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Protective Value of Elevated Levels of Gamma Interferon in Serum against Exoerythrocytic Stages of *Plasmodium falciparum*

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In vitro experiments suggested that gamma interferon, CD8$^+$ T cells, and anticircumsporozoite antibodies inhibited the exoerythrocytic stages of malaria parasites. To assess the role played in vivo by these factors, we conducted a prospective study in Madagascar. Forty individuals with a negative blood smear were followed for 8 weeks to detect the appearance of parasites in blood. Nineteen subjects remained negative for malaria, whereas 21 individuals became positive during follow-up. Among these, seven presented with blood parasites within the first 2 weeks and were excluded, as they probably were infected by sporozoites at enrollment. When measured at enrollment, antibodies to the synthetic peptide $(NANP)_5$, lymphocyte proliferation with $(NANP)_5$, and various lymphocytes subsets were similar among individuals that later presented with a *Plasmodium falciparum* blood infection or were not infected. Conversely, the level of gamma interferon in serum was higher in individuals that did not present with a *P. falciparum* infection during follow-up. These data suggest that gamma interferon may inhibit the malaria exoerythrocytic stages of development under in vivo conditions, as it does in vitro.

After a bite by a female anopheline mosquito, sporozoites pass into the bloodstream. Some of these sporozoites reach the liver, invade hepatocytes, and undergo a cycle that leads to the asexual blood cycle, which is responsible for malarial disease. The first target of the immune response is the sporozoite. Recently, tremendous efforts have been made to understand the mechanisms involved in this response. Experimental data have shown that gamma interferon (IFN-γ) (4), CD8$^+$ T cells (15), and anti-circumsporozoite (CS) protein antibodies (9) are potent inhibitors of sporozoite invasion or hepatic schizont maturation. However, initial vaccine trials in humans demonstrated that anti-CS protein antibodies were only partially effective in blocking the completion of the hepatic cycle (1, 3).

To assess the role played in vivo by these factors, we conducted a prospective study designed to determine whether the humoral or cellular response to CS protein, the lymphocyte subpopulations, and/or the level of IFN-γ in serum would be predictive of protection against *Plasmodium falciparum* infection in an endemic area during the peak of malaria transmission.

(This work has been presented at the VIIth International Congress of Parasitology, 20 through 24 August 1990, Paris, France [2a].)

MATERIALS AND METHODS

Subjects and field methods. The study took place in Manarintsoa, a village from the central highlands of Madagascar, 20 km from Antananarivo, from January to March 1988, at the peak of the malaria transmission season. Falciparum malaria reappeared in this area in the mid-1980s and currently is hyperendemic and transmitted seasonally from December to May (6, 10). Forty individuals older than 10 years old were enrolled and followed for 8 weeks. Enrollment criteria were (i) a negative thick blood smear, (ii) no clinical cachectic syndrome, (iii) permanent residency in the village for at least 3 years, and (iv) informed consent to participate in the study. To allow identification of malaria attacks occurring in each subject during the follow-up, a physician conducted weekly clinical examinations and thick blood smears. The physician lived in the village, and sick or febrile individuals of the cohort were free to visit him at any time between weekly examinations. Subjects who never presented with a positive thick blood smear during the follow-up were considered to be protected against exoerythrocytic forms of falciparum parasites, as they were very likely to have been bitten by sporozoite-infected anophelines. All individuals of the cohort lived in similar houses, and none used mosquito nets. Jointly conducted entomological studies failed to demonstrate variations in the sporozoite inoculation rates among various areas of the village (5, 6).

Blood sample collection. Venous blood samples were drawn in all individuals at enrollment (week 0) into Vacutainer tubes for the measurement of antibody and IFN-γ levels (dry tubes), the lymphocyte proliferation assay, and the determination of the absolute number of leukocytes and lymphocytes (tubes containing heparin).

Thick blood smears obtained during and between the weekly examinations were air dried, Giemsa stained, and observed against 1,000 leukocytes.

Anti-CS protein antibody measurement. Anti-CS protein antibodies were tested by the Falcon Assay Screening Test (FAST) enzyme-linked immunosorbent assay (ELISA) technique (2). Briefly, the polystyrene knobs of the FAST system were coated with the synthetic peptide $(NANP)_5$, in which the repeat sequence of the CS protein is reproduced, diluted to 5 μg/ml. The assay was performed by treating the solid-phase peptide sequentially with serum diluted 1:50, peroxi-

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dase anti-human immunoglobulin G, and tetramethyl-benzidine substrate. Between incubations, the solid-phase peptide was extensively washed. The A_{690} values were recorded. From previous studies, A_{690} of ≥ 0.18 were considered to be a positive reaction (2).

IFN-γ in serum. IFN-γ was assessed in serum samples by using a commercially available sandwich immunoassay (Holland Biotechnology bv, Leiden, The Netherlands) with two monoclonal antibodies that recognized different epitopes on the IFN-γ molecule. The resulting A_{405} was measured. A standard curve was determined with five reference samples and used to calculate the IFN-γ concentration (in units per milliliter) in the serum samples. According to the IFN-γ levels in the serum of nine healthy European individuals (mean + 3 standard deviations), the threshold of positivity was fixed at 4 U/ml.

T-cell proliferative response. The T-cell proliferation response to sporozoite major antigen was assessed as previously described (2) with the synthetic peptide $(NANP)_5$. Briefly, peripheral blood mononuclear cells were isolated on Ficoll-Hypaque and suspended in buffered RPMI 1640 supplemented with 10% pooled human serum at a concentration of 10^6 cells per ml. Aliquots of 100 μl were plated in triplicate in flat-bottom 96-well tissue culture plates. $(NANP)_5$ (1 μg/well) or RPMI 1640 alone (50 μl) was added to each well. The plates were incubated at 37°C for 6 days, and 1 μCi of [^{3}H]thymidine (specific activity, 1 Ci/ml; CEA, Gif sur Yvette, France) was added to each well. After an additional 16-h incubation, cells were collected and the radioactivity was counted in a scintillation counter. Data were expressed as stimulation indexes, calculated by dividing the mean counts per minute of antigen-stimulated cultures by the mean counts per minute of unstimulated cultures. The threshold of positivity was fixed to a stimulation index higher than or equal to 2.5, according to the response observed with cells from 13 healthy European individuals (2).

Flow cytometry analysis of blood lymphocytes. Leukocytes were counted in an hemacytometer. The percentage of lymphocytes was assessed on thin blood smears stained with May-Grünwald-Giemsa, and the absolute number of lymphocytes was then calculated.

Aliquots (1 ml) of isolated peripheral blood mononuclear cells (10^6 cells per ml) were frozen at −80°C until analysis (lymphocyte subsets have been shown to be similar in frozen samples and fresh blood [13]). By using standard procedures, samples were thawed at room temperature and lymphocyte surface markers were measured with fluorescein- and phycoerythrin-conjugated monoclonal antibodies anti-HLA-DR (B marker and activated T cells), anti-Leu-4 ($CD3^+$ lymphocytes), anti-Leu-3a ($CD4^+$ lymphocytes), anti-Leu-2a ($CD8^+$ lymphocytes), and anti-Leu-7 ($CD57^+$ natural killer-like cells) (Simultest immune monitoring kit; Becton Dickinson, Mountain View, Calif.). After the cell pellet was washed with phosphate-buffered saline, it was resuspended in 200 μl of 1% Formalin. The lymphocyte-associated fluorescence was detected with a Becton-Dickinson FACS analyzer.

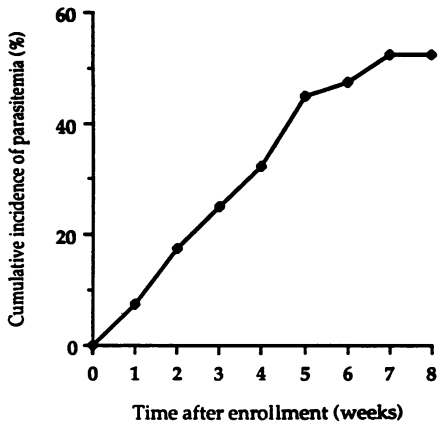

FIG. 1. Cumulative incidence of *P. falciparum* infection during the 8-week follow-up, in the group of 40 villagers of Manarintsoa, Madagascar, in 1988.

Statistical analysis. Differences in proportions were tested by the Fisher's exact test. Nonparametric relations were assessed by the Mann-Whitney *U* test. *P* values less than 0.05 were considered significant.

RESULTS

The 40 enrolled individuals had a mean (± standard deviation) age of 29.8 (±13.4) years. Nineteen never presented with a positive thick blood smear during the follow-up and were considered to be protected against the exoerythrocytic forms of falciparum parasites. Twenty-one became infected before the end of the follow-up. Among those, seven presented with parasites in the blood within the first 2 weeks and were excluded from subsequent analysis, because they probably were already infected by sporozoites at the time of enrollment. The 14 other individuals developed malaria infection between weeks 2 and 8 (Fig. 1). The ages of the individuals who developed *P. falciparum* parasites in blood and those who did not were similar (29.5 ± 12.3 versus 30.1 ± 14.9 years).

In blood samples obtained at enrollment, the positivity rates and mean levels of antibodies to CS protein central repeats were not statistically different between the 14 individuals who developed *P. falciparum* parasites in the blood between weeks 2 and 8 and the 19 individuals who did not (Table 1). Similarly, the rates and mean stimulation indexes

TABLE 1. Antibody and cellular responses and IFN-γ levels in serum[a]

Test group	Antibody response		T-cell proliferation		Serum IFN-γ	
	No. positive/total	Mean OD ± SEM	No. positive/total	Mean SI ± SEM	No. positive/total	Mean level (U/ml) ± SEM
Presented *P. falciparum*	2/14	0.11 ± 0.03	5/12	2.12 ± 0.30	1/14	2.4 ± 1.1
Remained negative	4/19 (0.49)	0.16 ± 0.23 (0.20)	6/16 (0.56)	2.65 ± 0.80 (0.38)	9/19 (0.015)	7.3 ± 2.9 (0.05)

[a] Test groups included 14 individuals who became infected and 19 who did not. The antibody response to $(NANP)_5$ is expressed as the mean optical density (OD), as detected by FAST-ELISA. SI, stimulation index. The thresholds of positivity are as follows: optical density of 0.18; stimulation index of 2.5; serum IFN-γ level of 4 U/ml. *P* values (given within parentheses) were deduced from the Fisher exact test and from the Mann-Whitney *U* test. The cellular response was not investigated in five subjects.

TABLE 2. Number of lymphocytes and flow cytometry analysis of blood at enrollment[a]

Cell type	Mean cells/μl in the following test group:	
	Presented *P. falciparum* (n = 8)	Remained negative (n = 11)
Lymphocytes	2,999 (2,476–3,639)	2,844 (2,208–3,664)
$CD3^+$	1,679 (1,340–2,104)	1,706 (1,330–2,188)
Activated T cells	275 (163–465)	239 (138–413)
$CD4^+$	800 (601–1,064)	782 (607–1,007)
$CD8^+$	906 (669–1,228)	991 (759–1,294)
NK-like cells (anti-$CD57^+$)	237 (121–464)	176 (114–273)
Unstained lymphocytes	547 (340–879)	367 (204–662)
B lymphocytes	327 (283–377)	372 (281–493)
$CD4^+/CD8^+$ ratio	0.88[b] (0.61–1.26)	0.79[b] (0.60–1.04)

[a] Results are expressed as geometric means and, within parenthesis, 95% confidence intervals of the means. Analysis by the Mann-Whitney U test did not demonstrate any difference between the two groups ($P > 0.15$).
[b] Results are given as ratios.

of the cellular responses in presence of $(NANP)_5$ were similar in both groups, as were the various cells subsets, as determined by flow cytometry analysis (Table 2). However, individuals who did not develop *P. falciparum* malaria presented with a mean IFN-γ level in serum that was higher than that in individuals who did develop malaria (7.3 versus 2.4 U/ml; Mann-Whitney U test; $P < 0.05$). Elevated IFN-γ serum levels were observed more frequently in individuals who did not develop *P. falciparum* malaria than in individuals who did (9 of 19 versus 1 of 14; Fisher's exact test; $P < 0.015$).

DISCUSSION

In our prospective study we did not observe any difference in anti-$(NANP)_5$ specific humoral or cellular immune response or in lymphocyte subsets between individuals that may be considered as protected and those who were not. However, a high level of IFN-γ in serum was associated with protection against organisms in the sporozoite or the hepatic stage of maturation.

For several years antisporozoite antibodies, most of which recognize the CS protein, have been considered to play a crucial role in antimalarial protection (9). It has been demonstrated more recently that these anti-CS protein antibodies were insufficient to confer complete protection against the exoerythrocytic stages of the parasite. This was the case for both antibodies acquired naturally in endemic areas (8) and after immunization with synthetic peptides (3) or a recombinant protein (1). In view of this incomplete protection of the humoral response, cellular immunity is now considered to play a major role, although the effector mechanisms involved are still poorly understood. We observed no difference in T-cell proliferation in response to $(NANP)_5$ or in lymphocyte subpopulations between protected and nonprotected individuals. However, this does not demonstrate that the cellular immune response is ineffective in preventing the maturation of exoerythrocytic stages. The central repeat of the CS protein constitutes the main target of the humoral response, but other T-cell epitopes are present in the sporozoite (7) or in the infected hepatocyte that may induce a more effective cellular response. In addition, T cells may not proliferate but may respond to stimulation by other means, such as IFN-γ or interleukin-2 production (18). On the other hand, cytotoxic T cells were reported to be essential for protection under experimental conditions (15, 17). However, the proportion of malaria-primed lymphocytes is probably too small to allow the detection of an increase in the overall population of $CD8^+$ T cells by flow cytometry analysis.

Our most striking finding is that elevated levels of IFN-γ in serum are associated with protection under natural conditions of exposure. In vitro experiments demonstrated that small amounts of IFN-γ inhibited the development of the liver stage of rodent (4, 16), and human (12) parasites. Similarly, there was a delay in the onset of parasitemia when IFN-γ was administered to rodents, monkeys, or chimpanzees before sporozoite challenge (4, 11), demonstrating that IFN-γ also reduced exoerythrocytic forms proliferation in vivo. However, the very high doses of IFN-γ required to eradicate the liver stages indicated that IFN-γ alone may not be sufficient to provide complete protection. On the other hand, the requirement of IFN-γ for protection was demonstrated in immunized mice and rats, in which the administration of monoclonal antibody to rodent IFN-γ after sporozoite challenge abolishes immunity (17). Although the level of IFN-γ within the liver is unknown, it might be expected that elevated levels in serum also reflect an increased level in the proximity of the developing exoerythrocytic stages. In our subjects the mechanism of production of IFN-γ is unknown. It has been demonstrated that IFN-γ can be secreted in vitro by blood stage-stimulated T cells (18); endogenously produced IFN-γ may persist in blood for several months after an acute malaria attack (14) and could act against the liver stages produced by a subsequent sporozoite challenge to limit superinfection. In Madagascar, the elevation of IFN-γ in the serum of aparasitemic villagers living in a malaria-endemic area probably reflects massive T-cell activation and seems to be associated with protection against the exoerythrocytic stages of falciparum malaria.

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