using trypan blue staining, and it was higher than 95% for all blood samples. A geometric mean of 89·3% $CD20^+$ cells (95% confidence interval: 83·9–95·1), as checked by analysis on a Becton Dickinson FACScan, using an anti-CD20 MoAb conjugated to FITC (Coultronics, Margency, France), was found in the final cell suspension.

T cell supernatant preparation

Units of peripheral blood cells from four healthy donors were kindly provided by the Hôpital Avicenne, Bobigny, France. PBMC were separated by the standard Ficoll–Paque gradient method, and T cells were then isolated by rosetting with 2-aminoethylisothiouroniumbromide-treated sheep erythrocytes (Sigma, St-Quentin-Fallavier, France; Biomerieux, Craponne, France). T cells from the four donors were then pooled, adjusted to 10^6/ml in RPMI–10% FCS, and cultured for 36 h in the presence of both phytohaemagglutinin (PHA) (5 μg/ml; Sigma) and phorbol-12-myristate-13-acetate (PMA) (10 ng/ml; Sigma). Cultures were centrifuged, and the supernatant collected, filtered and stored at −80°C. Its IgG concentration, assessed by ELISA, was < 2 ng/ml.

B cell limiting dilution assay

Limiting dilution assays (LDA) were conducted as previously described [9]. Cultures were performed at 37°C, in 5% CO_2, in RPMI 1640 medium supplemented with 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol (2-ME), penicillin-streptomycin (100 U/ml to 100 μg/ml), and 10% FCS. Varying numbers of B cells were added to flat-bottomed microwells (96-well plates; Falcon, Grenoble, France) containing in the same 50 μl volume, 5×10^4 irradiated (50 Gy) mutagenized murine EL4 thymoma cells (clone EL4 OUrBUr 6.1.5.5), kindly provided by Professor R. H. Zubler (Hôpital Cantonal Universitaire, Geneva, Switzerland). EL4 cells constitutively activate murine and human B cells via direct cell interaction, leading them to differentiate polyclonally into antibody-secreting cells (ASC) [10]. Prepared T cell supernatant was added at 15% in a final volume of 200 μl. After 10 days of incubation, culture supernatants were collected and stored at −30°C.

Quantification of total ASC was assessed from 28 replicate cultures for each of three B cell concentrations (1, 2 or 4 B cells/well). Quantification of anti-*P. falciparum* ASC was assessed from 32–40 replicate cultures at five B cell concentrations (37, 111, 333, 1000 or 3000 B cells/well). Higher numbers of cells were not used as they did not allow us to obtain linear B cell dose-response curves for both cell proliferation and IgG secretion.

ELISA for total and P. falciparum*-reactive IgG*

Total and *P. falciparum*-reactive IgG were assayed by ELISAs using successively (i) a goat anti-human F(ab)-specific IgG (1 μg/ml; Sigma) or a crude antigen from asexual blood stages of *P. falciparum* (Palo Alto strain, 7 μg/ml), obtained by sonication of *in vitro* cultures; (ii) undiluted culture supernatants; (iii) an anti-human IgG (Fc specific) alkaline phosphatase (Sigma). Bound enzyme was detected with *p*-nitrophenylphosphate and the absorbance read at 405 nm. For each ELISA plate, positive and negative wells were defined according to the mean OD + 3 s.d. of 12 control wells containing supernatants of cultures conducted without any B cells. Purified human IgG (Sigma) was used to establish standard curves with a sensitivity limit of 0·46 ng/ml.

Plasmodium falciparum-reactive antibodies were also measured by the same ELISA in 200-fold diluted sera from all subjects, including additional serum samples obtained from 1991 to 1993 for the 14 Madagascan subjects. The threshold of positivity was set at OD (405 nm) $\geqslant$ 0·421, according to the mean + 3 s.d. of anti-*P. falciparum* IgG from 72 non-immune subjects.

Statistical analysis

LDA were analysed by simple regression between the logarithm of the fraction of negative cultures and the numbers of B cells per well, using the SYSTAT statistical software (Evanston, IL). ASC frequency and 95% confidence intervals were interpolated from fitted parameters (slope, intercept). The probability that the data conformed to a regression line was assessed at the 95% level, indicating whether the data conformed to a Poisson distribution, which predicts that when 37% of the test cultures are negative, there is an average of one antibody-secreting

precursor cell per well [11,12]. Non-parametric comparisons were computed with Statview 4.02 software (Abacus Concepts, Inc., Berkeley, CA). $P \leqslant 0{\cdot}05$ was considered significant.

RESULTS

Only in samples C5 and C6 from Cameroon could parasites be detected microscopically (2000 and 38 000 *P. falciparum* per μl of blood, respectively). Table 1 shows the anti-*P. falciparum* serum antibody levels of all subjects in 1994, as well as those of all Madagascans in 1991, 1992 and 1993. Anti-*P. falciparum* antibodies were higher in Cameroonians (mean OD 1·302, s.d. 0·077) than in Madagascans (mean OD 0·889, s.d. 0·373) (Mann–Whitney *U*-test, $P < 0{\cdot}01$). All non-immune subjects were negative for *P. falciparum* antibodies (mean OD 0·076, s.d. 0·038). Among Madagascans, anti-*P. falciparum* antibodies decreased during the 4-year follow-up period (Friedman test, $P < 0{\cdot}0001$), confirming the absence of malarial antigenic stimulation in the area during this period, as checked in March 1992 by a prevalence rate of 0·5% in the population of Manarintsoa [5]. All annual thick blood smears during the 4-year follow up were negative. This did not exclude possible malarial infections between two annual samplings, as for M1, who was the only Madagascan to have presented an increased OD value between 1992 and 1993.

Table 1. Reactivity in 1994, in an anti-*Plasmodium falciparum* ELISA of sera from Cameroonians (C), Madagascans (M) and non-immune French subjects (F), and additional annual OD values observed from 1991 to 1993 for Madagascans

	OD values			
Subjects	1991	1992	1993	1994
Cameroonians				
C1				1·227
C2				1·262
C3				1·399
C4				1·347
C5				1·362
C6				1·217
Madagascans				
M1	1·269	1·274	1·510	1·475
M2	1·503	1·382	1·315	1·219
M3	1·386	1·406	1·255	1·115
M4	1·053	1·020	0·826	0·765
M5	1·167	1·062	1·030	0·900
M6	1·267	1·141	1·174	1·110
M7	0·922	0·663	0·593	0·536
M8	1·208	1·272	1·206	1·176
M9	1·109	0·922	0·865	0·804
M10	1·155	0·947	0·961	0·914
M11	0·648	0·588	0·556	0·461
M12	0·427	0·323	0·279	0·250
M13	0·519	0·463	0·397	0·397
M14	1·519	1·454	1·444	1·327
French				
F1				0·116
F2				0·101
F3				0·108
F4				0·059
F5				0·026
F6				0·044

Threshold of positivity was set at OD (405 nm) ⩾ 0·421, according to the mean + 3 s.d. of anti-*P. falciparum* IgG determinations of 72 non-immune subjects; OD values under threshold are underlined.

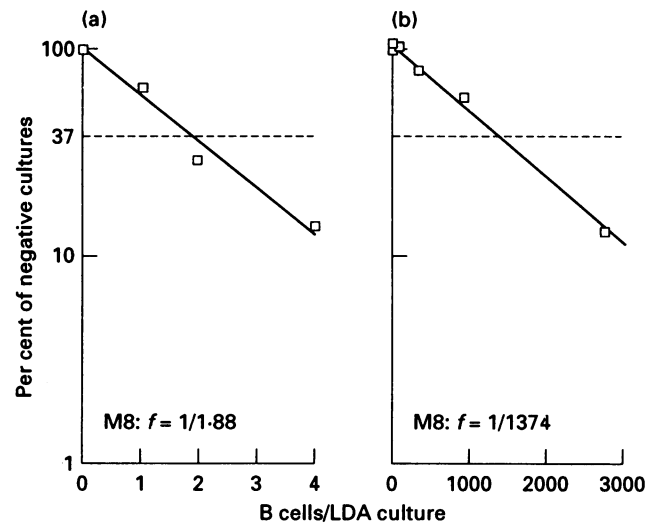

Fig. 1. Limiting dilution assay (LDA) applied to the determination of the frequencies of total (a) and anti-*Plasmodium falciparum* antibody-secreting cells (ASC) (b) in the peripheral blood B cells from one representative subject (M8). (a) Twenty-eight replicate cultures per data point. (b) Thirty-two to 40 replicate cultures per data point.

We assessed for all subjects the responder frequencies of B cells that proliferated and differentiated into total and anti-*P. falciparum* ASC in the EL4 culture system. For all regression lines, r^2 values were between 0·85 and 1, indicating that the data conformed to a Poisson distribution, as shown in Fig. 1 for one representative subject in each type of assay. The first part of Table 2 shows that the total IgG responder B cell frequencies were similar in all groups of subjects, whatever their immune *P. falciparum* malaria status (Kruskall–Wallis test, $P > 0{\cdot}05$). Overall, the geometric mean frequency was one IgG precursor B cell in 3·8 to 2·3 peripheral blood B cells. This result, in agreement with previous work [9], reveals that our study group is homogeneous and free of bias due to differences between groups in overall IgG production, and allows us to further evaluate anti-*P. falciparum* ASC.

The second part of Table 2 presents the geometric mean ASC frequencies for anti-*P. falciparum* antibodies in the three groups. For seven subjects, including all six non-immune French subjects and one Madagascan (M7), the study of the regression line led to a slope not different to zero, indicating that no B cell was detectable in our system, corresponding to a B cell responder frequency < 1/20 086. Mean anti-*P. falciparum* ASC frequency was twice as high in Cameroonians as in Madagascans, but the difference was not significant (Mann–Whitney *U*-test, $P > 0{\cdot}05$). Individual anti-*P. falciparum* responder B cell frequencies are shown in Fig. 2, where plots represent the 37% values (medium lines) and 95% confidence intervals (upper and lower lines) obtained for each subject. Plots can be divided into four groups according to anti-*P.*

Table 2. Geometric mean antibody-secreting cell (ASC) frequencies observed for total and anti-*Plasmodium falciparum* antibodies in Cameroonian ($n = 6$), Madagascan ($n = 14$) and French ($n = 6$) groups

	Geometric mean ASC frequency	CI 95%
Total IgG		
Cameroonians ($n = 6$)	1/3·8	(1/7·2–1/2·1)
Madagascans ($n = 14$)	1/2·3	(1/3·0–1/1·8)
French ($n = 6$)	1/2·4	(1/3·8–1/1·5)
*Anti-*P. falciparum *IgG*		
Cameroonians ($n = 6$)	1/1403	(1/1870–1/1052)
Madagascans ($n = 13$)*	1/2992	(1/5402–1/1657)
French ($n = 6$)	ND	

*No anti-*P. falciparum* ASC were detectable for one Madagascan subject (M7).

ND, Not detectable.

falciparum ASC frequencies. Group 1 corresponding to ASC frequencies > 1/2500 included all Cameroonian plus five Madagascan donors. Group 2 (ASC frequencies between 1/7000 and 1/2500) and group 3 (calculable ASC frequencies < 1/7000) included only Madagascans. Group 4 (no calculable ASC frequency) included, as described above, all non-immune subjects plus one Madagascan.

We investigated the relation between anti-*P. falciparum* ASC frequencies and serum anti-*P. falciparum* antibodies, a common marker of humoral responses. In 1994, all subjects from groups 1 and 2 had anti-*P. falciparum* antibody. OD values measured in sera from groups 3 and 4 were either negative (all F group, M12, M13) or weakly positive (M7, M11). These results suggest a strong relation between the frequencies of anti-*P. falciparum* ASC and the anti-*P. falciparum* antibody OD values, that was attested by the regression study between the two variables for the 19 subjects with calculable ASC frequencies (r^2 0·67; Fisher's r to z P value $< 10^{-4}$).

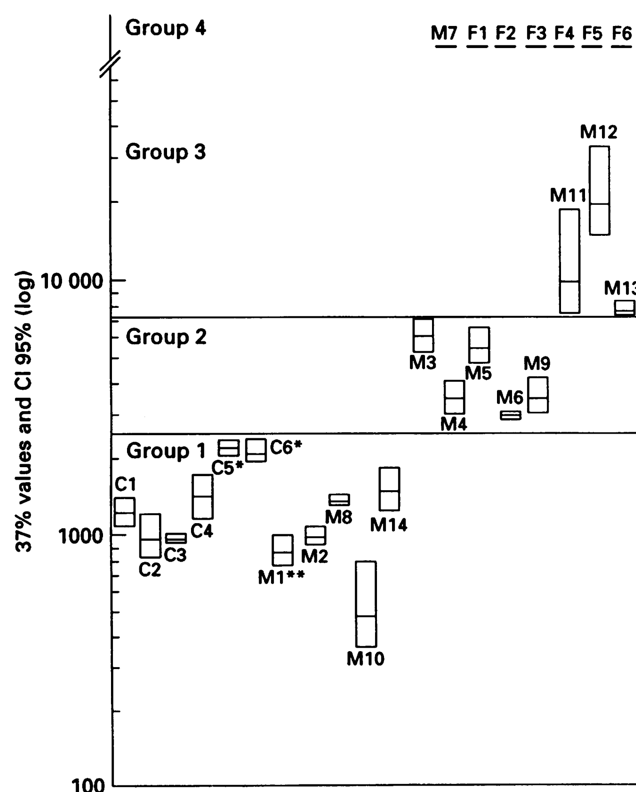

Fig. 2. Frequencies (and CI 95%) of anti-*Plasmodium falciparum* antibody-secreting cells (ASC) in peripheral blood B cells of Cameroonian (C, $n = 6$), Madagascan (M, $n = 14$) and non-immune French (F, $n = 6$) subjects. *Positive thick blood smears at sampling. **Higher anti-*P. falciparum* OD value in 1993 than in 1992.

DISCUSSION

In order to quantify the number of circulating B cells able to secrete anti-*P. falciparum* antibodies, we used the EL4 culture system which leads to polyclonal activation, proliferation and differentiation into ASC of about 90% of B cells from peripheral blood [9]. This culture system involves a direct cell interaction between the human B cell surface CD40 activation marker and its ligand, expressed on EL4 cells [13], as on T helper cells [14,15]. Assessment of total and anti-malaria responder B cell frequencies was made by applying a LDA to the EL4 culture system [9,16]. First, we confirmed that most circulating B cells are immunocompetent, as a geometric mean of 1/2·5 B cell (26 subjects) was found to generate clones secreting total IgG.

The major aim of our study was to explore further the malaria B lymphocyte memory in Madagascan subjects who had been exposed to a *P. falciparum* outbreak 8 years ago. No anti-*P. falciparum* ASC was detectable in the peripheral blood B cells of six *P. falciparum*-unprimed French control donors, plus one Madagascan subject. The anti-malaria ASC frequency ranged from 0·005% to 0·2% for all other subjects, and is similar to the 0·1–1% range found in a previous report that applied the same EL4 culture system to the quantification of anti-malaria ASC from six subjects who had just experienced a malaria infection [9]. Another report using the enzyme-linked immunospot assay (ELISPOT), found the range of ASC frequency in response to *P. falciparum* extract or single epitopes from Pf155/RESA, among 15 *P. falciparum*-primed Cameroonian donors, to be 0·04–0·29%. This was an approximate rate, since cultures were assessed using unseparated populations of mononuclear cells [17].

All Cameroonians presented with high anti-malaria ASC frequencies, in parallel with high antibody OD values resulting from their intense exposure to malaria parasites. Madagascans were much more heterogeneous with regards to their anti-malaria ASC frequencies, varying from high (as in Cameroonians) to nul (as in non-immune subjects) ASC responders, via intermediate and low ASC responders. These immunological disparities among Madagascans may reflect the very low level of malaria transmission in the central highlands, which peaked during the 1986–87 epidemic with an inoculation rate of 1·5 infective bite/person per year [18]. Thus, small differences in the number of infective bites received could have led to markedly different anti-malarial immune responses. This heterogeneity may also be related to variation in ability to maintain or not a

circulating pool of malaria-primed B cells for subjects who experienced malaria in their childhood. Indeed, we previously reported that, in this area, individuals older than 40 years in 1988 were more protected against clinical falciparum malaria, and had higher humoral and cellular responses to major epitopes of distinct parasite antigens than younger subjects [2]. This age shift was attributed to a difference in past exposure to malaria parasites, older subjects having spent their childhood in a hyperendemic area where they probably experienced numerous infections and developed an immunological memory. The present study included four subjects who were more than 40 years old in 1988: one belonged to each of the four groups of ASC responders that we defined. In the present study, there was no relationship between the distribution of the ASC frequencies and age, suggesting that the responder B cells revealed in our system are essentially a reflection of the recent malaria B cell memory acquired during the last epidemic.

Using a LDA applied to the EL4 culture system, we were able to quantify the *P. falciparum* ASC in the blood of individuals differing in their past and present exposure to malaria parasites. This sytem did not allow us to define precisely whether the B lymphocytes are effector cells, memory cells maintained in a semi-activated state, or a mixture of both types, for even surface marker studies may fail to distinguish between these groups [19]. The strong relationship between the *P. falciparum* ASC frequencies and the serum antibodies indicates a progressive decrease of the anti-malaria ASC, parallel to the decrease of the reactive antibodies since the outbreak of 1986–87. The number of circulating anti-malaria ASC may not necessarily decrease, however, since it has been reported that the maintenance of B cell memory depends on the persistence of stimulating antigen [20]. In the case of *P. falciparum* malaria, although recrudescent parasites may lead to malaria attacks over a period of more than 1 year, sequestration for prolonged periods of *P. falciparum* parasites in deep organs is unlikely, due to the absence in this species of the hypnozoite stage. Although other plasmodium species were transmitted during epidemic years, but at much lower rates than *P. falciparum*, none of the 14 investigated Madagascans was found to be infected by *P. vivax*, *P. ovale* or *P. malariae*. Thus, potential cross-reactivity might play a role in generating anti-malaria ASC, but this is considered unlikely. It is possible that cross-reactivity occurred with environmental antigens or even self antigens [21,22], so contributing to the immunological memory. In our study, the putative persistence of a very low level of *P. falciparum* transmission might also contribute to the maintenance of a residual immunity. The last clinical and parasitological data obtained in 1992 in the study area revealed a prevalence rate of 0·5% among 426 examined subjects (two asymptomatic subjects presenting with low parasite densities) [5]. The same paper presented entomological data collected in 1992, that assessed the absence of infected mosquito vectors, as determined by ELISA for the detection of *P. falciparum* antigens. Lastly, sequestered B lymphocytes that are not available for sampling [23] may also regenerate the circulating pool of specific B lymphocytes.

Sudden malaria reappearance in the central highlands of Madagascar is likely to occur in the future, due to the instability of malaria in this area. An immunological B memory is maintained in most individuals 8 years after the last murderous epidemic. Whether the anti-malaria ASC we found will be sufficient to confer some level of protection in the case of a new malarial epidemic remains questionable.

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