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ORIGINAL PAPER

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Malaria associated apoptosis is not significantly correlated with either parasitemia or the number of previous malaria attacks

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Abstract The occurrence and intensity of lymphocyte apoptosis in blood samples from 79 outclinic patients with uncomplicated *Plasmodium falciparum* or *Plasmodium vivax* malaria and 30 healthy individuals were investigated. No difference in apoptosis percentages was detected between healthy individuals and malaria patients when ex vivo lymphocytes were analyzed. However, significantly increased apoptosis levels were observed in lymphocytes from both *P. falciparum*- and *P. vivax*-infected patients when the cells were cultured for 24 h. $CD4^+$ and $CD8^+$ T cells were affected to a comparable extent in *P.falciparum*- and *P.vivax*-infected patients. However, when we compared apoptosis values in infected and non-infected individuals it appeared that $CD4^+$ T cells were more susceptible than $CD8^+$ T cells. A significant increase in the sIL-2R plasma levels was observed in malaria patients when compared with healthy individuals and a positive correlation was observed between sIL-2R levels and apoptosis rates in infected patients presenting increased rates of apoptosis. An increased expression of Fas antigen was recorded after stimulation with *P. falciparum* antigen or anti-CD3 monoclonal antibody. These data show that a consistent proportion of the lymphocyte population dies by apoptosis during a malaria infection and that a period of time is necessary before in vivo activated cells can express the apoptotic process in vitro.

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Introduction

Apoptosis is a process by which cells die in a controlled and programmed manner in response to specific stimuli. Besides participation in defining the T-cell repertoire (Blackman et al. 1990; Sprent 1993; Nossal 1994) and preventing autoimmune diseases (Lorenz et al. 2000; Navratil and Ahearn 2000; Newton and Strasser 2000, Piliponsky and Levi-Schaffer 2000) apoptosis is an efficient regulatory feedback mechanism in the immune system that may help to control cell numbers under conditions of high antigen load (Raff et al. 1993). However, apoptosis may also prevent and/or abolish the lymphocyte response (Krammer et al. 1994; Van Parijs and Abbas 1998) and by so doing, be involved in (or lead to) immunosuppression and autoimmunity. In parasitic infections, apoptosis can be observed both in fresh splenocytes and in in vitro-cultured spleen cells from mice infected with *Schistosoma mansoni* (Estaquier et al. 1997; Fallon et al. 1998), *Trypanosoma cruzi* (Lopes et al. 1995) or *Toxoplasma gondii* (Khan et al. 1996). This phenomenon has been implicated as a mechanism by which parasites can escape the immune response. Malaria infection induces drastic changes in the immune system, such as an important T-cell activation associated with raised levels of the soluble plasma interleukin-2 receptor (sIL-2R) (Ho et al. 1988; Josimovic-Alasevic et al. 1988; Hviid et al. 1991; Riley et al. 1993), a marked degree of polyclonal B-cell activation (Freeman and

Parish 1978; Rosenberg 1978; Banic et al. 1991; Burger-Rolland et al. 1992), a low level of in vitro proliferative response of peripheral blood mononuclear cells (PBMC) to malaria antigens (Deloron et al. 1989; Jakobsen et al. 1994), a decreased response to vaccines (Theander et al. 1986; Hviid 1995) in addition to a reduction in the number of circulating lymphocytes, particularly of the T-cell population (Wyler 1976; Strickland et al. 1979; Wells et al. 1980; Troye-Blomberg et al. 1983; Ho et al. 1986; Worku et al. 1997). The pathophysiology of the observed lymphopenia is still unclear (Hviid and Kemp 2000); however, apoptosis may be considered a possible mechanism leading to decreased T-cell counts, since African patients with acute *Plasmodium falciparum* infection have an increased ratio of lymphocyte apoptosis levels (Toure-Balde et al. 1995, 1996).

In this paper we present a study on the association of malaria infection with increased levels of apoptosis of T cells obtained from Brazilian malaria patients acutely infected with either *P. falciparum* or *Plasmodium vivax*, and evaluate the relationship between these increased levels and the levels of parasitemia and sIL-2 receptors as well as the number of previous malaria infections.

Materials and methods

Subjects and blood samples

A total of 79 blood samples from outclinic patients with uncomplicated *P. falciparum* ($n=34$) or *P. vivax* ($n=45$) malaria were collected at the Fundação de Medicina Tropical (FMT/IMT-AM) in Brazil. All of these patients presented positive, thick blood smears, with a parasitemia ranging from 143 to 60,572 (mean = 12,498) parasites/μl for *P. falciparum* and 341–19,720 (mean = 6,713) parasites/μl for *P. vivax* cases. Human peripheral blood samples were also obtained from 30 individuals living in the same area but with no history of current or previous malaria episodes. These samples were collected from members of the First Jungle Infantry Battalion, Amazon in Brazil. The ethics committee of the Fundação de Medicina Tropical and Fundação Oswaldo Cruz permitted this study. Both the patients and healthy individuals provided written, informed consent. The population studied was predominantly composed of young male individuals; 60% were 10–30 years old. The majority of patients reported from two to five previous malaria infections. All subjects were tested for the presence of HIV, *Trypanosoma cruzi* and *Leishmania major* IgG antibodies. None of the samples were positive for HIV, but two were positive for *T. cruzi* and two for *L. major*. These were excluded from our experiments and the individuals were immediately treated.

Standard laboratory examinations

The parasitemia was determined by routine clinical laboratory procedures. Thin and thick blood films were prepared and further stained by the May-Grünwald-Giemsa method. The blood films were examined for malaria parasites by an experienced microscopist from the FMT/IMT-AM. The parasitemia was quantified by examination of at least 300 leukocytes in the thick film.

Peripheral blood mononuclear cells

A venous blood sample (20 ml) was collected from each individual. PBMC were isolated from heparinized whole blood by ficoll-hypaque density gradient centrifugation. Cells were washed twice in RPMI-1640 (Sigma) medium containing 15 mM glutamin (Sigma), 10 mM Hepes, 200 U/ml penicillin (Sigma), 200 µg/ml streptomycin (Sigma), 3 mg/ml gentamicyn (Sigma) and 2 g/l sodium bicarbonate (Grupo Química), and resuspended in RPMI supplemented with 10% inactivated fetal calf serum (W.L. Imunoquímica). Due to the difficulties in performing the assays in field conditions, the cells were cryopreserved according to the method described by Ichino and Ishikawa (1985). Briefly, cells were suspended in 4°C RPMI-1640 supplemented with 40% fetal calf serum with an equal volume of cold RPMI-1640 containing 20% dimethyl sulfoxide and transferred to cryotubes that were immersed in a cold ethanol bath and placed in a −0°C freezer for at least 4 h. They were then transferred to a liquid nitrogen storage tank. After freezing for up to 30 days, the cells were thawed and the viability was assessed using trypan blue staining.

P. falciparum strain and antigenic extract preparation

The S20/87 *P. falciparum* strain (Rondônia, Brazil) was cultivated in vitro according to the method described by Trager and Jensen (1976). The antigenic *P. falciparum* extract used for the apoptosis induction assay was prepared when the parasitemia was higher than 6%. Parasitized erythrocytes with a predominance of schizonts were washed three times with a buffer solution (PBS/0.15 M, pH 7.2). The lysis of infected erythrocytes was done with the addition of 0.1% saponin with gentle shaking for 15 min. The lysates were ultrasonicated in the presence of 1 mM phenylmethylsulfonyl fluoride and centrifuged (7,000 *g* for 15 min at 4°C) in order to eliminate the cellular debris.

Apoptosis induction assay

Cell cultures were prepared in duplicates in 96-well flat-bottom microtiter plates (Falcon) at 37°C in 5% CO_2 in a final volume of 200 µl of culture medium alone or with medium plus a determined stimulant for 24 h. The cultures were performed at a cell density of 2.5×10^5 cells. The stimuli used were: 10 µg/ml phytohemagglutinin (PHA), 20 µg/ml *P. falciparum* antigen and 50 µg/ml monoclonal antibody (mAbs) anti-CD3 (Sigma). As a control, non-cryopreserved PBMC from healthy volunteers treated exactly in the same way as the cells from malaria patients or non-infected individuals were used in each experiment.

Determination of apoptotic cells using 7-aminoactinomycin D by flow cytometry

We used a rapid and sensitive method that allows the discrimination of live cells from apoptotic cells or necrotic cells (Schmid et al. 1994a). The recognition of viable, apoptotic or necrotic cells was done using 7-aminoactinomycin D (7-AAD), a fluorescent cytochemical probe, using a single laser cytometry. While live cells were not stained with 7-AAD, the cells with an apoptotic or necrotic pattern were discriminated based on low or high 7-AAD incorporation, respectively. Briefly, the cells were incubated for 20 min. at 4°C with 10 μg/ml of 7-AAD (Sigma) in PBS containing 2% fetal calf serum and 0.1% sodium azide. Labeled samples were analyzed with an EPICS XL-MCL flow cytometer with a single argon laser at 488 nm (Coulter, Healeah, Fla., USA) and red fluorescence from 7-AAD was filtered through a 675 nm long pass filter. More than 10,000 events were analyzed for each sample.

Principles of drawing the regions (gates)

Blood cell populations have sizes and distinct organelle densities that enable their differentiation through cellular size (forward

scatter, FS) versus inner granularity (side scatter, SS). These parameters generate an image (region A) that perfectly distinguishes the blood subpopulations (Fig. 1). Thus, the leukogate and the use of antibody for labeling were not needed.

Fresh PBMC were used for defining the size of the gates corresponding to viable (region C), apoptotic (region D) and necrotic (region E) PBMC in cytometry flow (Fig. 1). In order to better define these gates, we used necrotically induced PBMC by heating to 65°C for 5 min (Schmid et al. 1994b), and apoptotically induced PBMC using a known inducer of lymphocyte apoptosis, 4 μM staurosporine (Sigma), an inhibitor of kinase C protein isolated from *Streptomyces* (Omura et al. 1977; Tamaoki et al. 1986; Kiyoto et al. 1987). After defining the gates, PBMC from infected and non-infected individuals were analyzed for the percentage of viable, apoptotic and necrotic cells present within a constant region (A) of a similar size to that used for the definition of apoptotic (Fig. 1, panel II, region D) and necrotic cells (Fig. 1, panel III, region E).

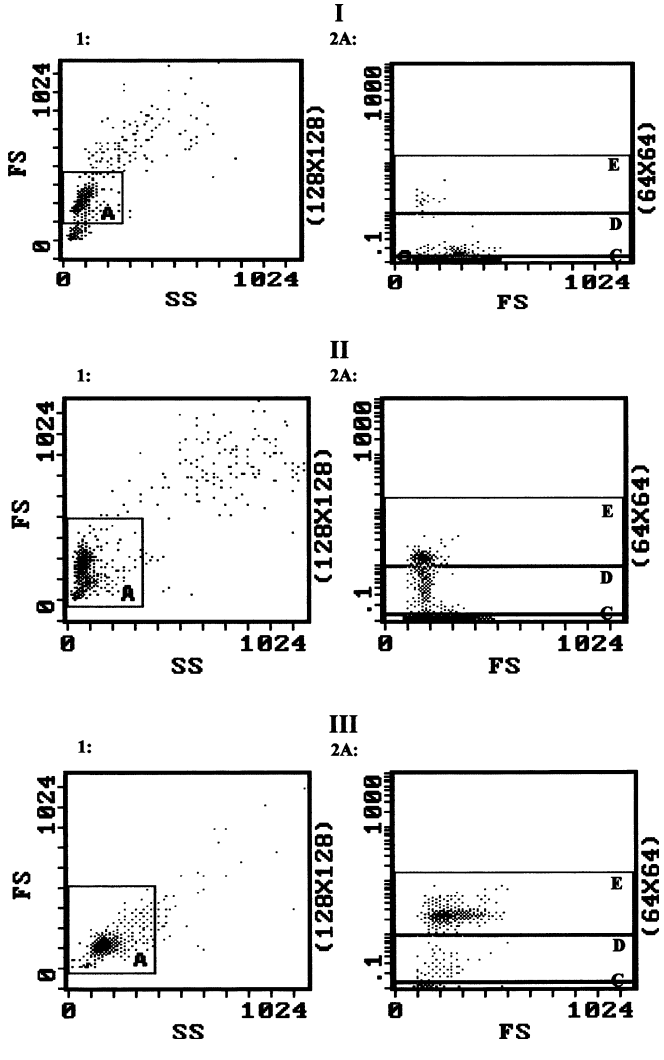

Fig. 1 Cytometric flow analysis of fresh peripheral blood mononuclear cells (PBMC) from one healthy individual. Necrosis was induced by heating (65°C for 5 min), and apoptosis by 24-h culture with 4 μM staurosporine: *I* normal PBMC; *II* apoptosis-induced PBMC; *III* necrosis-induced PBMC. *FS* Forward scatter; *SS* side scatter; *A* analyzed region of PBMC; *C* viable cells; *D* apoptotic cells, and *E* necrotic cells

Analysis of apoptosis in T-cell subsets

Briefly, 5 µl of anti-CD3-FITC/anti-CD4-RD1 mAbs or 5 µl anti-CD3-FITC/anti-CD8-RD1 mAbs surface antigens (Coulter) in 95 µl of PBS containing 2% fetal calf serum and 0.1% sodium azide were added to 5×10^5 cells/ml followed by incubation for 30 min at 4°C. After centrifugation (5 min at 600 *g*), the cells were incubated for 20 min with 10 μg/ml of 7-AAD (Sigma) at 4°C in the dark. Triple-labeled samples were analyzed with an EPICS XL-MCL flow cytometer with a single argon laser at 488 nm (Coulter). Red fluorescence from 7-AAD was filtered through a 675 nm long pass filter, while orange (RD1) and green (FITC) fluorescence was measured with 575-nm BP and 525-nm BP filters, respectively. More than 10,000 events were analyzed for each sample.

Fas antigen expression

The Fas antigen expression was analyzed by flow cytometry as follows: 10 µl of anti-CD95 mAbs (Coulter) were added to 2.5×10^5 cells/ml followed by incubation for 15 min at 4°C. After incubation, the cells were centrifuged (10 min at 350 *g*) and 10 µl of anti-human IgG-FITC mAbs (Sigma) were added to the pellet and kept on ice for approximately 15 min. After washing (5 min at 350 *g*), cells were labeled with 7-AAD in a 10 µg/ml PBS solution during 20 min at 4°C.

Gel electrophoresis of fragmented DNA

Cells (4×10^6) cultured with or without stimulus were washed three times with PBS and centrifuged (5 min at 400 *g*) and the pellet was suspended in 400 µl lysis buffer (200 µg/ml proteinase K pH 7.5 (Sigma), 100 mM NaCl (Merck), 1 mM EDTA (Sigma), 10 mM Tris-HCl pH 8.0 and 1% SDS (ICN). After incubation at 50°C for 30 min, DNA samples were extracted with phenol-chloroform and treated with 100 µg/ml of Rnase A (Sigma) for 30 min at 36°C. The DNA was precipitated by the addition of 3 M sodium acetate (1/10), 2.5 vol. ETOH (−20°C) (Sigma) and 3 µl glycogen 0.02 mg/ml (Sigma).

Electrophoresis was carried out through a 2% agarose gel in Tris-borate-EDTA (TBE) buffer. DNA bands were visualized by staining with ethidium bromide (0.5 µg/ml) and photographed. Oligonucleosomal fragments appeared as ladders of band whose molecular sizes were approximate multiples of 200 bp.

Electron microscopy

The morphology was assessed by transmission electron microscopy. The thawed PBMC were washed in PBS and fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 1 h at 4°C, washed three times in the same buffer and fixed with 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 hr at 4°C. The cells were washed in buffer, removed with a cell scraper, and then the suspension was dehydrated in graded acetone and embedded in Epon. Ultrathin sections were collected in copper grids, stained with uranyl acetate and lead citrate and observed with a Zeiss EM-10C transmission electron microscope.

Soluble IL-2 receptor determination

sIL-2R was evaluated by ELISA, using the sIL-2R kit (Immunotech) according to the manufacture's instructions.

Statistical analysis

Nonparametric Kruskal-Wallis and Mann-Whitney tests were used to compare the variables between the groups. The Wilcoxon paired

test was performed to determine the significance of differences in the apoptosis percentages between ex vivo samples and after 24 h of cell culture. Correlations were determined by the Spearman rank correlation coefficient. A P value of <0.05 was considered significant.

Results

Levels of apoptosis determined ex vivo and after short-term culture of PBMC

When we compared the level of ex vivo apoptosis of PBMC obtained from non-infected individuals with that of PBMC isolated from *P. falciparum*- or *P. vivax*-infected patients, no significant difference was found (Fig. 2). The apoptosis percentages in both groups were low compared to those observed when the cells were in vitro cultured for 24 h.

After cultivating PBMC from infected individuals for 24 h, a consistent increase in the apoptosis levels was found ($P<0.05$). No significant difference was detected between the cells treated with anti-CD3 mAb or with *P. falciparum* antigen and even cells cultured without any stimulus. Furthermore, similar apoptosis levels were found among *P. falciparum*- and *P. vivax*-infected patients (NS). Conversely, in individuals living in the same area but without a previous history of malaria, the ex vivo apoptosis profile was similar to that observed after short-term culture in the presence or absence of stimulus (Fig. 2).

When PBMC were cultivated with PHA mitogen, both infected (19% and 22% for *P. falciparum* and *P. vivax*, respectively) and non-infected individuals (16%) presented similar percentages of apoptosis (NS).

Once increased PBMC apoptosis in malaria-infected individuals was demonstrated, we looked for a potential association between the importance of the phenomenon and the intensity of parasitemia, but no significant relationship was observed. The same lack of association was verified when apoptosis levels were related to the number of past malaria attacks (NS).

Detection of malaria-associated apoptosis on electrophoresis gel

After thawing, intact DNA was observed in samples from infected as well as from non-infected individuals. However, in samples treated with anti-CD3 mAb or *P. falciparum* antigen, or in samples cultured without any stimulus, the DNA profile of PBMC from infected individuals was easily differentiated from that of non-infected individuals by the presence of DNA fragments characteristic of the apoptosis process (Fig. 3).

Morphology of malaria-associated apoptosis by electron microscopy

The great majority of PBMC from non-infected individuals (endemic control) had a normal morphology showing nuclei with normal chromatin appearance, maintenance of membrane integrity and distinct cytoplasmic organelles. In contrast, the activated PBMC from malaria patients undergoing apoptosis presented nuclei with highly condensed chromatin, loss of nuclear membrane integrity and cytoplasmic vacuolization (Fig. 4).

Malaria-associated apoptosis in $CD4^+$ and $CD8^+$ T-cell subpopulations

The analysis of apoptosis values in $CD4^+$ and $CD8^+$ T-cell subsets was performed using the combination of anti-$CD4^+$ or anti-$CD8^+$ mAbs and 7-AAD. $CD4^+$ and $CD8^+$ T cells were equally altered by apoptosis in both *P. falciparum*- and *P. vivax*-infected individuals. No significant difference was found for $CD4^+$ and $CD8^+$ subsets after in vitro cultures without stimulus (13% and 16% for $CD4^+$; 13% and 13% for $CD8^+$) or stimulated with anti-CD3 (14% and 15% for CD4+; 13% and 12% for $CD8^+$) or even with *P. falciparum* antigenic extract (16% and 15% for $CD4^+$; 14% and

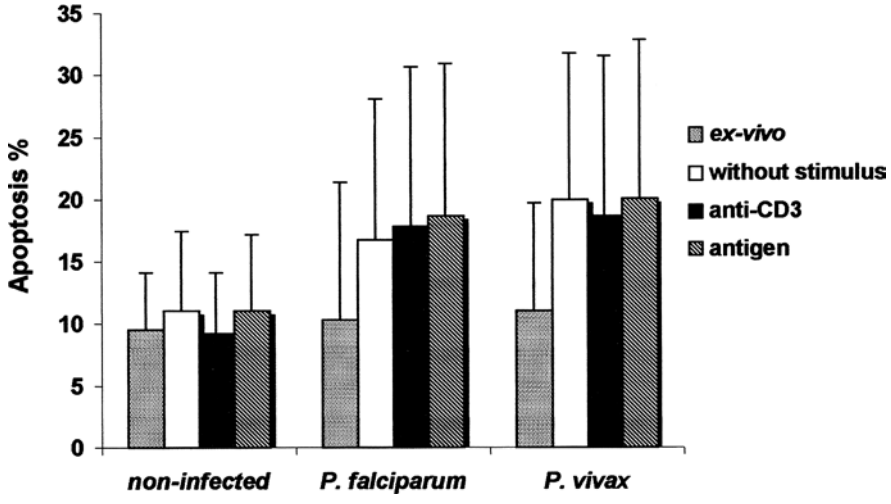

Fig. 2 Percentage of apoptotic cells from infected and non-infected individuals directly after thawing (ex vivo) and after 24 h culture with or without stimulus. Cultures containing 2.5×10^5 cells/well were cultivated for 24 h in duplicate in 96-well, flat-bottom microtiter plates at 37°C in 5% CO_2, with medium alone, with 20 μg/ml *Plasmodium falciparum* antigen or with 50 μg/ml monoclonal antibody anti-CD3. The *error bars* indicate SD

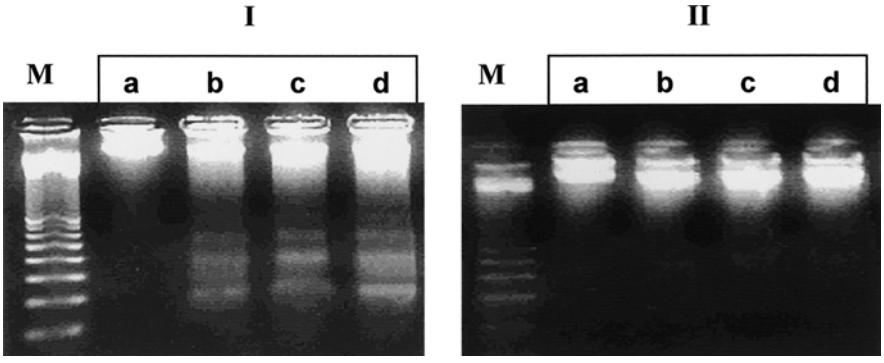

Fig. 3 Apoptosis evaluation by electrophoresis gel. DNA samples were extracted with phenol-chloroform and precipitated with sodium acetate and glycogen. Electrophoresis was carried out through a 2% agarose gel and the DNA bands were visualized by staining with ethidium bromide. Oligonucleosomal fragments appeared as ladders of bands, the molecular sizes of which were approximate multiples of 200 bp. *I* DNA of peripheral blood mononuclear cells (PBMC) from one malaria infected patient. *II* DNA of PBMC from one healthy individual: *m* molecular weight 100 bp; *a* ex vivo; *b* after culture without stimulus; *c* in the presence of anti-CD3 monoclonal antibody and; *d* in the presence of *P. falciparum* antigen

13% for $CD8^+$) in *P. falciparum*- and *P. vivax*-infected individuals, respectively. We also compared T-cell apoptosis percentages from non-infected individuals with the values found in *P. falciparum*- or *P. vivax*-infected patients (Figs. 5, 6). Although $CD4^+$ and $CD8^+$ T-cell subsets are equally susceptible to apoptosis in *P. falciparum* and *P. vivax* patients, when we compared the $CD4^+$ T-cell subset from infected patients with those from non-infected individuals, the differences observed were always significant independently of the presence or absence of the stimulus or even the plasmodial species ($P < 0.05$). In contrast the $CD8^+$ T-cell subset was more susceptible to apoptosis only in cells from *P. vivax*-infected patients cultured without stimulus or in the presence of anti-CD3 and in *P. falciparum*-infected patients in the cells cultured in presence of antigen. As a result, it seems that in malaria patients $CD4^+$ T cells are more susceptible to apoptosis than $CD8^+$ ones.

Evaluation of sIL-2R plasma concentrations in different individuals

We evaluated the sIL-2R plasma levels in malaria patients and non-infected individuals either residing or not in malaria endemic areas. A significant increase in the sIL-2R plasma levels, reflecting the activation of T cells, was observed in malaria patients (*P. falciparum:* 227.8 ± 105.4 and *P. vivax:* 328.6 ± 214). As expected, in non-infected individuals the sIL-2R plasma levels were low (126.5 ± 155.3 IU/ml) and sIL-2R plasma levels were similar in both groups of non-infected individuals (Fig. 7). No relationship was observed between sIL-2R levels and apoptosis rates when all of the infected patients were considered. However, a positive correlation was observed ($P < 0.05$) when only patients presenting apoptosis rates above the mean were analyzed.

CD95 expression in activated cells from malaria patients

Fas antigen expression was evaluated by flow cytometry using anti-CD95 mAbs in three infected and two non-infected individuals. The results showed a raised expression of Fas antigen levels in malaria patients ($P < 0.05$) after cell culture in the presence of *P. falciparum* antigen (mean = 59%) or anti-CD3 mAb (mean = 56.5%) in comparison to the levels found in non-infected individuals after cell culture in the presence of *P. falciparum* antigen (mean = 15.5) or anti-CD3 mAb (mean = 33.5) (Fig. 8).

Discussion

The present study shows that the simple and short-term incubation of PBMC resulted in the apoptotic cell death of PBMC and most notably, T cells from *P. falciparum*-infected patients. We also demonstrated for the first time that the same phenomenon could be observed in *P. vivax*-infected individuals. The apoptosis process was visualized by different technical approaches including flow cytometry analysis, the observation of morphological and characteristic cellular alterations such as a chromatin condensation and nuclear segmentation as well as the typical electrophoresis pattern of DNA fragmentation.

Since *P. falciparum* or *P. vivax* species are usually associated with different pathological effects, it may be somewhat surprising to find that very similar levels of apoptosis were found when cells from malaria patients were cultivated for a short period, either with or without stimulus. We think that, to some extent, these observations could be explained by the fact that the individuals enrolled in this study only included patients with uncomplicated but acute malaria.

Only few malaria patients presented elevated levels of an ex vivo apoptosis process —five were infected by *P. falciparum* and eight by *P.* vivax —an observation

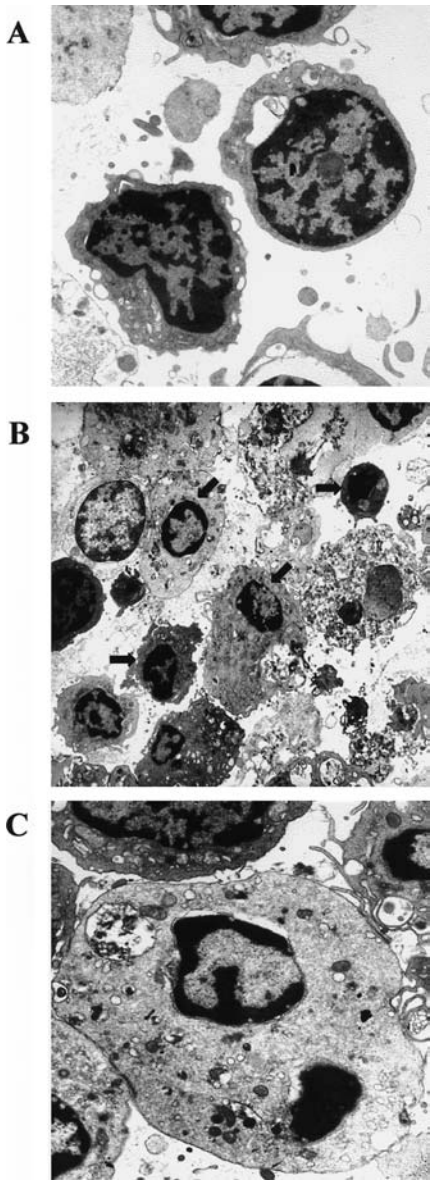

Fig. 4 Electron micrograph of ultrathin sections of PBMC from: **A** a non-infected individual (10,400×), **B** from *P. vivax* (4,700×) and **C** from *P. falciparum* (11,200×) infected patients. **A** shows cells presenting nuclei with normal chromatin appearance and membrane integrity. The *arrows* indicate cells with highly condensed cromatin (**B**) and the preservation of cytoplasm and organelles (**C**). PBMC were fixed with glutaraldehyde and osmium tetroxide both in cacodylate buffer. The ultrathin sections were stained with uranyl acetate

that is in sharp contrast with the increased apoptotic rates recorded when the cells of these patients were cultured for a short period. Similar results were recorded in lymphocytes obtained from African malaria patients (Toure-Balde et al. 1995, 1996) as well as from patients infected either by Epstein-Barr virus (Uehara et al. 1992) or HIV (Gougeon et al. 1991; Meyaard et al. 1992; Michel et al. 2000) as well as in the Chagas' disease murine model (Lopes et al. 1995).

Considering *P. falciparum* infection, the apoptosis ratios observed by Toure-Balde et al. (1995, 1996) in cells cultured for 72 h were higher than those recorded in our experiments using 24 h-cultured cells. We decided to study 24 h-cultured cells because human T-cell apoptosis can be detected by cytometry flow after only 8 h of in vitro culture, with maximum values being observed between 12 and 18 h after stimulation (Wesselborg and Kabelitz 1993) and also because after 24 h a significant increase in necrosis levels can be detected (data not shown). But it is also conceivable that the differences observed in the apoptosis rates might be related to the *P. falciparum* antigenic preparation, since molecules with distinct abilities to induce apoptosis may originate from different antigenic preparations. In our investigations, we used a soluble antigenic fraction instead of the *P. falciparum* lysate used by Toure-Balde et al. (1996), which contained whole parasites and erythrocyte debris. Besides these methodological differences, the epidemiological characteristics of the areas studied could also greatly contribute to the differences in intensity of apoptosis rates. From this point of view, the higher levels of apoptosis found by Toure-Balde et al. (1996) could reflect a massive and repeated in vivo lymphocyte stimulation induced by continuous *P. falciparum* infections, as the individuals studied by these authors were Africans living in holoendemic areas. This is consistently different from our situation in which samples were collected from Brazilian patients who had been living either in meso- or hypoendemic areas and who reported at most five past malaria episodes. In addition, the group that we studied only involved patients with uncomplicated malaria, contrasting with some of those tested by Toure-Balde et al. (1996) who were hospitalized.

Unexpectedly, the lymphocytes of malaria patients presented similar apoptosis levels after short-term culture, either in the presence or absence of stimuli. These results probably reflected the fact that the lymphocytes tested in the present study had already been activated and committed to cell death by an in vivo signal and/or an associated mechanism, and that a certain period of latency instead of a determined stimulus was required to trigger the cellular apoptotic process.

Apoptosis seems to have affected in similar proportions the $CD4^+$ and $CD8^+$ T-cell subpopulations in both *P. falciparum* and *P. vivax* infected patients. However, when comparing infected patients with non-infected individuals, the $CD4^+$ T cells appear to be more susceptible to apoptosis than $CD8^+$ T cells. This view is strengthened by the fact that significant differences between the rates of apoptosis in $CD8^+$ T cells from infected patients and non-infected individuals have only been detected for *P. vivax* patients after in vitro culture without stimulus and after CD3 stimulation, but not after antigenic stimulation in either *P. falciparum* or *P. vivax* patients. These findings suggest that despite in *P. falciparum*- and *P. vivax*-associated apoptosis there is a potential susceptibility to apoptosis in both the Th1

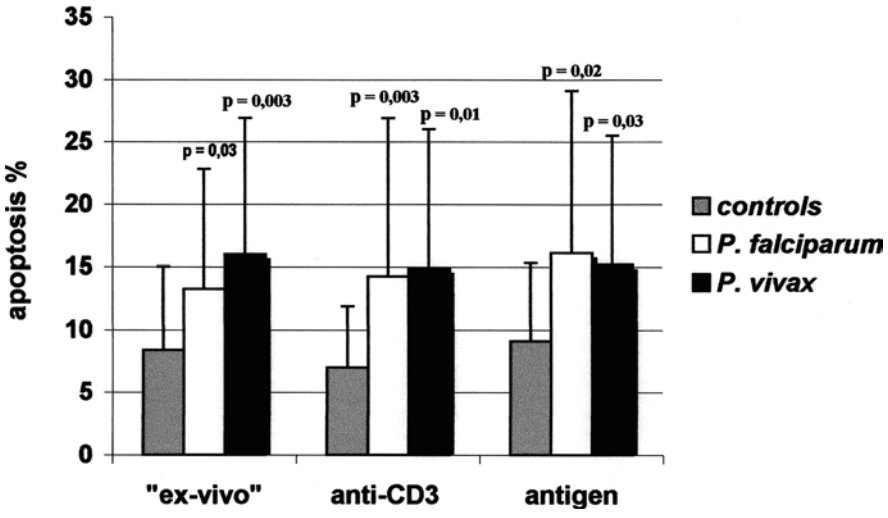

Fig. 5 Comparison of apoptosis percentages of $CD4^+$ T cells from 34 *P. falciparum*- and 45 *P. vivax*-infected patients and 30 non-infected individuals after 24 h-culture with or without stimulus. Apoptosis mean values from non-infected individuals were compared with those values found in infected patients. The cells were labeled with anti-CD4 monoclonal antibody plus 7-amininoactinomicy D. The *error bars* show the SD and *P* values were obtained using a Mann-Whitney U-test

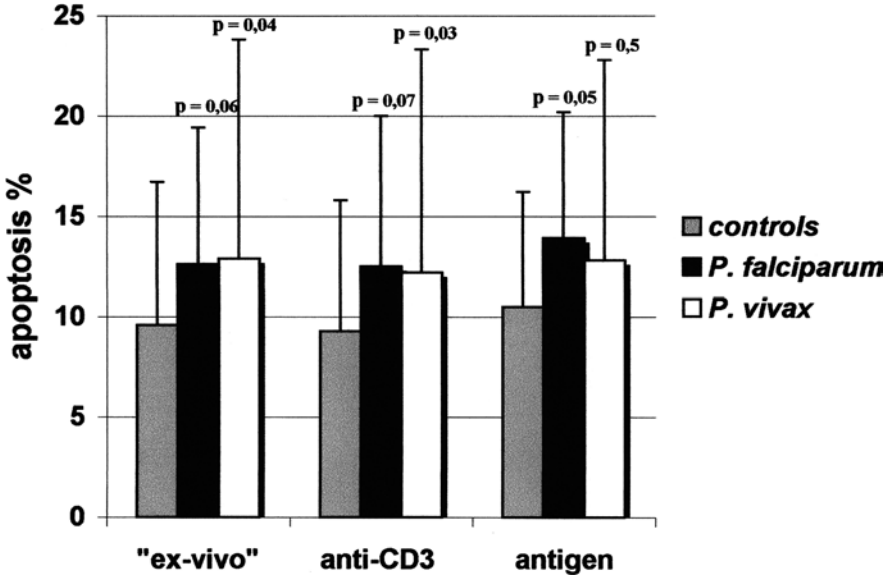

Fig. 6 Comparison of apoptosis percentages of $CD8^+$ T cells from 34 *P. falciparum*- and 45 *P. vivax*-infected patients and 30 non-infected individuals after 24 h-culture with or without stimulus. Apoptosis mean values from non-infected individuals were compared with those values found in infected patients. The cells were labeled with anti-CD8 monoclonal antibody plus 7-amininoactinomicy D. The *error bars* show the SD and *P* values were obtained using a Mann-Whitney U-test

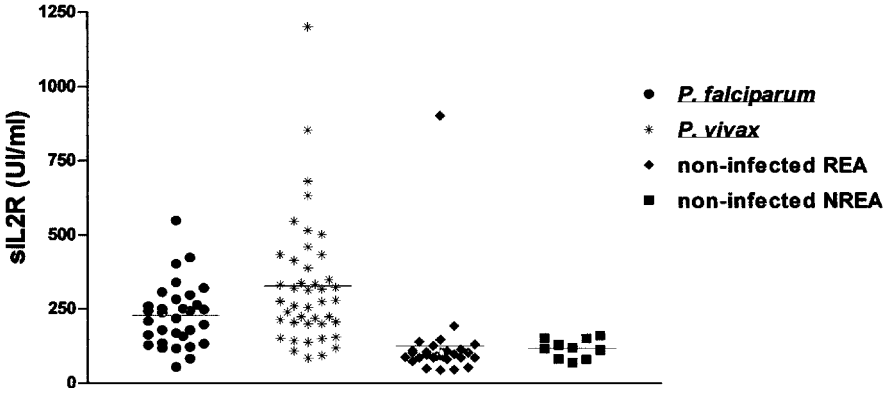

Fig. 7 Plasma interleukine-2 soluble receptor (sIL-2R) levels in *P. falciparum*- or *P. vivax*-infected patients and in non-infected individuals resident (*REA*) or not resident (*NREA*) in the endemic area. sIL-2R was evaluated by ELISA using a commercial sIL-2R kit

and Th2 subsets of lymphocytes (Janssen et al. 1991; Russel et al. 1991, 1992; Ucker et al. 1992), it seems that the malaria-associated apoptosis of lymphocytes is directly related to a determined T-cell cytokine spectrum.

We also detected increased plasma levels of sIL-2R in *P. falciparum* as well as in *P. vivax* malaria patients. Increased concentrations of this soluble receptor in *P. falciparum* malaria patients have been already reported (Ho et al. 1988; Josimovic-Alasevic et al. 1988;

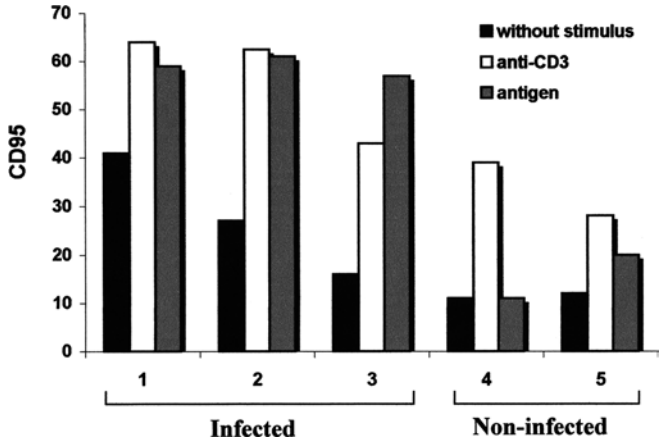

Fig. 8 CD95 Fas antigen expression in three infected and in two non-infected individuals resident in endemic area, after 24 h culture. Fas antigen expression was analyzed by flow cytometry using an anti-CD95 monoclonal antibody

Hviid et al. 1991; Riley et al. 1993). However, a positive correlation between sIL-2R plasma and apoptotic levels was observed only when we tested the subgroup of patients with increased apoptosis percentages ($P < 0.05$). Therefore, raised apoptosis levels were not a universal phenomena and it is possible that the apoptosis events could be fluctuating in the same individual, particularly during the progression of the disease. A longitudinal study could help estimate the actual frequency of the phenomenon at any moment of the acute infection.

In our study, cells activated in vitro (either by antigenic stimulus or anti-CD3 mAb) from short-term *P. falciparum* cultures expressed increased levels of Fas antigen and, at the same time, were more susceptible to apoptosis, contrasting with the results found among healthy individuals. Although we did not inhibit the Fas pathway, it is conceivable that the apoptotic process that we observed was, at least in part, mediated by and under the regulation of the Fas/Fas L system, a powerful modulator of cell death by apoptosis of previously activated T cells. Furthermore, in falciparum malaria it was shown that in vitro exposure of PBMC to *P. falciparum* extract induced a marked increase in the cell membrane expression of functional CD95 antigen and that the increased serum levels of the soluble Fas ligand (sFasL) significantly declined during the course of the disease (Toure-Balde et al. 2000; Kern et al. 2000). In addition, sFasL levels that were elevated before anti-malarial treatment began correlated significantly with depressed total lymphocyte and T-cell counts, suggesting that Fas-induced apoptosis might play a role in malaria-associated lymphopenia (Kern 2000).

We have demonstrated that infections by *P. falciparum* or *P. vivax* could result in a consistent amplification of PBMC apoptosis. On the one hand, it is comprehensible that this phenomenon could be essential for the regulation of the energetic immune response that occurs as a consequence of the marked peripheral B cells activation associated with infection by malaria parasites. On the other, one consequence of cell death might be the reduction of the critical immune responses to plasmodial antigens. From this point of view, apoptosis could act as an evasion mechanism to the host immune response and be involved in the successful persistence of the parasite in its host.

In summary, this study reveals that during malaria infection a proportion of the lymphocyte population undergoes a process leading to in vitro cellular death by apoptosis and that a period of time is required before in vivo activated cells can express the apoptotic process in vitro. These data suggest that apoptosis may contribute to the lymphopenia characteristic of this disease as previously reported (Kemp et al. 2002). However, in our study the lack of correlation between the levels of apoptosis and parasitemia and the number of previous infections, together with the lack of antigen-specificity in the system, suggests that, at least in part, the malaria-associated apoptosis may reflect a normal response of the immune system to an exogenous infection rather than a pathological phenomenon specifically induced by the disease.

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