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Immune response to *Plasmodium falciparum* liver stage antigen-1: geographical variations within Central Africa and their relationship with protection from clinical malaria

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Abstract

Two populations of schoolchildren from Gabon and Cameroon were tested in 1995 for their immunological reactivity to synthetic peptides (LSA-Rep, LSA-J and LSA-CTL) from *Plasmodium falciparum* liver stage antigen-1 (LSA-1). The prevalence and levels of both cellular (lymphocyte proliferation, tumour necrosis factor α (TNFα), interferon γ (IFNγ), and interleukin-10 (IL-10)) and humoral (immunoglobulin G) responses were determined. Protection from clinical malaria, determined after a prospective 1 year study in both sites, was associated with elevated proliferative responses to LSA-Rep and LSA-CTL in the Gabonese children, as well as with higher antibody levels to both schizont extract and LSA-Rep. The prevalence of peptide-stimulated TNF-α secretion was higher in the Cameroonian group, but higher levels of antibodies to LSA-Rep and LSA-J were found in the Gabonese children. The immunological differences observed between children in the 2 study sites are discussed in the context of both epidemiological and individual host factors.

Keywords: malaria, *Plasmodium falciparum*, immune response, T cells, TNFα, IFNγ, IL-10, liver stage antigen-1, Gabon, Cameroon

Introduction

The search for subunit vaccines against malaria has included liver stage antigens in recent years, as various immune responses are active against this stage of the parasite's life cycle. Infected hepatocytes may be destroyed by MHC class I-restricted cytolytic $CD8^+$ T cell responses (NARDIN & NUSSENZWEIG, 1993) as well as by antibody-dependent cellular cytotoxicity (RENIA *et al.*, 1990). However, the best described response against infected human liver cells involves the production *in vitro* of cytokines, in particular interferon γ (IFNγ), which binds to surface receptors on infected hepatocytes (SCHOFIELD *et al.*, 1987), and tumour necrosis factor α (TNFα), which acts by inducing the synthesis of interleukin-6 (IL-6) (NUSSLER *et al.*, 1991) as well as nitric oxide (DOOLAN *et al.*, 1996). Hepatocytes infected with *Plasmodium falciparum* express antigens derived from sporozoites (HOFFMAN *et al.*, 1989), a heat shock protein designated Pfhsp70 (RICHMAN *et al.*, 1989), and liver stage antigens including liver stage antigen-1 (LSA-1) of *P. falciparum*, which was first characterized in 1987 (GUÉRIN-MARCHAND *et al.*, 1987) and then sequenced (ZHU & HOLLINGDALE, 1991). The potential importance of LSA-1 as a vaccine candidate has been indicated by numerous studies. Mice immunized with a peptide derived from *P. falciparum* LSA-1 were partially protected from *P. berghei* sporozoite challenge (HOLLINGDALE *et al.*, 1990). LSA-1 has been found to elicit in humans a wide range of immune responses *in vitro*, including reactivity against T and B cell epitopes (FIDOCK *et al.*, 1994; CONNELLY *et al.*, 1997). The repeatless domains of the *LSA-1* gene were inserted into the genome of the highly attenuated NYVAC vaccinia virus, in order to generate the multiantigen, multistage NYVAC-Pf7 vaccine candidate, which was safe, well tolerated and able to elicit humoral immune responses when inoculated into rhesus monkeys (TINE *et al.*, 1996). A vaccine trial recently conducted among human volunteers proved that NYVAC-Pf7 was safe and well tolerated, but variably immunogenic (OCKENHOUSE *et al.*, 1998). A mixture of peptides from LSA-1 induced long-lasting immune responses when inoculated to *Aotus lemurinus* monkeys (PERLAZA *et al.*, 1998). LSA-1 is therefore a vaccine candidate of some interest (HOLLINGDALE *et al.*, 1998), and further information concerning its immunogenicity as well as its influence on clinical protection under conditions of natural transmission may be useful. In the present study, 2 cohorts of children were enrolled in 2 endemic regions of Central Africa in which the epidemiology of malaria is different (DELORON *et al.*, 1999). The cellular (lymphocyte proliferation, TNFα, IFNγ and IL-10 cytokine release) and humoral (anti-peptide plasma antibodies) reactivity to 3 synthetic peptides of LSA-1 were measured in these cohorts in order to investigate in each site the associations between specific immune responses and susceptibility to disease, and also to compare the immunological status in children from both sites.

Materials and Methods

Subjects

The villages studied were Dienga in south-east Gabon and Pouma in central Cameroon, where a clinical, biological and parasitological follow-up was being carried out during the whole malaria transmission season of 1995 among the primary school-going population of 300 children in Dienga and 186 children in Pouma, using similar procedures in the 2 villages (DELORON *et al.*, 1999). Clinical and parasitological data were used to distinguish between protected and unprotected children. Briefly, protected children were defined as those who never presented, during the whole survey, with a febrile episode (defined as axillary temperature $> 37{\cdot}5°C$) associated with either *P. falciparum* parasitaemia $> 400/\mu L$ or the presence of 4-aminoquinoline metabolites in urine, and unprotected children were defined as those who presented at least 1 malaria attack, defined as the association of fever and parasitaemia $\geq 5000/\mu L$; other children were unclassified. Based on these criteria, 76 children from the cohort in Dienga, including 38 protected and 38 unprotected children, were classified and selected for the immunological study. Similarly, 43 children from the cohort in Pouma, including 32 protected and 11 unprotected children, were selected for the immunological study. The same children had previously been enrolled in a similar study aimed at determining the

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relationships between immune responses directed against *P. falciparum* asexual blood-stage antigens and disease susceptibility, which led to the observation in Dienga of a positive association between clinical protection and lymphocyte proliferation to peptides from antigens MSA-1 and MSA-2 (merozoite surface antigens 1 and 2) as well as antibody levels to peptides from MSA-2 and RAP-1 (rhoptry-associated protein-1) (MIGOT-NABIAS *et al.*, 1999). Blood was drawn at the end of the follow-up period, with the informed consent of the parents. Ethical clearance for the study was given by the ethics committees of the research institutions in Gabon (CIRMF) and Cameroon (IRD/OCEAC).

Parasitological measurements

During the follow-up, thick blood films were prepared twice a month in Dienga and Pouma (16 and 10 films, respectively) for the detection of asymptomatic infections. After staining the films with Giemsa's stain, malaria parasites were counted against 1000 leucocytes, and the mean geometric parasite density (MGPD) of the positive slides was determined.

Antigens

Two synthetic peptides were used as antigens, $(LAKEKLQEQQSDLEQER)_2LAKEKLQ$ (residues 187–227), referred to as LSA-Rep, and ERRAKEKLQEQQRDLEQRKADTKK (residues 1613–1636), referred to as LSA-J, representing B and T cell epitopes from conserved regions of *P. falciparum* LSA-1, as well as the peptide KPIVQYDNF (residues 1786–1794), referred to as LSA-CTL, representing a conserved cytotoxic lymphocyte epitope from LSA-1 (FIDOCK *et al.*, 1994). All peptides were purchased from the Institut Pasteur (Paris, France) and were used at a final concentration of 1 μM in the cultures *in vitro*. Control antigens were the mitogen leucoagglutinin (Sigma, St Louis, Missouri, USA; final concentration 10 μg/mL) and the recall antigen tuberculin purified protein derivative (PPD, Statens Seruminstitut, Copenhagen, Denmark; final concentration 10 μg/mL).

Lymphocyte proliferative assay (LPA)

Within 16 h after bleeding, peripheral blood mononuclear cells (PBMC) were isolated on Ficoll–Paque (Pharmacia, Uppsala, Sweden), and cell viability was confirmed by trypan blue staining. Purified PBMC were suspended at 10^6 cells/mL in buffered RPMI-1640 supplemented with gentamicin (25 μg/mL), 2mM glutamine and 10% pooled human AB serum, and 100 μL aliquots were plated in triplicate in flat-bottomed 96-well plates. Control antigens, peptides or culture medium alone were added in 100 μL amounts at the indicated concentrations. Plates were incubated at 37°C in a humidified chamber with 5% CO_2. After 6 d, 110 μL of culture supernatants were removed and 50 μL of fresh medium containing 0.5 μCi of methyl-[^{3}H]thymidine (specific activity 2 Ci/mmol; Amersham, Les Ulis, France) were added to each well. After an additional 16 h, cells were collected on glass fibre filter paper and the radioactivity was determined. Stimulation indices (SI) were calculated by dividing the geometric mean counts/min of antigen-stimulated cultures by the geometric mean counts/min of unstimulated cultures. The threshold of positivity was set at SI > 2·0 and geometric mean count/min > geometric mean + 2 SD of the individual's own background (RZEPCZYK *et al.*, 1988).

Cytokine assays

Supernatants from each triplicate culture were pooled at 6 d and stored at −80°C. IFNγ was assayed in the undiluted supernatants using a two-site enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Mabtech, Stockholm, Sweden). Similar culture plates were incubated for 72 h, and triplicate supernatants were pooled and stored at −80°C for assaying TNFα and IL-10, using a two-site ELISA (Pharmingen, San Diego, California, USA). Cytokine concentrations were determined by reference to standard curves prepared with recombinant human cytokines (Pharmingen). The thresholds of sensitivity were 8 pg/mL for TNFα and 2 pg/mL for IFNγ and IL-10. For statistical purposes, values under the threshold were assigned a concentration of half this value. Mitogen and antigen-induced cytokine production was derived from the difference between the cytokine content in stimulated cultures and the spontaneous cytokine content in unstimulated cultures.

Antibody measurements

Anti-*P. falciparum* plasma antibodies were measured by ELISA using successively a schizont extract obtained by sonication of cultures *in vitro* of the Palo Alto strain of *P. falciparum* (7 μg/mL), 200-fold diluted plasma, and an anti-human immunoglobulin G (IgG) (Fc specific) conjugated to alkaline phosphatase (Sigma). Bound enzyme was detected with *p*-nitrophenylphosphate and the absorbance read at 405 nm (A_{405}). Anti-peptide antibodies directed against LSA-Rep and LSA-J, which both contain B-cell epitopes, were assayed by ELISA. For this purpose, peptides were used at a concentration of 2·5 μg/mL and plasmas were diluted 100-fold. Reference positive and negative control plasmas were included in each plate, and results were expressed in arbitrary units (AU) calculated from the following formula: $100\times$ [ln (A_{405} test plasma) − ln (A_{405} negative pool)]/[ln (A_{405} positive pool) − ln (A_{405} negative pool)] (RASHEED *et al.*, 1995). The thresholds for positivity were set at 21·1 AU for anti-*P. falciparum* IgG, and at 12·0 and 14·9 AU for anti-LSA-Rep and anti-LSA-J IgG respectively, as determined from the mean reactivities + 2 SD of >50 plasmas from non-immune subjects.

Statistical analysis

Differences in proportions were analysed using the χ^2 test. Differences in means were tested by Student's unpaired *t* test on linear or log-transformed values. When variable distribution was not normalized by log-transformation, the non-parametric Mann–Whitney *U* test was employed. Statview™ 4·5 (Abacus Concept, Berkeley, California USA) was used for these calculations. The association between specific immune responses and demographic characteristics (age, living area) that were found to be significant in the univariate analysis was investigated by logistic regression analysis using BMDP software (University of California, Los Angeles, California USA). For all tests, *P* values < 0·05 were considered significant.

Results

The cohort from Dienga in Gabon comprised 76 children (40 boys and 36 girls) with a mean age of 10·6 (SD = 2·5) years; the cohort from Pouma in Cameroon comprised 43 children (14 boys and 29 girls), with a mean age of 8·8 (SD = 2·4) years. As previously reported, although the children were attending equivalent school classes in the 2 study sites, they were significantly older in Dienga ($P = 0.0003$). Also, in Dienga, protected children were significantly older than unprotected ones ($P = 0.0009$) (MIGOT-NABIAS *et al.*, 1999). Over the survey period, the mean *P. falciparum* parasite prevalence in Dienga was 24%, with MGPD of positive slides = 192/μL (95% confidence interval [95% CI] = 148–248). In Pouma, 35% of slides contained *P. falciparum*, with a higher MGPD, 731/μL (95% CI = 559–957) than in Dienga ($P < 0.0001$). At the time of venous blood collection, 34% and 32% of the samples contained detectable *P. falciparum* blood stage parasites in Dienga and Pouma, respectively.

Immunological data used for analysis concerned individuals whose PBMC proliferated in response to leucoagglutinin and/or PPD (99%). Cellular responsive-

ness to control antigens and to peptides from LSA-1 is presented in the Figure. An average of 59% and 57% of donors from Dienga and Pouma, respectively, mounted a cellular response to at least 1 LSA-1 peptide, determined either by lymphoproliferation or cytokine production. More than 25% of individuals in both sites produced IL-10 in response to all peptides, whilst 20% or more of Gabonese children produced IFNγ and 25% or more of Cameroonian children produced TNFα. The levels of lymphoproliferative responses, as well as those of cytokines produced, were rather low and, after having pooled data from both sites and peptides, the mean SI value was 1·10 (range 0·39–3·7; 95th percentile 0·58), and the mean amounts of cytokines produced were 0·3 pg/mL (range 0–60·5 pg/mL) for IFNγ, 2·4 pg/mL (range 0–161·7 pg/mL) for TNFα and 2·5 pg/mL (range 0–157·5 pg/mL) for IL-10. Comparison of the proportions of responders in the 2 sites showed a higher prevalence of TNFα producers in Pouma (all tests, $P < 0·0004$). After adjusting for age, this difference persisted, the rate being higher in Pouma than in Dienga (logistic regression analysis, all tests $P < 0·005$, odds ratio = 7·9 for LSA-Rep, 11·1 for LSA-J, and 18·2 for LSA-CTL). No significant association was observed at the individual level, in either study site or when the data were pooled, between peptide-stimulated T cell proliferation and cytokine production or between the different cytokines produced to any of the LSA-1 peptides (Spearman rank correlation test: all tests, $P \geq 0·09$). The presence of detectable *P. falciparum* blood-stage parasites at the time of sampling was associated with higher T cell proliferative responses to all 3 LSA-1 peptides in Dienga (P values 0·007–0·047), but not in Pouma. In addition, cellular immune responses were related to clinical protection, as shown in Table 1, but again only in Dienga, where lymphocyte proliferative responses to LSA-Rep and LSA-CTL were significantly higher in protected than in unprotected children ($P = 0·024$ and $P = 0·036$, respectively). This association between higher responsiveness in Dienga and protection from clinical attack was not influenced by parasitaemia at the time of sampling, because in this site no relation was found between the presence of parasites in blood at the time of admission and clinical protection ($P \geq 0·5$).

Humoral responses are presented in Table 2. Similar prevalences of antibody responses to schizont extract, LSA-Rep and LSA-J were observed in both sites, but significantly higher levels of these antibodies were found in Dienga than in Pouma, independently of age (logistic regression analysis, all values of $P \geq 0·05$). No correlation was found between antibody responses to the peptides LSA-Rep and LSA-J when data from all 119 subjects were pooled (Spearman rank correlation test: $\rho = 0·14$, $P = 0·12$). Twenty-one per cent of those with an antibody response to LSA-Rep were non-responders to LSA-J. A positive association between antibody

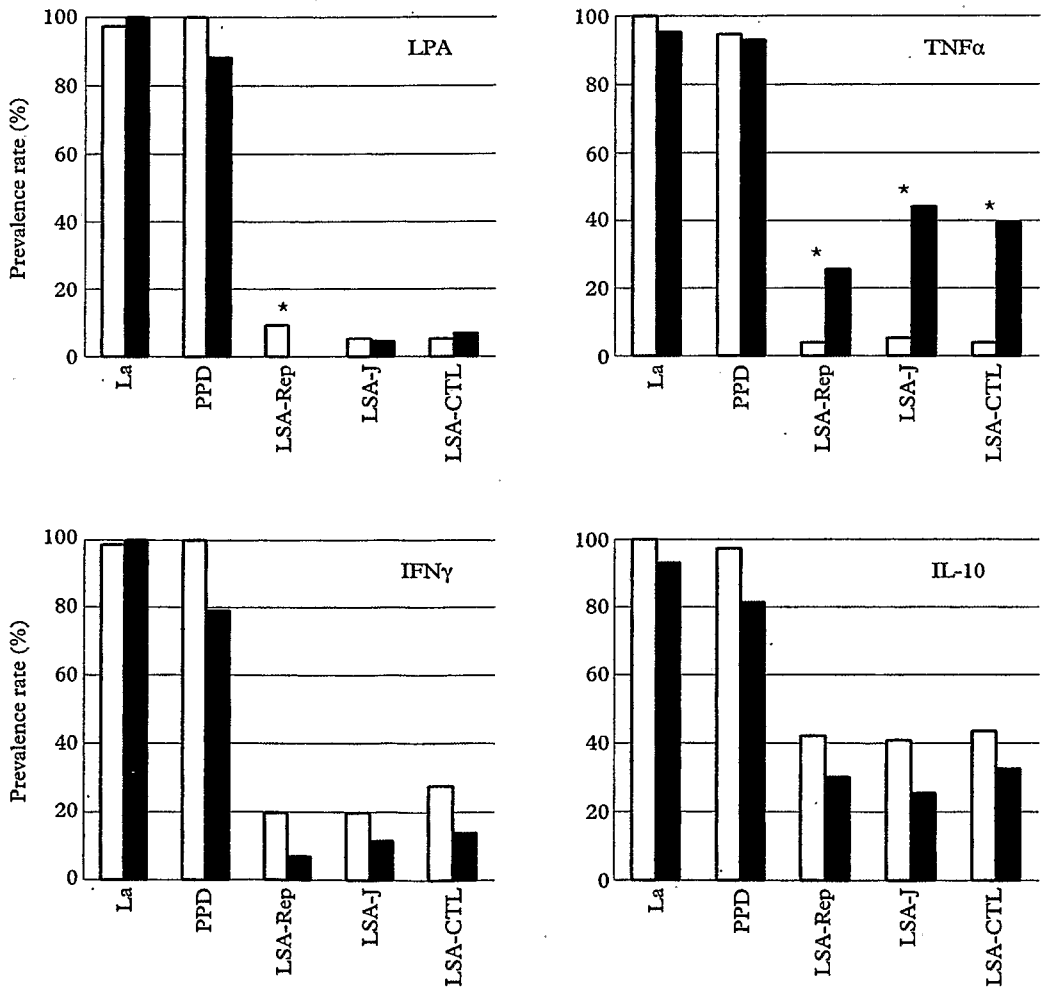

Figure. Percentages of T cell responders to leucoagglutinin (La), purified protein derivative (PPD) and synthetic peptides (LSA-Rep, LSA-J and LSA-CTL) from *P. falciparum* liver stage antigen-1 in 76 children from Dienga, Gabon (□) and 43 children from Pouma, Cameroon (■). Lymphocyte proliferation (LPA) was measured by thymidine incorporation. Cytokine release was measured by ELISA as the difference between stimulated and unstimulated culture supernatants. For thresholds of positivity, see text. * $P < 0·001$, χ^2 test.

Table 1. Differences in mean immune responses to schizont extract and synthetic peptides from *P. falciparum* liver stage antigen-1 between 38 protected and 38 unprotected children from Dienga, Gabon

	Geometric mean values[a]	P^b
Lymphoproliferative responses		
LSA-Rep[c]		
Protected	1·35 (1·22–1·51)	0·024
Unprotected	1·11 (0·99–1·25)	
LSA-CTL[c]		
Protected	1·03 (0·91–1·15)	0·036
Unprotected	0·88 (0·76–1·02)	
Antibody responses		
Schizont extract		
Protected	66·0 (46·5–93·8)	0·022
Unprotected	55·8 (39·3–79·3)	
LSA-Rep[c]		
Protected	12·7 (6·2–26·1)	0·065
Unprotected	4·5 (1·9–10·9)	

[a]95% confidence interval in parentheses. Lymphocyte proliferation was measured by thymidine incorporation and expressed as stimulation index (SI). IgG antibodies were determined by ELISA and expressed as arbitrary units (AU).
[b]Values of *P* determined using the Mann–Whitney test.
[c]Synthetic peptide.

Table 2. Prevalence rates and levels of humoral responses to schizont extract and synthetic peptides from *P. falciparum* liver stage antigen-1 in 76 children from Dienga (Gabon) and 43 from Pouma (Cameroon)

	Dienga		Pouma		
	Responders (%)	Geometric mean values[b]	Responders (%)	Geometric mean values[a]	P^b
Schizont extract	97	72·2 (67·6–77·2)	91	61·1 (54·2–68·8)	0·002
LSA-Rep	63	41·3 (34·8–49·0)	46	22·3 (18·0–27·5)	0·008
LSA-J	62	55·8 (47·0–66·3)	49	43·7 (33·6–56·7)	0·039

[a]Geometric mean values, including responders only, of IgG antibodies determined by ELISA and expressed as arbitrary units (AU).
[b]Values of *P* determined from all subjects by the Mann–Whitney *U* test.

responses and protection from clinical malaria was found only in Dienga (Table 1), where protected children had higher levels of antibodies directed against the schizont extract and LSA-Rep than did unprotected children ($P = 0·022$ and $P = 0·065$, respectively).

Discussion

The results of this study document the cellular and humoral immune responses to synthetic peptides from the LSA-1 antigen of 2 populations of children originating from different areas endemic for malaria. The prevalences of proliferative response and cytokine and IgG responses we observed are in agreement with previous findings concerning the responses of naturally infected individuals to the same peptides. Thus, in a study in a hyperendemic area of Burkina Faso, 64% of the study subjects had antibody to LSA-Rep (FERREIRA-DA-CRUZ *et al.*, 1995). In an area of Madagascar where malaria is endemic, a study using LSA-J or LSA-Rep peptides showed a dissociation between individual antibody responses to any 2 peptides. In the same study, stimulation *in vitro* by the same peptides showed that T cell proliferation and secretion of IFNγ were detected in PBMC of 6–20% and 22–28% of individuals, respectively (FIDOCK *et al.*, 1994). A recent study of Papua New Guinean adults, however, reported much higher frequencies of proliferative (76–82%) and IFNγ (33–88%) responses after stimulation with 3 different peptides located in the N- and C-terminal non-repeat regions of LSA-1 (CONNELLY *et al.*, 1997). An association between LSA-1 specific IFNγ responses and resistance to *P. falciparum* infection was suggested by these latter authors. In a separate study in Gabon, this association was definitely demonstrated: production of IFNγ in response to the LSA-J and LSA-CTL peptides was associated with resistance to reinfection in children with mild malaria (LUTY *et al.*, 1999). The results of the current study, showing a positive association between lymphocyte proliferation to LSA-Rep and LSA-CTL and clinical protection in Gabonese schoolchildren, extended these findings. Parasite clearance times, in children with mild malaria, were associated with the occurrence of IL-10 responses to LSA-J and LSA-CTL as well as with the levels of antibodies directed against LSA-Rep (LUTY *et al.*, 1998). The trend we found here, towards higher anti-LSA-Rep antibodies in protected children compared to those who were unprotected, suggests that immune responses to the antigen LSA-1, the expression of which is thought to be restricted to the hepatic phase of development of *P. falciparum*, may be associated not only with the control of parasitaemia but also with protection against clinical malaria attacks (HOLLINGDALE *et al.*, 1998; LUTY *et al.*, 1998). To account for this, it has been suggested that immunological cross-reactivity may occur between epitopes of LSA-1 and those of asexual blood-stage antigens (LUTY *et al.*, 1998). In this same context, we speculate that pre-erythrocytic immunity, by decreasing the number of *P. falciparum* strains emerging from the liver, might also limit the likelihood of blood-stage infections arising from strains against which acquired immunity has yet to be developed.

The prevalence of *P. falciparum* infections was twice as high in the Cameroon site as in the Gabon study site, whereas the monthly malaria attack rate was twice as high in the latter (DELORON *et al.*, 1999). Thus, the differ-

ences in T cell reactivity, reflected by inverse profiles of IFNγ and TNFα secretions in Dienga and Pouma, may be related to the higher rate of malaria attacks observed in the former site. Age is known to play a role in the development of naturally acquired antimalarial immunity (BAIRD, 1995). Thus, anti-LSA-J antibodies could be stratified by age, as demonstrated by a study conducted in a malaria endemic area in Burkina Faso, which revealed that their prevalence was high among subjects aged 1–20 years (>75%), and decreased thereafter (<30%) (FERREIRA-DA-CRUZ *et al.*, 1995). Nevertheless, age did not account for the differences in immune responsiveness observed in Dienga and Pouma, as these differences persisted after having excluded the oldest children from Dienga (those whose age exceeded the mean age + 1 SD of children from Pouma) (data not shown). Differences in the intensity or seasonality of malaria transmission, as well as in the genetic diversity of the *P. falciparum* strains circulating in the 2 sites, may also account for some of the apparent disparities in immunological activity between them (CREASEY *et al.*, 1990). In fact, the degree of polymorphism of the *MSP-1* and *MSP-2* genes of *P. falciparum* was similarly high in the 2 sites, although several alleles were observed in 1 site but not in the other (F. Ntoumi, unpublished data). Additionally, and perhaps most importantly in this context, the genetic background of populations has been shown to contribute to differences in immunological activity to plasmodial antigens (MODIANO *et al.*, 1996). We predict that the distribution of human leucocyte antigen (HLA) alleles, for example, probably differs significantly between the Gabonese, principally Nzebi ethnic group, and the Cameroonian Bassa populations we studied here. To date, the only known association between an HLA class 1 allele, HLA-B53, and protection from severe malaria was reported in Gambians (HILL *et al.*, 1991). This finding, however, may not be applicable to other population groups because of the striking differences which exist in the distributions of both HLA class 1 and class 2 among different sub-Saharan African populations (HAMMOND *et al.*, 1997).

The present study, by comparing the immune responses in 2 different populations sites, has confirmed the high immunogenicity of the LSA-1 antigen and given further encouragement to its use in malaria vaccines. The results point to an additional necessity, in the interpretation of immunological results, to consider confounding factors which might include differing specificities of both the parasite and the host in different geographical areas.

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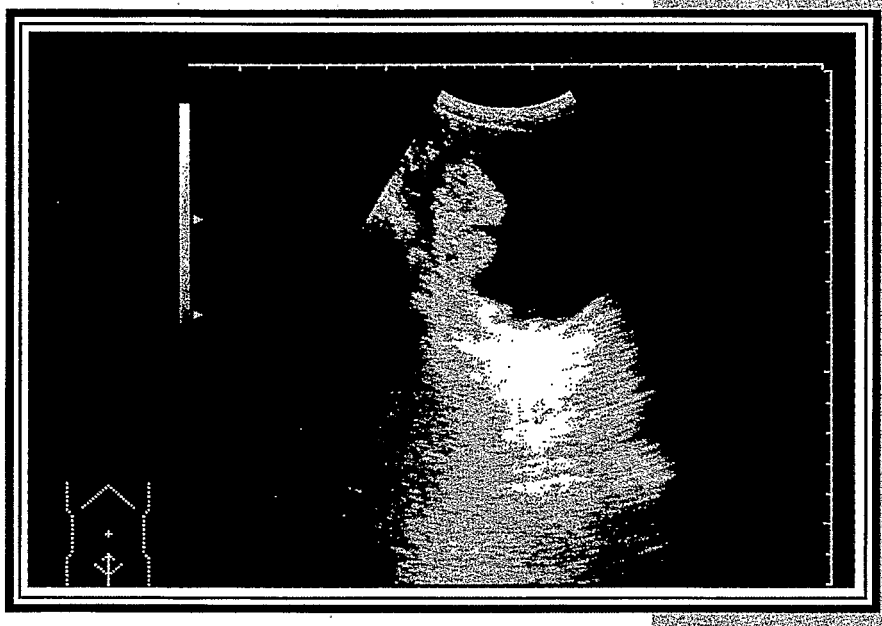

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