

PATTERN OF IMMUNOGLOBULIN ISOTYPE RESPONSE TO
PLASMODIUM FALCIPARUM BLOOD-STAGE ANTIGENS IN
 INDIVIDUALS LIVING IN A HOLOENDEMIC AREA OF SENEGAL
 (DIELMO, WEST AFRICA)

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Abstract. Three cross-sectional studies were conducted in a representative cohort of individuals living continuously in an area holoendemic for malaria in Senegal. Plasma from 145 children and adults were tested. The pattern of antimalarial immunoglobulin class (IgM and IgG) and subclass (IgG1 to IgG4) antibody distribution was determined by enzyme-linked immunosorbent assay using a crude blood-stage antigen of *Plasmodium falciparum*-infected red blood cells. Adults had higher levels of specific antibodies than children, and IgM, IgG2, and IgG3 accounted for the highest difference (2.9, 6.5, and 4.5 times, respectively). Differences in antibody levels were significant for IgG1 to IgG4 between the lowest and the highest transmission season. No particular isotype distribution pattern could be found associated with any given parasitemia level. The relationship between the optical density (OD) values of each isotype and the risk of clinical malaria attack was tested using a Poisson regression model. Only the IgG3 OD increases were found associated with a significantly reduced risk of malaria attack. These seroepidemiologic data suggest that whereas the total IgG-specific activity is not indicative of any given level of protection against malaria, the level of IgG3 was significantly associated with the relative susceptibility to clinical *P. falciparum* malaria attacks.

In recent years, increasing importance has been given to the immunoglobulin (Ig) classes and subclasses directed against extracts of various infectious agents, including parasitic infections.¹⁻⁵ The potential protective role of the humoral immune response to the erythrocytic stage of *Plasmodium falciparum* malaria has been suggested by different observations,⁶⁻¹² but the precise role of subclass antibodies in protection against human malaria is still incompletely determined. Nevertheless, a potential and preferential role for protection afforded by cytophilic IgG isotypes has been emphasized, both in animal models and in humans infected with *P. falciparum*.¹³⁻¹⁷ With regard to the hypothesis that the antibodies that are protective against malaria may act in collaboration with effector cells, in particular blood monocytes, the subclass determination of the specific antibodies produced in response to infection seems of particular interest.¹⁸⁻²³ Data also suggest that noncytophilic antibody subclasses inhibiting the activity of cytophilic antibodies are detectable during *P. falciparum* malaria.^{17, 24} Therefore, no clear pattern of association between isotypes and clinical immunity has so far emerged, but as a result of their functional and potentially opposite activity, the relative isotype distribution may differ among individuals living in endemic areas.

In the present study, we determined the extent to which the pattern of the whole *P. falciparum* antigen-specific immunoglobulin isotype distribution in the plasma of individuals exposed to continuous and intense parasite transmission was associated with and/or affected by age, transmission, parasitemia levels, and occurrence of malaria attacks in Senegal.²⁵ We evaluated by enzyme-linked immunosorbent assay (ELISA) the antibody responses directed against somatic antigens (representing mainly cytoplasmic schizont proteins, including some membrane antigens) that are indicative of the whole antibody response developed against mature schiz-

ont forms of the asexual erythrocyte stage of *P. falciparum*.^{26, 27}

MATERIALS AND METHODS

Study area and population, clinical and parasitologic monitoring, and collection of plasma. The village of Dielmo (13°45'N, 16°25'W) is situated in a rural area of Senegal 280 km southeast of Dakar, 15 km north of the Gambian border, and is located on the bank of a permanent fresh water river, as described elsewhere.^{25, 28}

Of the 250 inhabitants of Dielmo, blood was obtained from 145 individuals (mean \pm SD age 23.0 \pm 20.7 years) living permanently in the village during the survey. The subjects enrolled in this study included exclusively the inhabitants followed continuously during a two-year period. These individuals had not left the village for more than 10 days during the entire study period, and had spent 95-100% of their life in this village. Informed consent was obtained individually from all participants or their parents.

A malaria attack was defined by an episode of fever associated with a parasite density over an age-dependent pyrogenic threshold as described in this village. (The existence of a pyrogenic threshold effect allowed parasite density to be used to distinguish malaria attacks from other causes of fever. Its level varied by 2.45 trophozoites per leukocyte, maximum at one year of age, to 0.5 trophozoites per leukocyte, minimum at 60 years of age.²⁹) Malaria attacks were estimated by daily active case detection, and none of the subjects had a malaria attack or a febrile episode at the time of sampling.²⁵ Pregnant women and patients with a malaria attack during the previous two weeks were not included.

Capillary blood samples (n = 145) were obtained during the dry season in April 1991 (period 1 [P1], corresponding



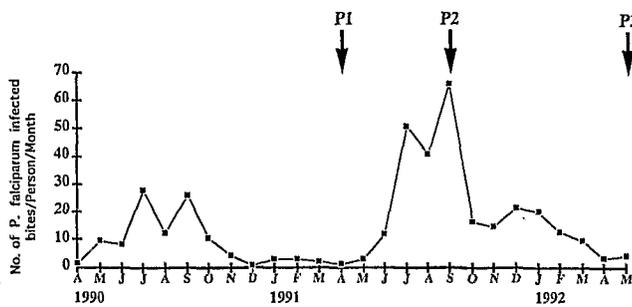


FIGURE 1. Monthly variations of inoculation rates of *Plasmodium falciparum* in Dielmo, Senegal from April 1990 to May 1992. P1, P2, and P3 correspond to the three periods of sampling.

to a low transmission period with 1.3 infected bites per person per month). Seventy-three samples randomly chosen from the 145 were sampled a second time during the wet season five months later in September 1991 (period 2 [P2], corresponding to the highest transmission season with 65.9 infected bites per person per month), and the remaining 72 were tested a second time in May 1992 (period 3 [P3], corresponding to a second low transmission season with 4.1 bites per person per month). The characteristics of the two subgroups of subjects enrolled at P2 and P3 were comparable either on the basis of age, sex ratio, or red blood cell polymorphism (AA or AS hemoglobin distribution). The three periods were selected after analysis of entomologic data and, as shown in Figure 1, allowed us to compare different levels of transmission.²⁸

Blood was drawn into heparinized capillary tubes (Liquemine®; Roche, Neuilly sur Seine, France) and thick blood films were prepared at the time of blood sampling. Thick smears were dried for 24–48 hr, dehemoglobinized, and stained with Giemsa. All blood film readings were independent from this ongoing study and standardized as previously described.^{30,31} Briefly, 200 microscopic oil-immersion fields were examined on each slide (approximately 0.5 µl of blood) and the identification of *P. falciparum*, *P. ovale*, or *P. malariae* was confirmed by a trained parasitologist. Parasitemias reported in this study referred only to the identification of asexual forms observed in each blood film.

Parasite strains used as antigens. The Dielmo strain of *P. falciparum* was isolated from an inhabitant of Dielmo in November 1991 and successfully cultivated thereafter, following the method of Trager and Jensen.³² The Banjul strain was a kind gift of Dr. K. Marsh (Medical Research Council, Banjul, The Gambia). It originated from the Gambia and has been cultivated for 13 years in our laboratory. The K+ strain was derived from the FUP/CB strain and had also been maintained for eight years in our laboratory. When the parasitemia was approximately 10%, sorbitol-synchronized cultures that had reached the schizont stage were concentrated by differential centrifugation, and the enriched mature schizont fraction was immediately isolated, aliquoted, and frozen at -70°C until use. The parasite extract was prepared by controlled lysis of schizont-infected erythrocytes with 0.1% saponin, 0.06 N NaCl, ultrasonication in the presence of enzyme inhibitors, centrifugation at 4°C, and filtration with a 0.2-µm Millex® filter (SA67; Millipore, Molsheim, France).

The IgM and IgG antibody levels detected against *P. fal-*

ciparum extracts obtained from different strains were compared. The lowest concentrations of *P. falciparum* antigen (5 µg/ml) and the highest dilution (1:200) of plasma samples that gave maximal absorbances in combination with the lowest background were chosen. The optical densities (ODs) obtained against the Dielmo extract were consistently higher than those observed for the extracts derived from the two other available strains. In a longitudinal study, 15 individuals were tested 4–12 times. The antibody responses observed against the extract produced with the Dielmo isolate were higher than those obtained with the other strains, both for IgM (in two of three cases) and IgG (in three of three cases) levels, while the negative controls were always low. Therefore, the signal (OD - background) was the highest with the *P. falciparum* Dielmo extract, which was selected for the following studies of isotype determinations.

Enzyme-linked immunosorbent assays (ELISAs) for anti-malaria antibody isotypes. The total *P. falciparum* antigen extract was used for analysis of reactivity with the plasma samples according to published and validated methods.^{15,23,33–36} Following preliminary experiments to optimize the ELISA conditions, the selected concentration of the *P. falciparum* antigen extract was 5 µg/ml. A total of 100 µl of a carbonate buffered (pH 9.6) solution containing the antigen was added to each well of 96-well, flat-bottomed polystyrene microtiter plates (Nunclon®; Nunc, Roskilde, Denmark) and incubated overnight at 4°C. After the plates were washed five times with phosphate-buffered saline (PBS)/1% Tween, 200 µl of 1% bovine serum albumin in PBS was added to each well and incubated for 1 hr at 37°C to block the antigen-free surface of the wells.

Thereafter, 1:200 dilutions of the plasma samples were added to the wells (100 µl/well) and incubated at 37°C for 90 min. Each plasma sample was tested in duplicate either in antigen-coated or control red blood cell-coated wells for IgM, total IgG, and each IgG subclass. After being washed, IgM and total IgG plates were incubated for 1 hr at 37°C in the presence of peroxidase-conjugated goat F(ab')₂ fragment to human IgG Fc (gamma chain-specific, at a dilution of 1:6,000) or human IgM Fc (mu chain-specific, at a dilution of 1:4,000) (Cappel Organon Teknica Corp., West Chester, PA).

For determination of the immunoglobulin isotypes, the monoclonal antibodies (MAbs) used were mouse anti-IgG1 (clone NL16) at a dilution of 1:2,000, anti-IgG3 (clone ZG4) at a dilution of 1:10,000, and anti-IgG4 (clone GB7B) at a dilution of 1:30,000. These three MAbs were obtained from Unipath (Bedford, United Kingdom). The MAb anti-IgG2 (clone HP6002), used at a dilution of 1:10,000, was obtained from Sigma (St. Louis, MO). When used at optimal concentrations, these MAbs gave subclass-specific results, and they were previously defined as correctly reflecting the immunoglobulin content of African or Asian individuals.^{17,23} By use of a panel of calibrated IgG1 to IgG4 purified myelomas (reference no. 1-3889, 1-4139, 1-4389, and 1-4639 for IgG1 to IgG4 respectively, Sigma; a gift from O. Mercereau-Puijalon, Institut Pasteur, Paris, France), the linear relationship between the OD and the concentrations of the different immunoglobulins (particularly that of IgG2) was confirmed using an ELISA and enzyme-linked immunosorbent dot-blot assays, and the titration curves were similar to those pub-

lished elsewhere.³⁷ Additional control standards were purified IgG from a pool of immune Africans and a purified IgM myeloma, a kind gift from Dr. J. L. Preudhomme (Poitiers, France), as previously reported.^{17, 20, 21}

Following incubation, the plates were washed, and 100 μ l/well of peroxidase-conjugated goat anti-mouse IgG Fabs specific at a dilution of 1:2,000 (Sigma) were added and the plates were incubated for 60 min at 37°C. After five washings, 100 μ l of citrate buffer (pH 4) containing 0.16 mg/ml of chromogenic substrate (*o*-toluidine) and 10% H₂O₂ (4 μ l/ml) was added, the reaction was then stopped with 4 N H₂SO₄, and OD values were read at 450 nm in a Titertek multiscan plate reader (Flow Laboratories, Ltd., Ayshire, Scotland).

The ELISA used in the present study proved to be sensitive and reproducible for our investigations after control of different parameters known to potentially influence ELISA measurements of IgG and IgG subclass antibodies to different antigens.³⁸⁻⁴² The optimal antigen coating concentration (5 μ g/ml) and the optimal dilution of plasma samples (1:200) were the same throughout our study and were chosen following checkerboard titrations. To minimize variations, plasma samples from a given individual were assayed on a single plate (i.e., different samples of a given individual were tested on the same plate, and the different isotypes were evaluated on the same day). Each plasma sample was analyzed in the presence of a control plasma known to be negative for malaria antibodies and a positive standard that consisted of a pool of positive plasma from Senegal. Results for IgG subclass activities were expressed on a relative basis using the patient's own plasma as an individual reference from one period (P1) to the other (either P2 or P3): each individual was tested twice, the first time at P1, and a second time either at P2 or P3.

In parallel, and for each determination, the background was determined as the OD obtained in the presence of control erythrocytes. These control erythrocytes corresponded to the red blood cells used for the parasite cultures, and they were maintained in the same incubator at 37°C for seven days and in a separate flask, aliquoted, and stored in conditions strictly comparable with those of the parasites. The mean OD \pm 1 SD of age- and ethnicity-matched healthy unexposed subjects was used for each immunoglobulin subclass as the cutoff value to determine positivity, and an OD value of 0 was given a value of 10⁻³ for statistical purposes.

Since the OD values for all immunoglobulins were evaluated in technically identical conditions, the OD variations within a given subclass could be directly compared as a function of age, particularly from one age group to the other, and/or as a function of transmission, from one malaria transmission season to the other, and/or as a function of changes in parasitemia, from one sampling period (P1) to another (P2 or P3). The OD levels of each antibody subclass were also individually tested for their potential association with a protective effect against malaria attacks.

Statistical analyses. The potential association between antibody levels and age was tested either by considering the age factor as a continuous variable or as a discontinuous one, when the group of 145 individuals was stratified into three age groups, on the basis of clinical follow-up and epidemiologic data reported elsewhere.^{25, 28, 43, 44}

The cohort was stratified into three critical age groups of similar size and defined as follows: group 1) \leq 7 years of age ($n = 38$ children); group 2) $> 7 - \leq 25$ years of age ($n = 57$ young individuals); group 3) > 25 years of age ($n = 50$ adults).

Each individual was tested twice: the first time at P1 and the second time at either a five-month (P2) or 13-month (P3) interval so that individual variations in antibody levels could be evaluated using paired-tests.⁴⁵ The statistical analyses included the paired *t*-test, the Wilcoxon test for quantitative data, Spearman's rank correlation, regression analysis, the Mann-Whitney U-test for comparisons between two groups (values dichotomized were sex, hemoglobin phenotype, i.e., either AA or AS, and thick blood smears as positive or negative values), and the Kruskal-Wallis test for categorical data (age groups, periods of analysis).⁴⁵ When *P* values were < 0.05 , they were considered significant.

The effect of age and period of study were estimated by a two-factor repeated measures analysis of variance test. This analysis allowed us to evaluate the effect of transmission level and its potential differential effect in the different age groups. The relationship between the pattern of isotype distribution at P1 and the risk of malaria attack during the six months before and after blood sampling (i.e., over a one-year period), was tested using a Poisson regression model taking into account the effect of age, hemoglobin phenotype AS, and gender. The OD values of each isotype were included as covariates in this model and they were individually tested by likelihood ratio statistics (LRS). Protective effects were determined as equal to 1 minus the estimated rate ratios.

RESULTS

IgM- and IgG-specific antibodies directed at the control red blood cell antigen. The level of recognition of the control red blood cell antigen was usually low in Dielmo. During the first period of sampling (period P1), most anti-red blood cell values were limited (mean \pm 1 SD = 0.119 \pm 0.108 and 0.393 \pm 0.163 for IgM and IgG, respectively). By comparison, these values were significantly more elevated during the second period of sampling at P2 (0.155 \pm 0.148; $P = 0.0014$ and 0.525 \pm 0.235; $P = 0.0001$ for IgM and IgG, respectively, by the Wilcoxon paired test). Between periods P1 and P3, the anti-red blood cell total IgG (but not IgM) were also different, with IgG1 and IgG2 values accounting for most of the differences observed ($P = 0.012$ and $P < 0.01$, respectively). However, among the individuals with elevated levels of anti-red blood cell antibodies, no correlation with hemoglobin levels was detected. These individual variations in anti-red blood cell immunoglobulins were systematically taken into consideration.

Age-dependent anti-*P. falciparum* isotype and subclass distribution. Among the 145 inhabitants of Dielmo enrolled at period P1, the mean specific IgM and IgG levels differed with age, and they were significantly different among the three age groups ($P = 0.0001$, by the Kruskal-Wallis test). Whereas this change was gradual for IgG, it was much more sudden after the age of seven years for the IgM levels, which were only slightly elevated later on, as illustrated in Figure 2. As a consequence, the IgM increase paralleled that of the

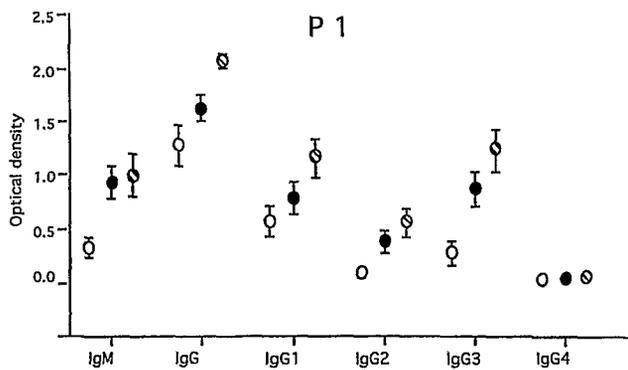


FIGURE 2. Pattern of *Plasmodium falciparum*-specific immunoglobulin optical density values (log mean \pm SEM) distribution in three different age groups during period P1 (April 1991, corresponding to a low transmission season). The original cohort was stratified into 38 children (open circles = ≤ 7 years), 57 young individuals (solid circles = > 7 - ≤ 25 years), and 50 adults (striped circles = > 25 years).

IgG among children and to a lesser extent among young individuals ($r = 0.542$ and $r = 0.331$, respectively), but this was no longer the case among adults more than 25 years of age. When the younger age group was compared with the older one, the difference in IgM and IgG OD values was 2.9-fold and 1.6-fold, respectively.

At P1, all *P. falciparum*-specific IgG1 to IgG4 subclasses were also found to differ as a function of age (Figure 2). The IgG2 and the IgG3 isotypes were more elevated (6.5-fold and 4.5-fold, respectively) than the IgG1 and IgG4 isotypes (2.0-fold and 1.9-fold, respectively) when comparing children less than seven years of age with adults more than 25 years of age. The IgG2 and IgG3 isotype OD values were significantly correlated with age among children ($r = 0.572$ and $r = 0.485$, respectively).

At P2 and P3, the same age-dependent changes were observed. Therefore, irrespective of the period of study, the isotype distribution pattern remained largely comparable over the 13-month study, and the main changes in OD values were those affecting IgM, IgG2, and IgG3.

Changes in isotype and subclass distribution as a function of malaria transmission. *Comparison of period P1 versus period P2.* Between these two periods, i.e., over an interval of five months, the number of infective bites per person per month was dramatically increased by a factor of 50.7. In our study group, the mean percentage of asymptomatic infected subjects (i.e., healthy people with a detectable level of asexual parasite forms at the time of sampling) was not significantly different between these two periods (54.3% at P1 and 54.5% at P2). Nevertheless, the mean parasitemia was increased from 2.9 (95% confidence interval [CI] = 0.8-5.1) to 7.7 (CI = 3.1-12.4). All immunoglobulin OD values (except total IgG) were significantly modified between these two different periods of transmission, and they were elevated at P2 (Figures 3-6).

The subclass OD values of all immunoglobulins were compared between P1 and P2 in the three age groups by a two-factor repeated measures analysis of variance. A significant season-related difference was detectable for IgG1 to IgG4 values with $P < 0.001$ for IgG1 and IgG3, $P < 0.03$ for IgG2, and $P < 0.005$ for IgG4.

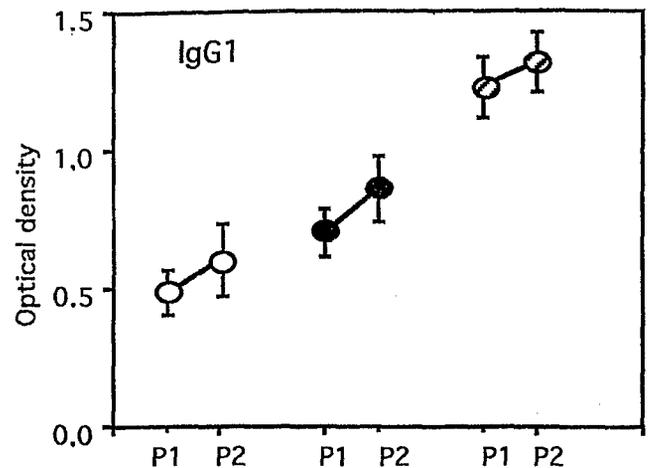


FIGURE 3. Pattern of *Plasmodium falciparum*-specific IgG1 optical density values (log mean \pm SEM) distribution in three different age groups during period P1 (April 1991, corresponding to a low transmission season) and period P2 (September 1991, corresponding to the highest transmission season). The original cohort was stratified into 17 children (open circles = ≤ 7 years), 28 young individuals (solid circles = > 7 - ≤ 25 years), and 28 adults (striped circles = > 25 years).

No major change was observed in those subjects less than seven years of age. In the second age group including 28 individuals, IgM levels were increased in 19 subjects, IgG1 in 22, and IgG3 in 19. The same three immunoglobulin isotypes were found to be increased in 17, 19, and 21 of 27 adults, respectively, and the mean IgG2 levels were also increased in 19 of 27 cases in the group of adults.

The impact of the transmission season at P2 was markedly different in the three age groups for the IgG3 values ($P < 0.05$): the OD increase at P2 was 8.8% in those less than seven years of age, 12.4% in the > 7 - ≤ 25 -year-old age group, and 17.9% in the > 25 -year-old age group. Therefore,

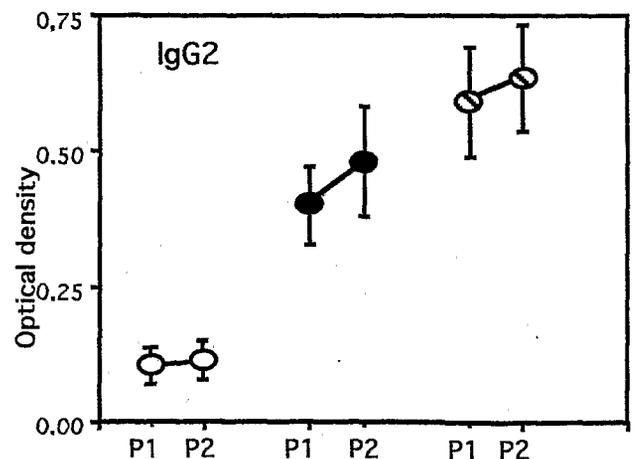


FIGURE 4. Pattern of *Plasmodium falciparum*-specific IgG2 optical density values (log mean \pm SEM) distribution in three different age groups during period P1 (April 1991, corresponding to a low transmission season) and period P2 (September 1991, corresponding to the highest transmission season). The original cohort was stratified into 17 children (open circles = ≤ 7 years), 28 young individuals (solid circles = > 7 - ≤ 25 years), and 28 adults (striped circles = > 25 years).

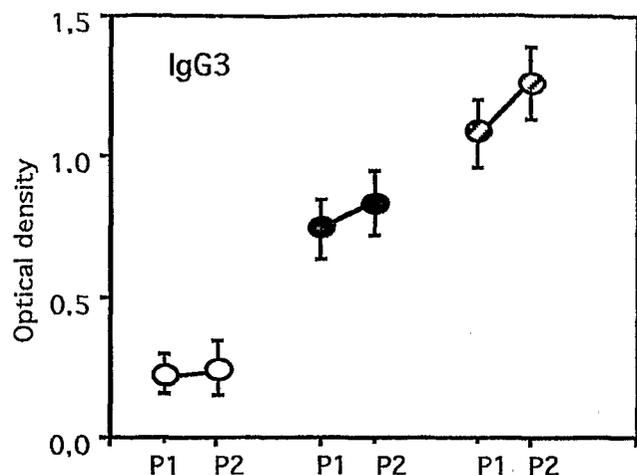


FIGURE 5. Pattern of *Plasmodium falciparum*-specific IgG3 optical density values (log mean \pm SEM) distribution in three different age groups during period P1 (April 1991, corresponding to a low transmission season) and period P2 (September 1991, corresponding to the highest transmission season). The original cohort was stratified into 17 children (open circles = ≤ 7 years), 28 young individuals (solid circles = > 7 - ≤ 25 years), and 28 adults (striped circles = > 25 years).

between P1 and P2, the most striking difference related to the dramatic increase in transmission level was observed for the IgG3 subclass values, which were significantly altered both as a function of season and age group. This differential impact of transmission season was not observed for any other parasite-specific immunoglobulin subclass.

Comparison of the two low transmission periods (P1 versus P3). Between these two periods of low transmission, i.e., over an interval of 13 months, the number of infected bites per person per month was comparable. The percentages of healthy asymptomatic subjects harboring parasite asexual blood forms were only slightly but not significantly different (67.6% and 52.1% at P1 and P3, respectively). The mean parasitemia at P1 (mean = 9.8, CI = 4.2-15.5) and at P3 (mean = 6.9, CI = 0.7-13.0) was not statistically different.

The two-factor repeated measures analysis of variance test did not allow the detection of a statistically significant difference in any isotype values between P1 and P3 for all age groups. Only limited variations were detectable between the two samplings: among children, the IgG2 and IgG4 isotypes were increased (in 17 and 14 of the 21 children tested at P3), and the IgG3 isotype was decreased (in 20 of 29 young individuals).

Therefore, at a one-year interval, the pattern of isotype distribution was largely comparable, particularly in the two older age groups, with the highest variability observed among children.

Relationship between parasitemia and the isotype distribution. At the individual level, daily changes in parasitemia were not directly associated with modifications in the pattern of humoral response. At the cohort level during period P1, parasitemia values were inversely correlated with age ($\rho = -0.464$, $P = 0.0001$). The age variable was controlled by studying independently two subgroups of subjects either less than or more than 25 years of age. As shown in Table 1, in each subgroup, the presence (in 73 of 94 and 14

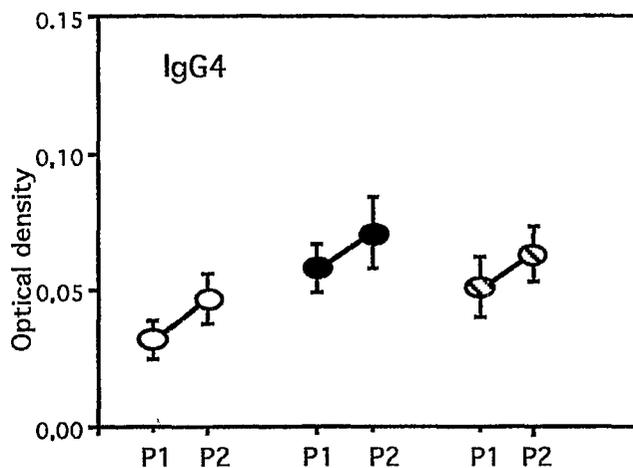


FIGURE 6. Pattern of *Plasmodium falciparum*-specific IgG4 optical density values (log mean \pm SEM) distribution in three different age groups during period P1 (April 1991, corresponding to a low transmission season) and period P2 (September 1991, corresponding to the highest transmission season). The original cohort was stratified into 17 children (open circles = ≤ 7 years), 28 young individuals (solid circles = > 7 - ≤ 25 years), and 28 adults (striped circles = > 25 years).

of 50 individuals less than or more than 25 years of age, respectively) or the absence of detectable parasitemia at an identical age was not associated with any given pattern of *P. falciparum*-specific class or subclass antibody distribution. Statistical analysis showed that the age factor and the presence or absence of parasites were highly correlated ($P = 0.0001$, by Kendall rank correlation), but for a given age, no correlation between specific humoral responses and parasitemia could be found.

Pattern of isotype distribution and incidence of malaria attacks. In Dielmo, the risk of malaria attacks and age were inversely correlated: the risk dramatically decreased after two years of age and reached the level found in adults at approximately 7-10 years of age. The mean number of malaria attacks observed over a one-year period was 4.8 (CI = 3.1-6.6) in the 0-2-year-old age group, 2.5 (CI = 1.3-3.6) in the 3-6-year-old age group, and 0.2 (CI = 0.1-0.4) in the older individuals. The risk of malaria attacks observed in children less than three years of age (0-2 years) was referred to as a baseline level of risk. According to the Poisson regression analysis, the protective effect of age was 39.2% (CI = 10.2-58.8) in the 3-6-year-old age group and 94.6% (CI = 91.3-96.6) in the oldest children and adults.

A series of Poisson models was fitted to test and estimate how much of the age protective effect could be accounted for by the OD level of each isotype. The IgG3 OD increases alone were found associated with a significantly reduced risk of malaria attacks (LRS = 11.3, $P < 0.001$). In contrast, the OD values of all other immunoglobulin classes or subclasses (parasite-specific IgM, total IgG, IgG1, IgG2, and IgG4) were not significantly associated with a reduced risk of malaria attack.

An IgG3 increase from 0.095 (mean value observed in the 0-2-year-old age group) to 1.015 (mean value observed among children more than seven 7 years of age) was associated with a 55.4% protective effect (CI = 22.1-74.5). An

TABLE 1
Relationships between parasitemia and *Plasmodium falciparum* specific class and subclass distribution found in the plasma of two subgroups of Dielmo, Senegal inhabitants greater than and less than 25 years of age*

Age (years)	Thick blood smear	N	Mean \pm SD parasite density	Mean \pm SD age (years)	Immunoglobulin class			Immunoglobulin subclass			
					IgM	IgG	IgG1	IgG2	IgG3	IgG4	
<25	0	21	0	9.1 \pm 8.9	0.603 \pm 0.141	1.369 \pm 0.149	0.658 \pm 0.115	0.355 \pm 0.105	0.510 \pm 0.132	0.051 \pm 0.01	
	>0	73	24 \pm 10	10.4 \pm 6.0	0.727 \pm 0.064	1.527 \pm 0.055	0.713 \pm 0.062	0.237 \pm 0.033	0.671 \pm 0.069	0.041 \pm 0.04	
>25	0	36	0	49 \pm 14	1.012 \pm 0.122	2.082 \pm 0.041	1.137 \pm 0.100	0.598 \pm 0.092	1.278 \pm 0.105	0.060 \pm 0.01	
	>0	14	5.8 \pm 3.2	43 \pm 20	0.941 \pm 0.164	1.985 \pm 0.067	1.187 \pm 0.173	0.449 \pm 0.063	1.103 \pm 0.219	0.047 \pm 0.01	

* Ig values are the mean \pm SEM optical density values.

IgG3 increase of 2 ODs was associated with a 82.7% protective effect (CI = 69.8–90.1). In this analysis, there was no significant interaction between IgG3 OD values and age effects.

When the IgG3 OD level was included as a covariate in the model and when the 0–2-years-old age group was referred as a baseline level of risk, the residual age protective effect was 25.4% (CI = 11.7–50.8) in the 3–6-years-old age group and 89.1% (CI = 80.5–93.9) in the older individuals. This indicated that the IgG3 OD could account for 35.2% (39.2 – 25.4/39.2 = 35.2%) of the age-associated protective effect in the 3–6-years-old age group and 5.8% in the older inhabitants of Dielmo.

DISCUSSION

In the present study, we evaluated the isotype distribution of malaria-specific antibodies directed against unfractionated schizont antigens derived from a local isolate recently adapted to in vitro cultures. Antibodies to somatic antigens were detected by ELISA, a methodology that allowed the evaluation of some aspects of the humoral immune response in different malarial infections.^{14, 15, 17, 22, 23, 46, 47} Comparable blood-stage extracts were already used in previous studies and considered as indicators of the response directed against the parasite.^{48–52} The precise nature of the different antigens recognized by the malaria-specific antibodies was not determined in this study, but immunoblot analysis of different plasma samples obtained from a given individual consistently revealed the same pattern of antigen recognition, and this is in agreement with previous work.⁵³ Indeed, part of the humoral response was occasionally found to be directed towards normal red blood cell contaminants, and this illustrated the necessity of the simultaneous use of red blood cell control antigen with the crude plasmodial antigen.⁵⁴ Such recognition of uninfected erythrocytes was described during chronic falciparum malaria and autoimmune anemia.^{55–57} However, it should be mentioned that in Dielmo, this red blood cell recognition was restricted mainly to the high transmission season, and no anemia was found, even in individuals with high levels of anti-erythrocyte antibodies.

In this initial evaluation of the antibody response in an endemic area, our data are indicative of differential isotypic responses, and this is in agreement with previous field studies.^{17, 23, 46} Our study design allowed for the control of the individual factor, giving an estimate of the age variable, seasonal impact (level of exposure to transmission), the effect of parasitemia detectable at the time of sampling, and the potential relationship between immunoglobulin levels and the occurrence of malaria attacks. This approach was only possible when evaluating the parasitemia at period P1 and/or the occurrence of malaria attacks during the entire study period and when comparing the pattern of isotype distribution in individuals with different levels of parasitemia or with different numbers of malaria attacks (provided that the occurrence of parasite-positive smears or malaria attacks were not altered by a recent treatment, i.e., less than one year). This was indeed the case since we carried out the active and continuous follow-up of all individuals for a long period, during which a specific program including daily

monitoring of the Dielmo inhabitants allowed the evaluation of their individual susceptibility to the parasite.^{25, 43}

When the level of specific antibodies against *P. falciparum* extracts was analyzed as a function of age, a gradual increase in levels of specific antibodies from the younger to the older age group was apparent. With age (thus exposure to the parasite), the transmission level tended to have a greater (but still transient) impact upon the isotype distribution, whereas the duration of exposure to the parasite had a maximum influence upon the isotype level of children. As a function of age, which was the major factor associated with the antibody specific isotype distribution pattern, the main changes detectable were those affecting the parasite-specific IgM, IgG2, and IgG3 isotypes.

Changes in IgM levels probably reflected the occurrence of successive infections by different strains in Dielmo where it was recently demonstrated that acquiring antiparasite immunity results in a decrease in both the parasite load and its apparent complexity (i.e., the number of potentially different strains participating in the infection).⁵⁸ Variations in specific IgM levels have been previously reported and were found to be potentially associated with immunoprotection of humans living in a hyperendemic area, but this was not confirmed in a different study carried out in Gabon.^{51, 59} In Dielmo, IgM levels were gradually and consistently increased during the highest transmission season. This suggested the recent contact with new strains of parasites, and either the immune recognition of these new strains of parasite by the young hosts⁵⁸ of an antigen-induced T cell-independent response.⁵¹

Comparisons between P1 and P2 and/or P3 illustrated the immunologic shifts associated with long-term exposure to *P. falciparum*. The dramatic increase in transmission level at P2 was associated with increased levels of IgM, IgG1, and IgG3 in the two older age groups, in which individuals progressively developed an efficient protective immunity.^{25, 43, 44} No significant difference was detectable in OD values between P1 and P2 in children less than seven years of age, and this was probably due to the large OD fluctuations observed in the younger age group or to constraints of physiologic maturation described in children.⁶⁰

In the intermediate age group, the transient increase in specific immunoglobulins at period P2 was much more marked than in those less than seven years of age. In particular, IgM, IgG1, and IgG3 values were significantly increased. In contrast, most OD levels were largely comparable between P1 and P3. Therefore, in this intermediate age group, the transmission season had a much higher impact than the time lapse between two samplings.

In the older age group, a dramatically marked but transient alteration in OD values was observed at period P2: most isotype levels, with the exception of IgG4, differed significantly between P1 and P2, whereas in contrast, all immunoglobulin classes and subclasses were largely comparable between P1 and P3. Therefore, in this age group, only slight differences could be detected after a 13-month interval whereas the transmission level had a marked but momentary impact, as was observed for the second age group. As previously described,²⁵ protection is progressively acquired in Dielmo, and it is probably of some relevance that in the two older age groups, both IgG1 and IgG3 (cytophilic antibodies) levels were found to be altered at a time of increased parasite

transmission. That IgG1 and IgG3 levels were found to be increased in association with an increase in malaria transmission is reminiscent of previous studies suggesting that cytophilic antibodies could participate in mechanisms of cellular antibody cooperation, which could play a prime role in the development of protection.^{17-21, 61, 62}

Parasitemia was not found to be directly associated with a given pattern of antibody distribution (i.e., it was not associated with an absolute specific antibody level). This observation is in agreement with a comparable study, and could be related to the fact that even a negative thick blood smear is no guarantee of parasite negativity.^{24, 25} Therefore, exposure to, more than infection by *P. falciparum*, tended to significantly alter the antiparasite isotype-specific distribution.

The relationship between the risk of malaria attack in Dielmo and the level of IgG3 was evaluated after taking into account the effect of age, using a Poisson regression model. This method of analysis indicated that irrespective of age, an increase in parasite-specific IgG3 was significantly associated with a decrease in the risk of malaria attack. The part of the age-associated protective effect that could be explained by the level of parasite-specific IgG3 was statistically greater during the earliest stages of acquisition of natural immunity in Dielmo (i.e., in children less than six years of age). Therefore, our study is indicative that the level of parasite-specific IgG3 is a critical factor and that its measurable contribution with regard to protection varies with age. On the one hand, this is evocative of a possible increase in the affinity and/or the fine specificity of these cytophilic antibodies, and in the development of a progressively more efficient functional IgG3 activity following repeated exposure to *P. falciparum*. On the other hand, this observation suggests that more than one mechanism could participate in the resistance against malaria attacks (in this case, the involvement of parasite-specific IgG3 would effectively differ between the young individuals and the older ones.

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REFERENCES

1. Hitch WL, Hightower AW, Eberhard ML, Lammie PJ, 1991. Analysis of isotype-specific antifilarial antibody levels in a Haitian, pediatric population. *Am J Trop Med Hyg* 44: 161-167.
2. Dhandayuthapani S, Izumi S, Anandan D, Bhatia VN, 1992. Specificity of IgG subclass antibodies in different clinical manifestations of leprosy. *Clin Exp Immunol* 88: 253-257.

3. Dafa'Alla TH, Ghalib HW, Abdelmageed A, Williams JF, 1992. The profile of IgG and IgG subclasses of onchocerciasis patients. *Clin Exp Immunol* 88: 258-263.
4. Yazdanbakhsh M, Paxton WA, Kruike YCM, Sartono E, Kuriawan A, Wout AK, Selkirk ME, Maizels RM, 1993. T-cell responsiveness correlates differentially with antibody isotype levels in clinical and asymptomatic filariasis. *J Infect Dis* 167: 925-931.
5. Ellassad AMS, Youni SA, Siddig M, Grayson J, Petersen E, Ghalib HW, 1994. The significance of blood levels of IgM, IgA, IgG and IgG subclasses in Sudanese visceral leishmaniasis patients. *Clin Exp Immunol* 95: 294-299.
6. Cohen SJ, McGregor IA, Carrington SC, 1961. Gammaglobulin and acquired immunity to human malaria. *Nature* 192: 733-737.
7. McGregor IA, 1964. The passive transfer of human malarial immunity. *Am J Trop Med Hyg* 13: 237-239.
8. Tobie JE, Abele DC, Wolff SM, Contacos PG, Evans CB, 1966. Serum immunoglobulin levels in human malaria and their relationship to antibody production. *J Immunol* 97: 498-490.
9. Cohen SJ, Butcher GA, 1971. Serum antibody in acquired malarial immunity. *Trans R Soc Trop Med Hyg* 65: 125-135.
10. Fandeur T, Dubois P, Gysin J, Dedet JP, Pereira da Silva L, 1984. In vitro and in vivo studies on protective and inhibitory antibodies against *Plasmodium falciparum* in the Saimiri monkey. *J Immunol* 132: 432-437.
11. Hoffman SL, Wistar R Jr, Ballou WR, Hollingdale H, Wirtz R, Schneider I, Marwoto H, Hockmeyer W, 1986. Immunity to malaria and naturally acquired antibodies to the circumsporozoite protein of *Plasmodium falciparum*. *N Engl Med* 315: 601-606.
12. Marsh K, Otoo L, Hayes RJ, Carson DC, Greenwood BM, 1989. Antibodies to blood stage antigens of *Plasmodium falciparum* in rural Gambians and their relation to protection against infection. *Trans R Soc Trop Med Hyg* 83: 293-303.
13. Green TJ, Kreier JP, 1975. Demonstration of the role of cytophilic antibody in resistance to malaria parasite (*Plasmodium berghei*) in rats. *Infect Immun* 19: 138-145.
14. Wahlgren M, Berzins K, Perlmann P, Persson M, 1983. Characterization of the humoral immune response in *Plasmodium falciparum* malaria. II. IgG subclass levels of anti-*P. falciparum* antibodies in different sera. *Clin Exp Immunol* 54: 135-142.
15. Wahlgren M, Berzins K, Perlmann P, Bjorkman A, 1983. Characterization of the humoral immune response in *Plasmodium falciparum* malaria. I. Estimation of antibodies to *P. falciparum* or human erythrocytes by means of microELISA. *Clin Exp Immunol* 53: 127-134.
16. Langhorne J, Evans CB, Asofsky R, Taylor DW, 1984. Immunoglobulin isotype distribution of malaria-specific antibodies produced during infection with *Plasmodium chabaudi adami* and *Plasmodium yoelii*. *Cell Immunol* 87: 452-461.
17. Bouharoun-Tayoun H, Druilhe P, 1992. *Plasmodium falciparum* malaria: evidence for an isotype imbalance which may be responsible for delayed acquisition of protective immunity. *Infect Immun* 60: 1473-1481.
18. Khusmith S, Druilhe P, 1983. Cooperation between antibodies and monocytes that inhibit in vitro proliferation of *Plasmodium falciparum*. *Infect Immun* 41: 219-223.
19. Lunel F, Druilhe P, 1989. Effector cells involved in nonspecific and antibody-dependent mechanisms directed against *Plasmodium falciparum* blood stages in vitro. *Infect Immun* 57: 2043-2049.
20. Bouharoun-Tayoun H, Attanath P, Sabchareon A, Chongsuphaisiddhi T, Druilhe P, 1990. Antibodies that protect humans against *Plasmodium falciparum* blood stages do not on their own inhibit parasite growth and invasion in vitro, but act in cooperation with monocytes. *J Exp Med* 172: 1633-1641.
21. Druilhe P, Bouharoun-Tayoun H, 1991. Natural immunities. *Res Immunol* 142: 637-643.
22. Bouharoun-Tayoun H, Oeuvray C, Lunel F, Druilhe P, 1995. Mechanisms underlying the monocyte mediated antibody-dependent killing falciparum asexual blood stages. *J Exp Med* 182: 409-418.
23. Dubois B, Deloron P, Astagneau P, Chougnnet C, Lepers JP, 1993. Isotypic analysis of *Plasmodium falciparum*-specific antibodies and their relation to protection in Madagascar. *Infect Immun* 61: 32768-4500.
24. Groux H, Perraut R, Garraud O, Poingt J, Gysin J, 1990. Functional characterization of the antibody-mediated protection against blood stages of *Plasmodium falciparum* in the monkey *Saimiri sciureus*. *Eur J Immunol* 20: 2317-2323.
25. Trape JF, Rogier C, Konate L, Diagne N, Bouganali H, Canque B, Legros F, Badji A, Ndiaye P, Brahimi K, Faye O, Druilhe P, Peirera da Silva L, 1994. The Dielmo project: a longitudinal study of natural malaria infection and the mechanisms of protective immunity in a community living in a holoendemic area of Senegal. *Am J Trop Med Hyg* 51: 123-137.
26. Boudin C, Sheik I, Chumpitazi B, Pazart L, Hogh B, Peyron F, Deloron P, Picot S, Ambroise-Thomas P, 1994. The multifactorial and multistage character of protective immunity to *Plasmodium falciparum* naturally acquired by an indigenous population in Burkina Faso. *Scand J Immunol* 39: 409-417.
27. Chumpitazi B, Lepers JP, Rason M, Meunier A, Boudin C, Ambroise-Thomas P, 1993. Circulating stable antigens at higher levels down-regulate antibody responses to *Plasmodium falciparum*. *Parasitol Res* 79: 163-167.
28. Konate L, Diagne N, Brahimi K, Faye O, Legros F, Rogier C, Petrarca V, Trape JF, 1994. Biologie des vecteurs et transmission de *Plasmodium falciparum*, *P. malariae*, et *P. ovale* dans un village de savane d'Afrique de l'ouest (Dielmo, Senegal). *Parasite* 1: 325-333.
29. Rogier C, Commenges D, Trape J-P, 1996. Evidence for an age-dependent pyrogenic threshold of *Plasmodium falciparum* parasitemia in highly endemic populations. *Am J Trop Med Hyg* 54: (in press).
30. Trape JF, Peelman P, Moreault-Peelman B, 1985. Criteria for diagnosing clinical malaria among a semi-immune population exposed to intense and perennial transmission. *Trans R Soc Trop Med Hyg* 79: 435-442.
31. Trape JF, 1985. Rapid evaluation of malaria parasite density and standardization of thick smear examination for epidemiological investigations. *Trans R Soc Trop Med Hyg* 79: 181-184.
32. Trager W, Jensen J, 1976. Human malaria parasite in continuous culture. *Science* 193: 673-675.
33. Fandeur T, Bonnefoy S, Mercereau-Puijalon O, 1991. In vivo and in vitro derived Palo Alto lines of *Plasmodium falciparum* are genetically unrelated. *Mol Biochem Parasitol* 47: 167-178.
34. Spencer HC, Collins WE, Chin W, Skinner JC, 1979. The enzyme-linked immunosorbent assay (ELISA) for malaria. I. The use of in vitro-cultured *Plasmodium falciparum* as antigen. *Am J Trop Med Hyg* 28: 927-932.
35. Voller A, Cornille-Broger R, Storey J, Molineaux L, 1980. A longitudinal study of *Plasmodium falciparum* malaria in the west Africa savanna using the ELISA technique. *Bull World Health Organ* 58: 429-438.
36. Quakyi I, 1980. The development and validation of an enzyme linked immunosorbent assay for malaria. *Tropenmed Parasitol* 31: 1-8.
37. Falconer AE, Carson R, Johnstone R, Bird P, Kehoe M, Clavert JE, 1993. Distinct IgG1 and IgG3 subclass responses to two streptococcal protein antigens in man: analysis of antibodies to streptolysin O and M protein using standardized subclass-specific enzyme-linked immunosorbent assays. *Immunology* 79: 89-94.
38. Hvatum M, Scott H, Brandtzaeg P, 1992. Pitfalls in determining IgG and IgG subclass antibodies to food antigens. *J Immunol Methods* 148: 77-85.
39. Venkatesan P, Wakelin D, 1993. ELISAs for parasitologists: or lies, damned lies and ELISAs. *Parasitol Today* 9: 228-232.
40. Eltiro F, Ye EY, Taylor MG, 1992. Evaluation of an enzyme-linked immunosorbent assay (ELISA) using *Schistosoma mansoni* soluble egg antigen as a diagnostic tool for *Schistosoma mansoni* infection in Ethiopian schoolchildren. *J Trop Med Hyg* 95: 52-56.
41. Riley EM, Olerup O, Troye-Blomberg M, 1991. The immune recognition of malaria antigens. *Parasitol Today* 7: 5-11.

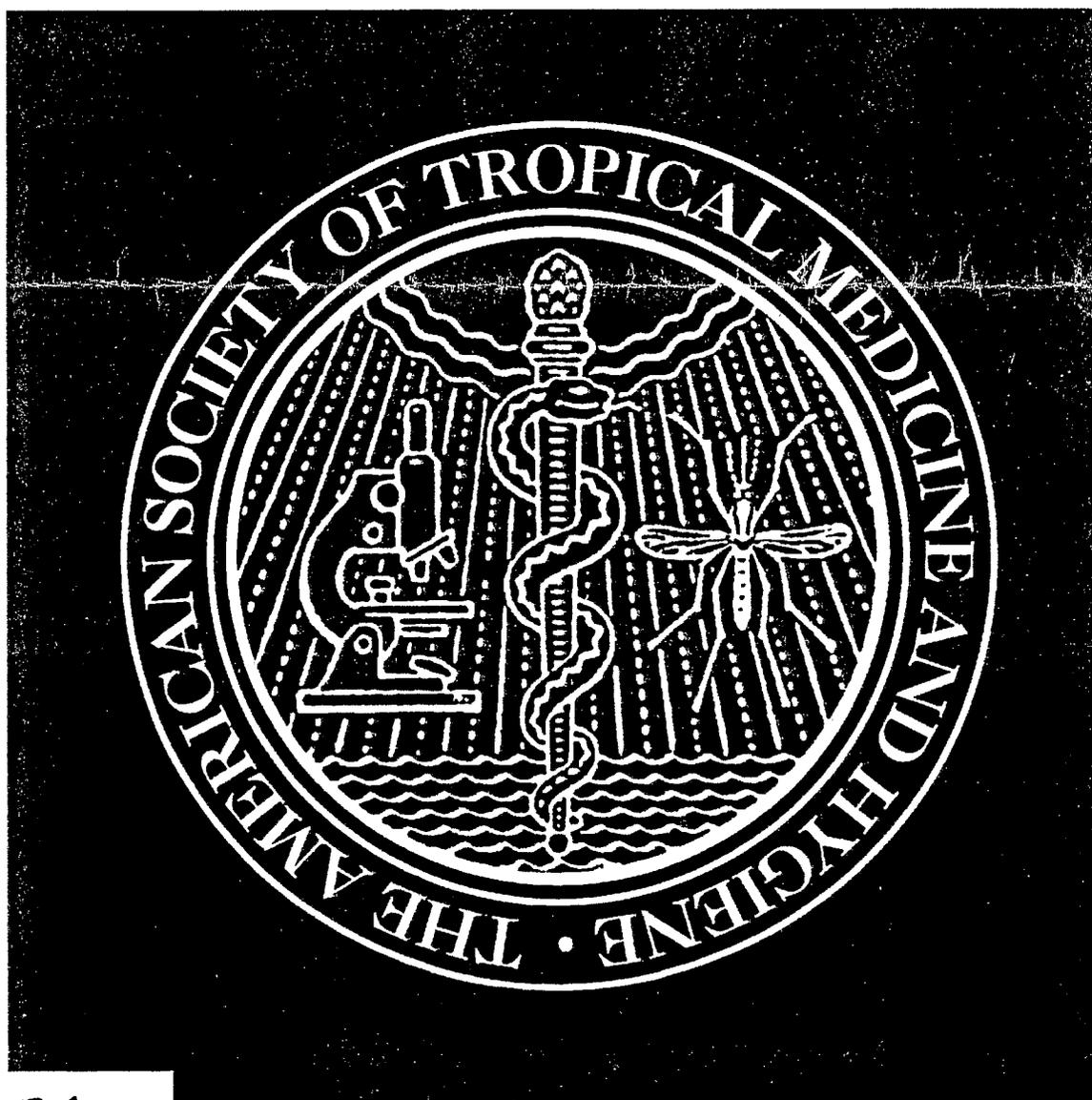
42. Chougnat C, Deloron P, Lepers JP, 1990. Humoral and cell-mediated immune responses to the *Plasmodium falciparum* antigens Pf155/RESA and CS protein: seasonal variations in a population recently reexposed to endemic malaria. *Am J Trop Med Hyg* 43: 234-242.
43. Rogier C, Trape JF, 1993. Malaria attacks in children exposed to high transmission -Who is protected? *Trans R Soc Trop Med Hyg* 87: 245-246.
44. Trape JF, Rogier C, 1995. Efficacy of SPf66 vaccine against *Plasmodium falciparum* malaria in children. *Lancet* 345: 134-135.
45. Siegel S, Castellan NJ, 1989. *Non Parametric Statistics for the Behavioral Sciences*. New York: MacGraw-Hill.
46. Wahlgren M, Perlmann H, Berzins K, 1986. Characterization of the humoral immune response in *Plasmodium falciparum* malaria. III. Factors influencing the coexpression of antibody isotypes (IgM and IgG-1 to 4). *Clin Exp Immunol* 63: 343-353.
47. Thelu J, Ambroise-Thomas P, Contat M, Kupka P, 1982. Antigènes excretés-sécrétés par *Plasmodium falciparum* en culture in vitro. Etude comparée avec les antigènes somatiques et les antigènes figures. *Bull World Health Organ* 60: 761-766.
48. McGregor IA, Williams K, Voller A, Billewicz WZ, 1965. Immunofluorescence and measurement of immune response to hyperendemic malaria. *Trans R Soc Trop Med Hyg* 59: 395-414.
49. Brasseur P, Ballet JJ, Druilhe P, 1990. Impairment of *Plasmodium falciparum*-specific antibody response in severe malaria. *J Clin Microbiol* 28: 265-268.
50. Erunkulu OA, Hill AVS, Kwiatkowski DP, Todd JE, Iqbal J, Berzins K, Riley EM, Greenwood B, 1992. Severe malaria in Gambian children is not due to lack of previous exposure to malaria. *Clin Exp Immunol* 89: 296-300.
51. Luty AJF, Mayombo J, Lekoulou F, Mshana R, 1994. Immunologic responses to soluble exoantigens of *Plasmodium falciparum* in Gabonese children exposed to continuous intense infection. *Am J Trop Med Hyg* 51: 720-729.
52. Wahlgren M, Bjorkman A, Perlmann H, Berzins K, Perlmann P, 1986. Anti-*Plasmodium falciparum* antibodies acquired by residents in a holoendemic area of Liberia during development of clinical immunity. *Am J Trop Med Hyg* 35: 22-29.
53. Roffi J, Portales P, Delgado G, Parent G, Chevassus-Agnes S, 1985. Specificité antigenique des anticorps antiplasmodiaux en zone d'endémie: essai de corrélation avec le développement de l'immunité protectrice. *Ann Inst Pasteur/Immunol* 136: 323-341.
54. Demedts P, Vermeulen-Van Overmeir C, Wery M, 1987. Simultaneous use of *Plasmodium falciparum* crude antigen and red blood cell control antigen in the enzyme-linked immunosorbent assay for malaria. *Am J Trop Med Hyg* 36: 257-263.
55. Lefrançois G, Le Bras J, Simmonneau M, Bouvet E, Vroklans M, Vachon F, 1981. Anti-erythrocyte autoimmunisation during chronic falciparum malaria. *Lancet* ii: 661-664.
56. Rosenberg EB, Strickland CT, Yang SL, Whalen GE, 1973. IgM antibodies to red cells and autoimmune anemia in patients with malaria. *Am J Trop Med Hyg* 22: 146-152.
57. Zouali M, Druilhe P, Gentilini M, Eyquem A, 1982. High titres of anti-T antibodies and other haemagglutinins in human malaria. *Clin Exp Immunol* 50: 83-91.
58. Ntoumi F, Contamin M, Rogier C, Bonnefoy S, Trape JF, Mercereau-Puijalon O, 1995. Age-dependent carriage of multiple *Plasmodium falciparum* merozoite surface antigen-2 alleles in asymptomatic malaria infections. *Am J Trop Med Hyg* 52: 81-88.
59. Boudin C, Chumpitazi B, Dzielge M, Peyron F, Picot S, Hough B, Ambroise-Thomas P, 1993. Possible role of specific immunoglobulin antibodies to *Plasmodium falciparum* antigens in immunoprotection of humans living in a hyperendemic area, Burkina Faso. *J Clin Microbiol* 31: 636-641.
60. Goddard EA, Malan EH, Beatty DW, 1992. IgG subclass values from normal children in Cape Town. *Scand J Immunol* 36: 210-214.
61. Perignon JL, Druilhe P, 1994. Immune mechanisms underlying the premunition against *Plasmodium falciparum* malaria. *Mem Inst Oswaldo Cruz* 89: 51-53.
62. Oeuvray C, Bouharoun-Tayoun H, Grass-Masse H, Lepers JP, Ralamboranto L, Tartar A, Druilhe P, 1994. A novel merozoite surface antigen of *Plasmodium falciparum* identified by cellular-antibody cooperative mechanism antigenicity and biological activity of antibodies. *Mem Inst Oswaldo Cruz* 89: 77-80.

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