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Plasmodium falciparum Induces Apoptosis in Human Mononuclear Cells

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The level of spontaneous apoptosis in short-term lymphocyte cultures was evaluated in different human immunodeficiency virus-negative groups of either healthy control individuals or patients with clinical malaria. The mean percentage of spontaneous apoptosis found in patients during a malaria attack was significantly higher than in sex- and age-matched healthy controls. The healthy asymptomatic controls were individuals with different degrees of exposure to *Plasmodium falciparum* as reflected by their various mean levels of specific anti-*P. falciparum* (immunoglobulin G and M) antibodies. The percentages of apoptotic nuclei were found to be significantly higher in lymphocytes from subjects living in an area where malaria is holoendemic than in lymphocytes from subjects less exposed. Concentrations of soluble plasma interleukin-2 receptor were also higher in subjects from areas where malaria is endemic than in other groups, revealing different levels of lymphocyte activation. Of particular relevance to the in vivo situation, a *P. falciparum* schizont-rich extract induced a systematic and significant elevation of apoptotic nuclei at day 6 in 87.5% (35 of 40) of the subjects tested. In additional studies with different concentrations of extract, [^{3}H]thymidine incorporation was concomitant with a low or limited level of apoptosis. Taken together, our results strongly suggest that acute as well as chronic asymptomatic *P. falciparum* infections were consistently associated with a marked increase in the level of mononuclear cell apoptosis. This process could be implicated in some of the alterations reported for the proliferative T-cell responses in areas where malaria is endemic.

Malaria is a disease for which a major activation of the immune system has been demonstrated to occur in subjects living in areas of high endemicity (8). This activation, evidenced by a raised level of soluble plasma interleukin-2 receptor (sIL2R), was suggested to play a major role both in the defense mechanism against the parasite and in pathogenesis (24). In some reports, it was demonstrated that high levels of circulating sIL2R were correlated with low in vitro lymphocyte spontaneous proliferation, suggesting that activated cells might have been withdrawn from the circulation (9, 16). Moreover, drastic changes induced by *Plasmodium falciparum* in several different parameters of immune function, such as a decreased number of circulating T lymphocytes (36, 37) and in vitro depression of the proliferative response of peripheral blood mononuclear cells (PBMC) to malaria antigens (23, 28), were reported.

Apoptosis is a widely studied mechanism of cell death involved in a large range of pathological as well as physiological events. The general characteristics of apoptosis are well established and occur through distinctive morphological and molecular characteristics, including chromatin condensation, fragmentation of DNA into oligonucleosome-size pieces, swelling, and progressive cell degradation (2, 7). Evidence shows that in most circumstances, apoptosis serves a biologically meaningful, homeostatic function mainly in development and growth regulation. Indeed, apoptosis was described in some situations as a protective mechanism against disease by eliminating unwanted (damaged, precancerous, or excessive) cells (2). But recent work suggested that in some cases, apoptosis may be involved in some pathological dysfunctions and diseases (6). One of the hypotheses put forward was that persistent exposure to activation may lead to immune dysfunction and either loss of ability to respond to an antigen (anergy) or induction of an abnormal program of cell death (1, 13, 19).

Following our initial observation of increased levels of apoptosis during and following a malaria attack (30), we have further investigated the possibility that the phenomenon of apoptosis is associated with some of the perturbations described above. In this study, we first confirm and extend our previous report showing that subjects with acute *P. falciparum* infection have elevated percentages of in vitro lymphocyte apoptosis in comparison with healthy individuals. These high percentages of apoptosis detected in vitro could persist for at least 1.5 months. The levels of spontaneous apoptosis, i.e., apoptosis detected in short-term cultures of PBMC, from human immunodeficiency virus (HIV)-negative, healthy African individuals living in areas with different malaria transmission levels were evaluated and compared with those of PBMC of healthy Caucasian subjects with limited exposure to malaria. In the present study, we demonstrated that apoptosis levels were significantly correlated with exposure to *P. falciparum* which was associated with high levels of sIL2R. Finally, the effect of *P. falciparum* antigens (a schizont-enriched extract obtained after adaptation and cultivation of a local parasite isolate) was also evaluated, and we observed that the parasite was responsible for an increase in the level of in vitro apoptosis of PBMC. These observations are discussed in relation to the different effects potentially induced by the parasite upon the host's immune system.

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MATERIALS AND METHODS

Subjects and blood samples. A total of 26 patients (8 Caucasians and 18 Africans) with a clinically defined *P. falciparum* attack were studied. Most of these patients were hospitalized in the Hôpital Principal de Dakar.

Human peripheral blood samples were also obtained from 95 healthy adult donors (control groups) sampled during the dry season. These donors were age- and sex-matched groups of subjects differing only by the degree of exposure to *P. falciparum*. Parameters such blood formula were controlled and found comparable among all groups. The first group of African subjects ($n = 37$, mean age ± standard deviation = 35.6 ± 9.6 years) lived in greater Dakar (Senegal, West Africa), which is an area of low and seasonal malaria transmission (33). Two other groups of African subjects originated from two different villages: Dielmo ($n = 32$, mean age of 39.5 ± 20.7 years) and Ndiop ($n = 14$, mean age of 33.5 ± 13.6 years). These two villages are situated in a rural area of Senegal, 270 km southwest of Dakar. Entomological and parasitological surveys showed that in Dielmo (an area where malaria is holoendemic) there was a high and perennial parasite transmission, whereas in Ndiop (an area where malaria is mesoendemic), parasite transmission was clearly seasonal (33). Means of 15.6 and 3.2 infective bites per person per month were recorded in Dielmo and Ndiop, respectively, during our study period (1992 to 1993). In Dakar, the mean number of infective bites ranged from 0.382 to 0.014 per person per year, according to a recent study (32). The fourth group was composed of Caucasian subjects ($n = 12$, mean age of 41.1 ± 9.5 years) who had spent generally a few months in Dakar and had no history of clinical malaria infection.

Venous blood samples were collected in dry evacuated tubes to which 250 UI of heparin (Liquemine; Roche) was added per 10 ml of blood. The blood samples of the subjects from Dielmo and Ndiop were transferred by road to the Pasteur Institute in Dakar in less than 5 h, using isotherm boxes in order to maintain the temperature between 20 and 25°C. The subjects of the other groups were sampled directly in the Pasteur Institute facilities. We determined that the levels of apoptosis of lymphocytes from the subjects living in the two rural areas where malaria is endemic were not altered by the transfer, per se, of the blood samples to Dakar. For this control, the blood samples from different volunteers were taken in Dielmo or Ndiop and then transferred to Dakar. The same volunteers came to Dakar on the same day, and they were sampled a second time. When transferred and freshly drawn blood samples were subsequently treated simultaneously, the percentages of apoptosis detected in each paired samples had systematically comparable values.

We also sampled 56 subjects from areas where malaria is endemic (14 subjects from Dielmo and 42 subjects from Ndiop) during the rainy season to evaluate the influence of the rate of parasite transmission.

With their informed consent, all subjects were tested for the presence of HIV infection, and none was found positive in the cohort enrolled for this study.

Preparation of the *P. falciparum* extract. The Dielmo isolate used in this study was obtained from an inhabitant of Dielmo in November 1991 and successfully cultivated thereafter as previously described (31). *P. falciparum* extracts were prepared by different methods, depending on later use.

For the extract used in cellular culture, schizont enrichment was performed by Plasmagel (Laboratoire Roger Bellon, Neuilly sur Seine, France) sedimentation, yielding preparations containing >95% schizont-infected erythrocytes. Both parasitized and control noninfected erythrocytes were washed three times in RPMI 1640 (Gibco Laboratories, Grand Island, N.Y.). After centrifugation, the final pellet was resuspended in 4 volumes of distilled water, aliquoted, and frozen at −70°C until use. The lysate was used in the different tests at a final dilution of 3/2,000, which was found to induce a peak of cellular [^{3}H]thymidine incorporation between day 6 and 7. In some experiments, the effects of differents dilutions (from 0.5/2,000 to 6/2,000) of the parasite extract on the percentage of detectable apoptotic nuclei and on the level of [^{3}H]thymidine incorporation were evaluated in parallel. An extract of another strain of *P. falciparum* (Palo Alto, FUP/66 Marburg strain) was then tested simultaneously with the Dielmo parasite crude extract.

The extract used for quantitation of antimalaria antibodies was prepared by another method. When the parasitemia was around 10%, sorbitol-synchronized cultures which 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 thawed only once, and the extraction procedure was carried out immediately. A *P. falciparum* extract was prepared by controlled lysis of schizont-infected erythrocytes with 0.1% saponin in 0.06 M NaCl, ultrasonication in the presence of enzyme inhibitors, centrifugation at 4°C, and filtration on a 0.2-μm-pore-size Millex-GV filter (Millipore S.A., Molsheim, France).

Detection of antimalaria antibodies by ELISA. Quantitation of immunoglobulin M (IgM) and IgG antimalaria antibodies in plasma samples was performed by using an enzyme-linked immunosorbent assay (ELISA) according to published methods (27, 35). In these assays, the total *P. falciparum* antigen extract was used at the selected concentration of 5 μg/ml in a pH 9.6 carbonate-buffered solution. One hundred microliters of this preparation were added to each well of 96-well flat-bottom polystyrene microtiter plates (Nunclon; Nunc, Roskilde, Denmark), and the plates were incubated at 4°C overnight. After the plates were washed five times with phosphate-buffered saline (PBS)–1% Tween, 200 μl of 1% bovine serum albumin (BSA; Sigma, Chemical Company, St. Louis, Mo.) in PBS was added to each well, and the plates were incubated for 1 h at 37°C to block the antigen-free surface of the wells. Thereafter, the diluted (1/200) plasma samples were added to the wells (100 μl per well) and incubated at 37°C for 90 min. Each plasma sample was tested in duplicate either in antigen-coated or control erythrocyte-coated wells for IgM and total IgG. After being washed, the plates were incubated 1 h at 37°C in the presence of peroxidase-conjugated goat $F(ab')_2$ fragments to human IgG Fc (at 1/6,000) or human IgM Fc (at 1/4,000) (Cappel, Organon Teknika Corporation, West Chester, Pa.).

Cell preparation and cultures. PBMC were isolated from heparinized whole blood by Histopaque-1077 density gradient (Sigma Diagnosis, St. Louis, Mo.) and washed three times in PBS. Cultures were prepared in flat-bottom 24-well Nunclon plates at a concentration of 10^6 cells per ml in culture medium containing RPMI 1640 supplemented with 10% (vol/vol) heat-inactived pooled human AB serum (CNTS, France), 1 mM glutamine, 35 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 1% sodium pyruvate, 2 g of sodium bicarbonate per liter, and 10 mg of gentamicin per liter. The plates were thereafter incubated at 37°C in a water-saturated atmosphere containing 5% CO_2. The percentages of apoptotic nuclei were determined 72 h after the initiation of the culture, a delay chosen after preliminary experiments. When the effect of parasite antigens was tested, 10 μl of the mature-form-enriched parasite extract was added to the cell suspensions, and the apoptosis levels were evaluated both at day 3 and day 6. Systematic control cultures consisted of nonparasitized erythrocytes treated exactly in the same way as the parasitized erythrocytes. To compare the effect of the *P. falciparum* extract with that of a more classical stimulus, we studied in parallel the effect on apoptosis levels of purified protein derivative (PPD), used at a final concentration of 2.5 μg/ml. When lymphocyte proliferative responses were studied, cultures were simultaneously prepared in 96-well round-bottom Nunclon plates at a concentration of 10^6 cells per ml in a total volume of 200 μl. [^{3}H]thymidine (1 μCi per well) was added after 6 days of culture, 16 h before harvesting, and incorporation of radioactivity was measured by liquid scintillation counting.

Quantitation of apoptosis. (i) PI staining technique. Apoptosis was quantitated by staining nuclei with propidium iodide (PI; Sigma) and analyzing fluorescence with a FACScan (Becton Dickinson, Immunocytometry Systems Inc., San Jose, Calif.) as originally described by Nicoletti et al. (22) on thymocytes and as adapted by Gougeon et al. (11) on human cells. Briefly, following either 72 h or 6 days of incubation, the cells were collected after centrifugation at 200 × *g* for 10 mn. The pellet was gently resuspended in 1.5 ml of a hypotonic fluorochrome solution of PI: 50 μg of PI per ml was diluted in 0.1% sodium citrate with 0.1% Triton X-100 (Sigma). The suspension was incubated overnight at 4°C and analyzed with a FACScan flow cytometer to determine the PI fluorescence of individual nuclei. Cytometric analysis of the red fluorescence channel allowed us to distinguish the different types of nuclei expressed as the percentage of gated nuclei in each region. Apoptotic nuclei appeared as a broad hypodiploid DNA peak that was easily discriminated from the narrow peak of nuclei with normal (diploid) DNA content and from nuclei originating from activated cells with hyperdiploid DNA content.

(ii) 7AAD staining technique. In some experiments, another technique of quantification of apoptotic cells was used. This technique consisted of staining the cells with 7-amino-actinomycin D (7AAD; Sigma) for discrimination between live and early apoptotic cells. The advantage of the latter method is that the spectral characteristics of 7AAD permit the combination of this DNA dye with fluorescein isothiocyanate and phycoerythrin cell surface staining. This technique was performed as described by Schmid et al. (25). In short, each of the fluorescein isothiocyanate- or phycoerythrin-stained monoclonal antibodies (Becton Dickinson) was diluted at 1/100 in PBS containing 1% BSA (Sigma) and 0.1% sodium azide (Sigma) (PBS-BSA-Az). The diluted monoclonal antibodies were added to 5×10^5 cells, which were then incubated for 15 min at 4°C. After two washes in PBS-BSA-Az, the supernatant was removed and the cells were incubated with 20 μg of 7AAD per ml for 20 min at 4°C. The cells were centrifuged once, and the supernatant was removed and replaced by a solution of 20 μg of actinomycin D (Sigma) per ml in PBS-BSA-Az to which was added 1% paraformaldehyde. After overnight incubation at 4°C, the samples were analyzed on a FACScan flow cytometer.

In several different experiments, the 7AAD staining technique was used simultaneously with the PI staining technique, and the results obtained with the two techniques were highly comparable (Spearman rank correlation coefficient [r] = 0.893, $P = 0.029$). Therefore, these two labeling techniques could be independently used to determine and confirmed that B cells as well as the different T-cell subpopulations ($CD4^+$ and $CD8^+$) were potentially affected by the process of apoptosis.

Evaluation of sIL2R. sIL2R was evaluated by ELISA, using an Immunotech sIL2R kit (Immunotech S.A., Luminy, Marseille, France) according to the manufacturer's instructions.

Statistical analysis. Nonparametric Kruskal-Wallis and Mann-Whitney tests were used to compare the continuous variables between the different groups. Correlations were studied by the Spearman rank correlation coefficient test. To analyze the results obtained for each individual at days 3 and day 6, the Wilcoxon nonparametric paired test was used. A P value of <0.05 was considered significant.

RESULTS

Unusually high levels of spontaneous apoptosis are detected in short-term cultures of lymphocytes from individuals with clinical malaria and from healthy sensitized asymptomatic subjects. Table 1 shows characteristics of the three group of subjects studied (Caucasians, Africans living in an area with low malaria transmission, [Dakar], and Africans from areas where malaria is holoendemic [Dielmo] and mesoendemic [Ndiop]) sampled during the dry season. In each group, we analyzed the percentages of apoptosis and the levels of specific anti-*P. falciparum* antibodies in healthy subjects and in individuals with clinical malaria.

When comparing healthy subjects with those with clinical malaria, we found that the mean percentages of spontaneous apoptosis determined at day 3 (i.e., the percentage of apoptotic nuclei found after 3 days of lymphocyte culture without any exogenous stimulus) were higher in short-term lymphocyte cultures from individuals with malaria than in those of the corresponding matched healthy HIV-negative controls ($P < 0.001$). It is notable that the level of apoptosis was evaluated in some cases ex vivo (i.e., just after mononuclear cell isolation), and high levels of apoptosis (up to 16% of apoptotic nuclei) were already detectable in patients with malaria. Such a marked increase of spontaneous apoptosis was long lasting and was detectable for up to 6 weeks.

When considering the asymptomatic subjects of the different groups, we found very different mean levels of specific anti-*P. falciparum* antibodies, which reflected the differences in malaria transmission. The highest mean levels of parasite-specific antibodies were found in subjects from areas where malaria is holoendemic (Dielmo) and mesoendemic (Ndiop), while lower mean levels were observed in subjects from a low-malaria-transmission area (Dakar). The lowest levels were found in Caucasians. Concurrently, we analyzed the mean levels of spontaneous apoptosis in the different groups of asymptomatic subjects. The following observations confirmed that the parasite infection was very likely potentially associated with elevated levels of apoptosis. First, following 3 days of in vitro culture, the mean percentage of spontaneous apoptosis was lower ($P \leq 0.001$) in lymphocytes obtained from Caucasian subjects (mean ± standard deviation = 8.2% ± 2.9%) with low-level or no detectable specific antibodies than in lymphocytes originating from any of the sensitized African subjects with parasite-specific antibodies. Second, the mean percentages of apoptosis of lymphocytes obtained from African subjects living in the two villages located in areas with high malaria endemicity, Ndiop (mesoendemic) and Dielmo (holoendemic), were the most elevated (21.4% ± 5.5%). Third, there was no detectable difference between the mean percentage of apoptosis found in Dielmo (22.1% ± 5.6%) and that found in Ndiop (21.8% ± 5.8%) in the lymphocytes from individuals with comparable levels of specific antibodies. Fourth, these mean levels of apoptosis were, in contrast, significantly higher ($P \leq 0.0001$) than the mean percentage of apoptosis found in lymphocyte cultures obtained from age- and sex-matched healthy African subjects living in greater Dakar (13.7% ± 3.8%), an area of comparatively low and seasonal transmission where malaria is hypoendemic, as reflected by a low mean level of malaria-specific antibodies. The usual biological parameters such as hemoglobin phenotype or blood formula were largely comparable between these different groups of subjects, suggesting that the subjects differed only in the degree of exposure to *P. falciparum.*

Therefore, there existed an apparent potential relationship between the level of detectable spontaneous apoptosis and the

TABLE 1. Percentages of apoptotic nuclei and levels of malaria-specific antibodies in the different group of subjects

Group of subjects[a]	Clinical status	% Apoptosis at day 3 [range]	Specific anti-*P. falciparum* antibody optical density [range]	
			IgG	IgM
Caucasians living in Dakar	Healthy	8.2 ± 2.9 [4.0–13.0] (n = 12)	0.072 ± 0.086 [0.003–0.319] (n = 11)	0.082 ± 0.045 [0.013–0.181] (n = 11)
	With clinical malaria	22.5 ± 2.2 [19.5–25.4] (n = 8)	0.258 ± 0.187 [0.120–0.615] (n = 6)	0.205 ± 0.106 [0.067–0.336] (n = 6)
Africans subjects from Dakar	Healthy	13.7 ± 3.8 [7.0–24.0] (n = 37)	0.268 ± 0.315 [0.022–1.539] (n = 25)	0.132 ± 0.251 [0.021–1.289] (n = 25)
	With clinical malaria	29.7 ± 9.1 [14.7–43.0] (n = 15)	0.695 ± 0.622 [0.030–2.000] (n = 15)	0.855 ± 0.778 [0.003–2.750] (n = 15)
Subjects from Dielmo and Ndiop	Healthy (Dielmo)	22.1 ± 5.6 [12.5–33.2] (n = 32)	1.306 ± 0.575 [0.054–2.016] (n = 28)	0.787 ± 0.644 [0.010–2.264] (n = 28)
	Healthy (Ndiop)	21.8 ± 5.8 [15.6–35.5] (n = 14)	1.007 ± 0.522 [0.149–1.669] (n = 14)	1.044 ± 0.915 [0.024–2.262] (n = 14)
	With clinical malaria (Dielmo and Ndiop)	36.3 ± 10.0 [30.0–48.0] (n = 3)	1.091 ± 0.912 [0.351–2.110] (n = 3)	0.902 ± 0.715 [0.077–1.320] (n = 3)

[a] For description of the areas, see the text.

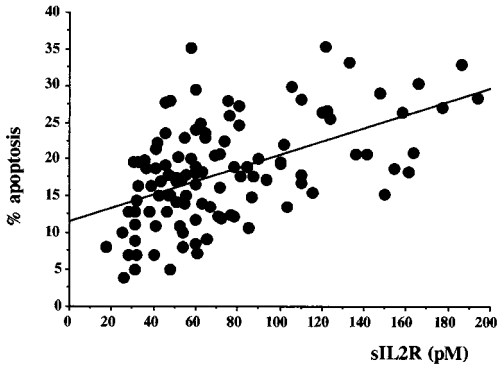

FIG. 1. Percentages of apoptotic nuclei in lymphocyte cultures at day 3 and corresponding sIL2R concentrations of subjects. A correlation between the two parameters ($r = 0.54$, $P = 0.001$) was found.

degree of exposure to *P. falciparum* of individuals living in areas differing in endemicity. As a consequence, when considering the relationships between specific antibody levels and the percentage of apoptotic nuclei detected, we found a strong correlation ($r = 0.56$, $P < 0.001$ for IgG; $r = 0.46$, $P < 0.001$ for IgM).

The concentrations of sIL2R were studied in the different groups. An apparent relationship existed between the level of sIL2R and the percentages of apoptotic nuclei in in vitro mononuclear cell cultures ($r = 0.54$, $P = 0.001$) (Fig. 1).

Influence of the rate of parasite transmission on the mean level of apoptosis and cellular activation in Dielmo and Ndiop. As shown in Table 1, the percentages of apoptotic nuclei were comparable during the dry season in Dielmo and Ndiop. This was also the case during the rainy season even if the percentages of detectable apoptotic nuclei were lower (Table 2). During both seasons, the mean percentage of apoptotic cells detected in vitro in lymphocyte cultures of individuals from these areas of endemicity remained significantly higher than that of individuals living in greater Dakar. Therefore, the consistently higher levels of apoptosis detected in lymphocytes from the subjects living in Ndiop and Dielmo were not accidental or transient. This observation of long-lasting, elevated percentage of apoptosis was confirmed when successive blood sampling of different individuals was performed throughout the year (data not shown). Our data did not show any correlation between the percentage of apoptotic nuclei and either the presence or absence of circulating parasites following blood smear analysis at the time of sampling (data not shown).

As shown in Table 2, during the rainy season, which is the season of maximal *P. falciparum* transmission both in Ndiop and Dielmo, no significant difference in the percentage of activated cell nuclei was observed between the two villages. In contrast, during the dry season, which corresponds to an almost complete absence of parasite transmission in Ndiop,

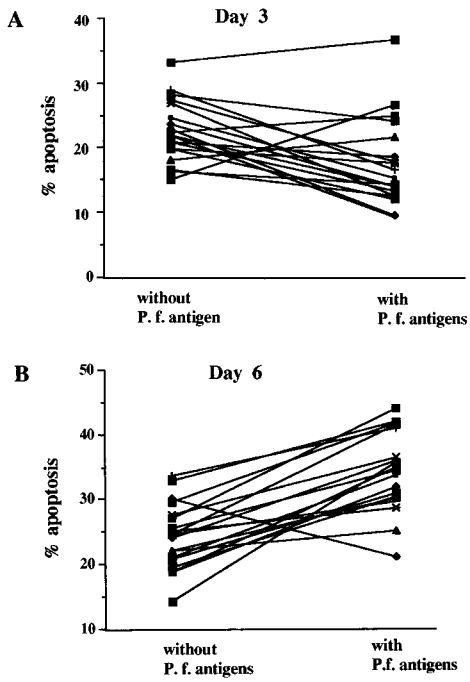

FIG. 2. Dynamics of the effect of *P. falciparum* extract on the levels of apoptosis. At day 3, *P. falciparum* (P.f.) extract induced a temporary decrease of apoptosis levels (A), whereas at day 6, the parasite antigens induced a consistent increase of apoptotic levels (B).

whereas transmission is perennial in Dielmo, a significant difference in the percentage of activated cell nuclei was noted between the two villages. The percentage of activated cell nuclei detected was lower in lymphocytes of subjects from Ndiop than that observed in the rainy season ($P < 0.001$), while in Dielmo, the percentages of activated cell nuclei detected were comparable during the two seasons.

The mean levels of sIL2R were evaluated on different occasions in Dielmo and Ndiop. Irrespective of the transmission season, the mean levels of sIL2R were comparable in the samples collected in the two villages (Table 2). During the dry season, the sIL2R levels were also evaluated in the other groups. They were significantly higher in samples of residents of Dielmo (95.9 ± 47.7 pM, $n = 23$) and Ndiop (82.1 ± 39 pM, $n = 14$) than in samples of individuals with reduced exposure to *P. falciparum* living in greater Dakar, either Africans (59.2 ± 43.1 pM, $n = 31$) or Caucasians (53.03 ± 48.3 pM, $n = 12$).

Effects of the *P. falciparum* extract on apoptosis levels. Figure 2A shows that the *P. falciparum* extract induced a slight but significant initial decrease of apoptosis at day 3 in 90% (36 of 40) of the mononuclear cell cultures. This was, in sharp contrast, followed by a subsequent rise in the apoptosis levels

TABLE 2. Percentages of apoptotic and activated cell nuclei and levels of sIL2R during different *P. falciparum* transmission seasons

Region	Season	Mean no. of infected bites/mo[a]	Mean % apoptotic nuclei ± SD	Mean % nuclei from activated cells ± SD	Mean concn of sIL2R ± SD (pM)
Dielmo	Rainy	1.7	17.5 ± 4.4 ($n = 14$)	2.26 ± 2.2 ($n = 14$)	102.6 ± 86.7 ($n = 14$)
	Dry	1.25	22.1 ± 5.6[b] ($n = 32$)	2.38 ± 1.9 ($n = 32$)	95.9 ± 47.7 ($n = 23$)
Ndiop	Rainy	2.6	18.7 ± 5.07 ($n = 42$)	2.09 ± 1.02 ($n = 42$)	70.8 ± 30.1 ($n = 13$)
	Dry	0	21.8 ± 5.8 ($n = 14$)	1.06 ± 0.4[b] ($n = 14$)	82.1 ± 39 ($n = 14$)

[a] Mean value of the month during which the samples were collected and those of the month before (10a).
[b] A significant difference in values ($P < 0.02$) was found between the two seasons.

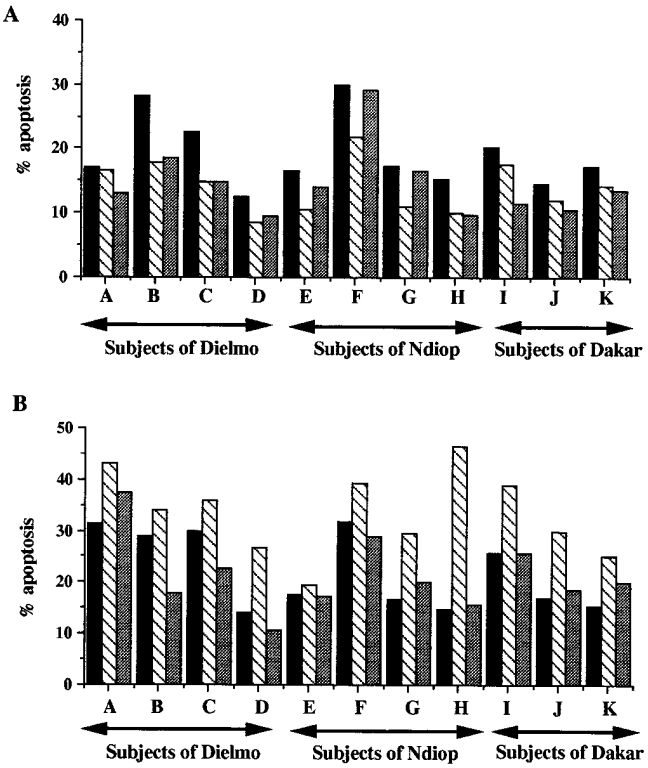

FIG. 3. Influence of *P. falciparum* extract (▨) and PPD (▩) on the percentages of apoptotic nuclei detected after 3 and 6 days of in vitro culture of PBMC in comparison with PBMC cultured in medium alone (■). A to K represent subjects of the groups studied. At day 3, either the parasite extract or PPD induced a decrease of spontaneous apoptosis (A), but the increase of apoptosis percentages at day 6 was observed only with the parasite antigens (B).

observed at day 6 in 87.5% of the lymphocytes tested (35 of 40 cultures) (Fig. 2B), at a time when the parasite-induced [^{3}H]thymidine uptake was maximal. The percentages of apoptosis observed at day 3 as well as at day 6 in cultures in which *P. falciparum* extract was present were markedly different from those observed in lymphocytes cultures without any exogenous stimulus ($P = 0.001$, Wilcoxon paired test). The effect of a classical stimulus, PPD, was simultaneously studied. At day 3, PPD induced a significant decrease ($P < 0.001$) in the percentages of detectable apoptotic nuclei, and this effect was largely comparable to that found in the presence of parasite antigens (Fig. 3A). In contrast, at day 6, there was no detectable difference between the level of apoptosis observed when the lymphocytes were cultivated alone or when they were cultivated in the presence of PPD (Fig. 3B). Finally, when added to the lymphocyte cultures, control nonparasitized erythrocytes (prepared in exactly the same conditions as the parasitized ones) induced no detectable difference in the percentage of apoptosis compared with that found for the lymphocytes alone. As noninfected erythrocytes were the true controls in our study, this observation led us to assume that the parasite itself was very likely responsible for the induction of apoptosis observed at day 6.

In three independent experiments, extracts of two different *P. falciparum* strains were added at various concentrations, and simultaneously with the evaluation of the levels of apoptosis, [^{3}H]thymidine incorporation was tested. Figure 4a shows that the lymphocytes of subject A, from Dakar, had low levels of apoptosis and incorporated [^{3}H]thymidine in a dose-dependent manner with each of the two parasite extracts. The lymphocytes of subject B, from Dielmo, showed a very high level of spontaneous apoptosis (Fig. 4b). A decrease of apoptosis levels with different concentrations of the Dielmo parasite extract (extract 1) was associated with an increase in [^{3}H]thymidine incorporation. The lymphocytes of subject C, also from Dielmo, presented always high levels of apoptosis, and no [^{3}H]thymidine incorporation was observed (Fig. 4c). Therefore, in these experiments, occurrence of [^{3}H]thymidine incorporation was concomitant with a low or limited level of apoptosis.

DISCUSSION

These results confirmed and extended our initial observation of elevated levels of apoptosis during and following malaria

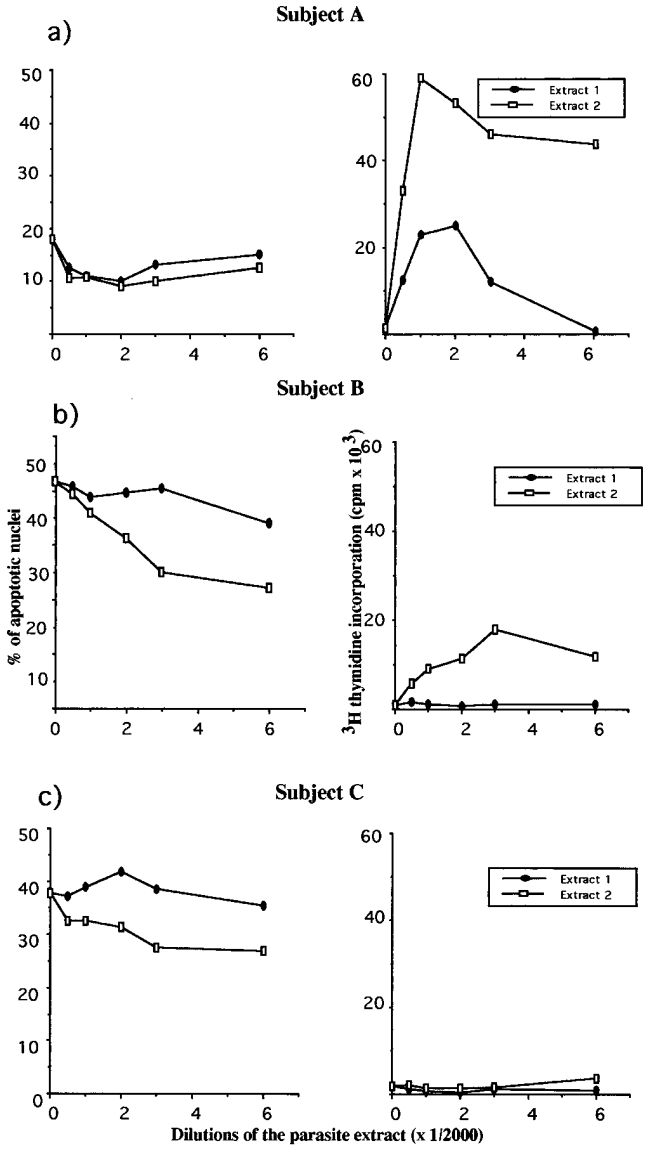

FIG. 4. Effects of different concentrations of two different parasite extracts on the percentages of apoptotic nuclei and the [^{3}H]thymidine uptake by lymphocytes. Extract 1 was prepared from the Dielmo parasite isolate, and extract 2 was prepared from a Palo Alto (FUP/66 Marburg) strain. The subject A is from Dakar, while the subjects B and C are from Dielmo. The lymphocytes of subject A showed [^{3}H]thymidine uptake in the presence of the two extracts, the lymphocytes of the subject B were reactive solely with extract 2, and none of the extracts induced [^{3}H]thymidine incorporation the lymphocytes of subject C.

attacks. Apoptosis was elevated not only in patients suffering from acute malaria infections but also in asymptomatic, healthy, HIV-negative individuals living in areas where malaria is endemic. In areas characterized by a high and permanent rate of parasite transmission, malaria represents a chronic infection wherein the parasite is continuously present following permanent reinfection. Malaria infection was observed to lead to a chronic state of activation (9, 24) but also to a state of anergy (23, 28). One can hypothesize that permanent exposure of the immune system to *P. falciparum* antigens might induce a deletion of reactive T cells as described for superantigens (17, 18). Our results tended to support such a possibility, as they clearly illustrated that a relationship could exist between *P. falciparum* exposure and the level of spontaneous apoptosis. Therefore, acute as well as chronic *P. falciparum* asymptomatic infection was very likely responsible for the induction and/or the amplification of the phenomenon of apoptosis.

The finding that the difference of transmission during the dry season in the two villages studied was not associated with a difference in the levels of detectable apoptotic nuclei was in agreement with our previous observation that malaria infection could have a long-lasting effect (up to several months) on in vitro lymphocyte viability (30). As a consequence, no direct correlation could be found between the level of spontaneous apoptosis detected in vitro and the presence or absence of parasites in the blood of subjects, at least when assessed by blood smears. Moreover, it must be pointed out that negative thick smears did not allow us to completely rule out the possible presence of the parasite at a very low level. In a previous intensive and longitudinal study with daily thick-smear analysis, we showed that during a 4-month period, up to 98% of individuals in Dielmo harbored parasites at least once (33). In addition, when PCR analysis was carried out, it was shown that a number of subjects with parasite-negative blood smears were in fact positive by the PCR technique (14). Therefore, most of the inhabitants of Dielmo and Ndiop probably experienced a permanent asymptomatic parasite infection even if it was not systematically detectable on a single blood film. As a consequence, the potential in vivo incidence of parasite antigens on lymphocytes was probably long lasting if not permanent.

In contrast to what was observed for apoptotic nuclei, significant differences in the percentages of activated cell nuclei were found between the two villages during the dry season. In Ndiop, the clear-cut reduction of malaria transmission observed during this season was associated with a significant decrease of detectable activated cell nuclei compared with what was found during the rainy season. This was not the case in Dielmo, where limited but permanent transmission occurred even during the dry season (33). Therefore, this observation strongly suggested the direct impact of the parasite on mononuclear cell activation, and this hypothesis was strengthened by the positive correlation found between the percentage of apoptotic nuclei and the concentration of sIL2R, a marker of activation (21) reported to be associated with malaria infection (9, 16, 24).

In vitro, the addition of parasite extract to cell cultures consistently affected the level of apoptosis of mononuclear cells. This indicated a direct potential impact of parasite antigens on the in vitro apoptosis because it was not observed either with the control antigen (nonparasitized erythrocytes) or with a ubiquitious control antigen such as PPD. Interestingly, upon exposure to various concentrations of parasite extract, [^{3}H]thymidine incorporation was found when the apoptosis levels were low or decreasing, suggesting that mononuclear cells could either respond to antigenic stimulation by proliferation or be driven to apoptosis.

Taken together, our in vitro observations strongly suggested that parasite-derived antigens could be responsible for the unusually elevated level of apoptosis found in areas where malaria is endemic. It was previously suggested that *P. falciparum* products could have mitogenic (12) or superantigenic (3) activity, and indeed such activity leads to PBMC apoptosis. There is also the possibility that malaria-associated oxidative stress accounts for the induction of unusual levels of apoptosis (5).

Of notice, the apoptosis percentages detected in lymphocytes from subjects living in areas of endemicity reached the levels of apoptosis found in asymptomatic African HIV-infected subjects living in Senegal (29). A potential consequence of our observation is that the level of in vitro apoptosis, which is considered by some authors as a marker associated with the progressive evolution of HIV infection, should be evaluated with particular caution in areas of endemicity where basic apoptosis levels can already be consistently increased.

This study strongly suggested that, in parallel to viral and bacterial infections (20, 34, 38) and as recently reported for experimental Chagas' disease (10), a parasite infection such as malaria is very likely to induce a significant long-lasting increase in spontaneous apoptosis levels. This phenomenon could be essential in maintaining a normal balance in the number and the density of renewing cell populations and in the regulation of the polyclonal activation observed in malaria. Apoptosis could also participate in the resolution of the parasite-induced acute inflammation as evoked in other diseases (15, 26). This finding raised the question of the various consequences of such a mechanism both in vivo and in vitro and, in particular, the validity of in vitro proliferative assays measured by [^{3}H]thymidine uptake and widely used in areas of endemicity to detect specific responses to *P. falciparum* antigens. We previously showed that different lymphocyte subpopulations were involved (30). But among these cells, it remains to determine if the mononuclear cells undergoing apoptosis belong to a particular group of sensitized and/or reactive cells eliminated by the parasite, thus enabling the pathogen to establish itself in the host. If this is the case, then parasite-induced apoptosis could participate in reducing the immune response directed toward critical antigens by increasing the fragility of potential cellular effectors. Complementary studies are being carried out to determine if defined antigens such as parasite-derived synthetic peptides can induce apoptosis in lymphocytes cultured in vitro. This analysis could have considerable impact on the final choice of molecules to include in a vaccine strategy.

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