The Spleen is an Important Site of T Cell Activation During Human Hepatosplenic Schistosomiasis

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We have undertaken a comparative immunephenotypic study of spleen cells from hepatosplenic patients (HS) and uninfected individuals (NOR) using flow cytometry. Our data did not show any significant differences in the mean percentage of T-cells and B-cells between the two groups. Analysis of activated T-cells demonstrated that HS present an increased percentage of CD3+HLA-DR+ splenocytes in comparison to NOR. Analysis of T-cell subsets demonstrated a significant increase on the percentage of both activated CD4+ T-splenocytes and CD8+ cells in HS. We did not find any difference in the mean percentage of CD28+ T-cells. Analysis of the B-cell compartment did not show any difference on the percentage of B1-splenocytes. However, the spleen seems to be an important reservoir/source for B1 lymphocytes during hepatosplenic disease, since after splenectomy we found a decreased the percentage of circulating B1-lymphocytes. We observed an increase on the percentage of CD2+CD3- lymphocytes in the spleen of HS suggesting that the loss of CD3 by activated T-cells or the expansion of NK-cells might play a role in the development/maintenance of splenomegaly.

Key words: schistosomiasis - spleen cells - flow cytometry

Analysis of the immune response in different compartments of the host organism during pathology is a new scientific strategy relevant for the study of human schistosomiasis. This approach allows simultaneous investigation to determine whether the immunological events observed in the peripheral blood are representative of those occurring in the lymphoid tissue and organs. Evaluation of the cellularity in different compartments during *Schistosoma mansoni* infection is a field of scarce investigation. Considering that adult worms, eggs and *S. mansoni* soluble antigens are of hematogenic nature, it becomes relevant to investigate the spleen as a site of cell activation during schistosomiasis. The white pulp is a major domain of lymphocyte activation in the spleen and its involvement in the immune response to blood circulating antigens has been well reported (Kopp 1990). Moreover, the spleen has been identified as an important site for lymphocyte proliferation and macrophage maturation (Tischendorf 1985). Considering the spleen as the main site of lymphoid cells interfaced into the systemic and portal blood stream and the fact that *S. mansoni* lives within the portal venous system, the splenic lymphoid cells are frequently in contact with blood circulating antigens from *S. mansoni*. Splenic cells under activation might respond to *S. mansoni* stimuli by multiple mechanisms. Although, the spleen commitment during acute phase and chronic hepatosplenic *S. mansoni* infection is documented, little is known about the immunological aspects of the spleen during human schistosomiasis. Phenotypic analysis of splenocytes during hepatosplenic schistosomiasis has been previously reported (Garcia et al. 1986). However, these studies did not present data of normal spleen cellularity, and no conclusions could be taken as far as the importance of the spleen as a immunological active site during *S. mansoni* infection. Functional studies of splenocytes have been conduced basically through antigen stimulation *in vitro*. Reiner et al. (1979) demonstrated that hepatosplenic patients could be grouped as responders and non-responders based on their splenocyte proliferative response to antigen-driven stimulation *in vitro*. The removal of plastic adherent cells from the splenocyte population could convert the low-responders into high-responders, suggesting the presence of regulatory macrophages within the splenocyte population (Garcia et al. 1986). The presence of non-adherent cells with suppressor activity of PHA-stimulated cultures has also been reported (Ellner et al. 1980).

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Considering these information it is still unclear the contribution of the spleen for the immune-mechanisms in the genesis of splenomegaly during human schistosomiasis. In order to address these questions we have developed a detailed phenotypic analysis of the spleen biopsies as well as peripheral blood samples from patients with severe hepatosplenic disease. Our study has also evaluated samples from healthy individuals as a control. The data presented here contribute to support the significance of immune mechanisms on the development/maintenance of the splenomegaly during the immunopathogenesis of schistosomiasis.

MATERIALS AND METHODS

Patients - In this study we have analyzed 13 spleen biopsies from hepatosplenic patients (HS), 9 males and 4 females age ranging from 13-63 years. Blood samples were also collected from HS, including 47 individuals with age ranging from 17-67 years. All HS were submitted to splenectomy or spleno-renal shunts to improve their clinical status and to prevent esophageal bleeding. Medical care was given at the Hospital das Clínicas, Universidade Federal de Minas Gerais (UFMG), Belo Horizonte, MG, Brazil under supervision of one of us (JRCM). Blood samples were also collected from 9 HS after splenectomy. This group of individuals was classified as splenectomized patients (SPL). Spleen biopsies from 9 uninfected individuals (NOR), 7 males and 2 females, age ranging from 16-40 years, were obtained after traumatic splenic rupture. Blood samples were obtained from 47 healthy donors from Belo Horizonte, with age ranging from 19-63 years. This study has the approval of the Ethical Committees from Fiocruz (Ministry of Healthy, Brazil) and Hospital das Clínicas, UFMG, Minas Gerais.

Isolation of splenic mononuclear cells and immunophenotyping - Tissue biopsies were minced in cold RPMI-1640 over a Petri dish, placed on ice. Minced tissues were filtered on stainless steel tamises. The mononuclear spleen cells were isolated by differential centrifugation on histopaque cushion. The cell suspension was washed twice in RPMI-1640 and ressuspended to 10 x 10^6 cells/ml. A sample of 500,000 mononuclear splenocytes was transferred to polysterene tubes previously filled with fluorocrome conjugated monoclonal antibody (mAbs) specific anti-cell surface markers. Cells were stained through double color immunocytrometric assays using simultaneously FITC and PE conjugated antibodies. In this study we have evaluated the phenotypes using antibodies to identify T and B cell populations and subpopulations as listed in Table I. After the staining procedure the cell suspensions were fixed using 1% paraformaldehide, 10.2 g/ml sodium cacodilate buffered saline. Cell phenotype was analyzed by flow cytometry, in a FACScan, using the software CellQuest for data acquisition and analysis. The results reported here are expressed as mean percentage of gated splenocyte or blood lymphocyte population and subpopulations. Comparative analysis of HS, SPL and NOR were performed by One Way Variance Analysis followed by Student's T Test.

RESULTS

Analysis of T cell, B cell and NK cells - T cell subsets and B cells were examined in the spleen mononuclear cell population. Our data showed that the mean percentage of total T cell population (CD3+) did not differ significantly between HS and NOR (Table II). Analysis of CD4+ and CD8+ T cell subsets as well as the mean ratio CD4+/CD8+ did not show any significant difference between HS and NOR (Table II). The analysis of B cells show a broad range of values in both groups, however, the mean percentage of B cells (CD19+) did not differ between HS and NOR (Table II). No differences on B1 cell population (CD5+CD19+) within CD19+ lymphocytes were observed between HS and NOR (Table II). A higher percentage of NK-like cells (CD2+CD3-) was found within the splenocytes from HS in comparison to NOR (Table II). However no differences were observed for the mean percentage of CD16+ splenocytes between HS and NOR (7.6±4.0% and 5.1±3.3%, respectively).

Analysis of the activated T cell population and subsets - Using double staining FACS analysis we have evaluated the percentage of T cell and T cell subsets (CD4+ and CD8+) that co-express the HLA-DR (Table II). Our data demonstrated a higher mean percentage of activated T cells (CD3+HLA-DR+ cells) in the spleen of HS in comparison to NOR (10.8±6.8%, 21.3±10.3%, respectively). Analysis of the mean ratio of activated T cells revealed that HS had over twice the number of activated T splenocytes within the T cells than did healthy individuals (Table II). Studies on activated T cell subsets demonstrated that severe chronic shistosomiasis is accompanied by increased activation of both CD4+ and CD8+ T cell subsets into the spleen in comparison to the healthy spleens (9.6±5.7% and 5.0±2.7% for CD4+ splenocytes; 13.4±7.8% and 7.8±3.5% for CD8+ splenocytes, respectively). Studies of the mean ratio of activated CD4+ and CD8+ cells within the T cell subsets demonstrated that schistosomiasis induces an increase on T splenocyte activation that is not restricted to a specific subset (Table II). It is interest-

TABLE I

Monoclonal antibodies/Cell population analyzed [a]

Monoclonal antibodies (mAbs)	Cell population analyzed
Isotypic control G1CL – FITC	-
Isotypic control G1CL – PE	-
Anti-human CD3 (Leu-4) –FITC	T cells
Anti-human CD4 (Leu-3a) – FITC	T helper/inducer cells
Anti-human CD8 (Leu-2a) – PE	T supressor/citotoxic cells
Anti-human CD8 (Leu-2a) – FITC	T supressor/citotoxic cells
Anti-human HLA-DR – PE	Activated T cell and subsets
Anti-human CD19 (Leu-12) – PE	B cells
Anti-human CD5 (Leu-1) – FITC	B1 cells
Anti-human CD28 (Leu-28) – PE	T cells and subsets

a: the antibodies were diluted 1:5 in FACS buffer (Phosphate buffered saline – PBS 0.015 M, 1% BSA, 0.01% sodium azide) prior to use. The mAbs used in this study were purchased from Becton-Dickinson (Mountain View, CA) and DAKO.

TABLE II

Comparative analysis of cell populations and subpopulations in the peripheral blood and spleen compartments of HS, SPL and NOR

Cell population	Peripheral blood			Spleen	
	NOR (n=47)	HS (n=31)	SPL (n=09)	NOR (n=09)	HS (n=13)
CD3+	69.1±9.8	57.4±14.2 [a]	50.3±12.8 [a]	41.1±9.6	37.9±13.4
CD4+	41.2±8.5	33.7±8.7 [a]	27.4±3.7 [a]	21.5±5.9	18.1±7.5
CD8+	31.8±6.9	26.6±9.5 [a]	37.6±9.2	18.6±6.9	20.6±10.9
CD19+	10.0±2.9	17.2±8.7 [a]	16.8±10.0 [a]	47.1±11.4	42.4±16.9
CD2+CD3-	11.1±7.7	16.9±12.0	ND	6.9±3.0	11.8±6.4
RatioCD19+CD5+:CD19+	15.1±4.4	29.6±14.2 [a]	15.3±9.1	11.7±8,1	19.2±20.3
RatioCD4+CD28+:CD4+	93.6±9.7	96.9±3.0	ND	95.7±3.9	91.9±9.5
Ratio CD8+CD28+:CD8+	47.2±14.6	37.4±13.2 [a]	ND	46.1±10.4	41.8±10.4
Ratio CD4+:CD8+	1.4±0.5	1.5±0.8	0.8±0.2	1.2±0,3	1.0±0.4
Ratio CD3+HLADR+:CD3+	13.2±7.7	24.6±14.9 [a]	15.3±9.2 [b]	23.9±11.6	52.9±13.3
Ratio CD4+HLADR+:CD4+	8.3±4.9	15.6±8.5 [a]	ND	21.8±7.2	50.9±19.7
Ratio CD8+HLADR+:CD8+	20.9±15.6	28.9±19.2 [a]	ND	43.7±17.3	72.1±14.5

HS:hepatosplenic patients; SPL: splenectomized patients; NOR: uninfected individuals; *a:* significantly differences in comparison to NOR; *b:* analysis of four patients; ND: not determined.

ing to observe that even in healthy conditions the ratio of activated CD8+ cells is higher than CD4+ cells into the spleen.

Analysis of CD28 expression on T cell subsets - The expression of the co-stimulatory molecule CD28 on T cell subsets was evaluated using a double-staining procedure. The data analysis did not show any significant difference between the mean ratio of CD4+CD28+ cells within CD4+ splenocytes of HS in comparison to NOR (Table II). Analysis of CD28 expression on CD8+ cells also did not demonstrated any significant difference between the two groups of individuals evaluated (Table II).

Study of cell phenotype in peripheral blood and spleen compartments of HS and healthy controls - The results of the phenotypic studies of cell populations in the peripheral blood and spleen from HS are presented in Table II. We observed a reduced mean percentage of T cell population and subpopulations (CD4+ and CD8+) in the peripheral blood of HS in comparison to NOR, whereas no difference was observed in comparative analysis of the spleen cells of HS and NOR. An increase in CD2+CD3- cells was observed only in the spleen of HS group with no significant difference in the peripheral blood of these patients when compared to NOR. Analysis of CD28 expression by CD4+

cells did not show any difference in the mean percentage of CD4+CD28+ cells in both peripheral blood and spleen of HS in comparison to NOR. However we found a reduced mean percentage of CD8+CD28+ cells only in the peripheral blood of HS in comparison to NOR, with no difference in the analysis of the spleen. Analysis of activated T cell population and subpopulations (both CD4+ and CD8+) demonstrated a pronounced increase of activated cells in both compartments (peripheral blood and spleen) of HS in comparison to NOR. Analysis of B1 lymphocytes demonstrated an increase of these cells in the peripheral blood of HS with no significant difference in the spleen compartment. In this study we have also analyzed the phenotype of blood leukocytes from splenectomized HS in order to identify the effect of splenectomy in the immune system. Our data demonstrated that splenectomy can restore some of the blood populations to normal levels. Our results indicated that SPL have lower percentage of T cell and CD4+ T cell subset and an increase of circulating B cells in comparison to NOR. Data of activated T cells demonstrated that splenectomy can bring these values back to normal as well as the mean percentage of CD8+ cells and CD5+CD19+ cells in comparison to healthy individuals.

DISCUSSION

Analysis of different cell populations in the human spleen is a field of very little investigation. Most of the work describing the cellularity of the spleen, the mechanism of cell activation and migration in this organ, as well as the cell distribution into the spleen are very conflicting and, in general, the hypothesis is elaborated using data from studies with different animals species (Jandl 1996). The basic literature related to the role of the spleen during infectious disease as schistosomiasis is still unclear. Early studies investigating the cellularity of the spleen during *S. mansoni* infection were developed by Garcia et al. (1986). However, that study offered, in the conception of its own authors, limited interpretation, since no parallel studies using spleen from uninfected individuals were developed. Our study presents itself as a pioneer work, analyzing comparatively the cell phenotype of HS and NOR spleens. Moreover, our data show a simultaneous analysis of cell phenotype in two different compartments: peripheral blood and spleen.

Considering the hematogenic nature of *S. mansoni*, it is expected that the spleen represents an important site for cell activation during schistosomiasis, since this organ is a major lymphoid compartment interposed into the systemic blood circulation. Our results did not show any difference in the mean percentage of T cell population and subpopulations (both CD4+ and CD8+) in comparison to NOR. However, we found that the spleen from HS presented an elevated percentage of activated T cell, including both subsets CD4+ and CD8+, in comparison to NOR. Our data demonstrated that the spleen of HS had an increased mean percentage of activated CD4+ T cells in comparison to NOR. We believe that cell activation in the spleen can have a direct effect on the development and/or maintenance of pathogeny of schistosomiasis, since cell homing of spleen cells in another organ can occur directly through the blood stream. Recent studies have demonstrated that CD4+ T lymphocytes are able to produce and secret a fibroblast stimulating factor, called fibrosin, which has been characterized as a major factor on the development of tissue fibrosis during experimental infection by *S. mansoni* (Prakashi & Wyler 1991, Prakashi et al. 1992). The biochemical analysis of the liver fibrose during schistosomiasis has demonstrated an elevated deposition of colagen and a latent production of colagenase into the liver (Takahashi et al. 1980). Considering these observations Prakashi et al. (1991) proposed a casual relationship between the production of fibrogenic cytokines and the development of liver fibroses during *S. mansoni* infection. Recently, Leite-Freitas (1995) demonstrated that colagen and fibronectin deposition are the most important mechanisms responsible for the splenic cords thickness observed in HS. Taken together these results reemphasize our hypothesis that the activation of CD4+ T lymphocytes is directly associated with the development of pathology during human schistosomiasis mansoni.

Considering the nature of the parasite, and the mechanism proposed for extracellular antigen processing and presentation, the activation of CD8+ cells specific to *S. mansoni* antigens would not be expected (Brodsky & Guagliardi 1991). However, the phenotypic analysis of CD8+ T lymphocytes demonstrated significant activation of these splenocytes in the spleen of HS. We suggest that this activation may be a consequence of a bystander effect or the result of peptide engagement on MHC-class I molecules during the process of continuous and prolonged antigen stimulation. Analysis of CD28 expression, by CD8+ T splenocytes did not demonstrate any significant alteration in comparison to healthy individuals, suggesting that the activation of CD8+ T cells at the spleen, would occur in the presence of co-stimulatory events, via CD28, resulting in an effective activation of these cells. Previous studies in our laboratory demonstrated that during severe hepatosplenic schistosomiasis, the loss of co-stimulatory mechanisms,

detected by a decrease on the percentage of circulating CD8+CD28+ T cells can be correlated with the development and/or maintenance of hepatosplenic chronic schistosomiasis. In this contest, it is possible that the loss of CD28 is a mechanism that takes place in the periphery an not at the spleen. Moreover, there is also a possibility that defective expression of CD28 natural ligant, the B7 molecule, expressed on macrophages and B lymphocytes (Lenschow et al. 1996), could be altered in the spleen cell population of HS. The study of B7 expression by different cell populations in the peripheral blood as well as splenocytes during schistosomiasis are under evaluation in our laboratory.

We have previously demonstrated that the expansion of circulating CD5+ B lymphocytes correlates with severe forms of human schistosomiasis. The data presented here did not show any differences between the mean percentage of CD5+ B splenocytes of HS in comparison to NOR. However, these results did not exclude the spleen as an important site for B1 lymphocyte expansion. Our results on blood cell phenotypes demonstrated that splenectomized patients present lower mean percentage of circulating B1 lymphocytes in comparison to HS and are in agreement with the hypothesis of the spleen being an important site for B1 cell origen and/or storage. Studies on B cell compartment in the spleen have demonstrated that the marginal zone of the spleen is an important site of specialized B cells, rich in IgM+ B lymphocytes, that respond to T-independent antigens (Van Ewijk & Nieuwenhuis 1985). Probably, the existence of a cell interchange between the spleen and the blood circulation could explain the increased mean percentage of B1 lymphocyte observed in the peripheral blood of HS. Studies developed using murine experimental model for schistosomiasis have demonstrated that the expansion of B1 lymphocytes is a phenomena restricted to peritonial cavity without an elevation in the percentage of these cells in the spleen of infected animals (Vellupillai et al. 1997). These authors suggested that in the spleen a selective expansion of CD23+ B lymphocytes occurs, which happens simultaneously with the decrease of B1 lymphocytes in the peritonial cavity. Although no studies correlating these two cell populations has been performed, these authors suggested that when B1 lymphocytes reach the spleen, they probably become CD23+CD5-, since these two cell populations presented similar functional activities and cytokine secretion profile (Velupilai et al. 1997). Considering this possibility we have performed a study of B CD23+ lymphocytes in the spleen of HS as well as NOR, however, no significant alterations were observed in this study (data not shown).

Our data demonstrated a significant increase in the percentage of CD2+CD3- cells in the spleen of HS in comparison to NOR. Although we observed a tendency in the increase percentage of CD2+CD3- cells in the peripheral blood of HS in relation to NOR, no significant difference were observed. Studies developed by Colley et al. (1983), analyzing T cell subpopulations in the peripheral blood of HS patients, demonstrated an absence of correlation between the mean percentage of T3+ (CD3+) cells in relation to cells able to rosette with sheep red blood cells T11+(CD2+) cells. These authors observed that the percentage of T11+ cells was higher in comparison to the percentage of T3+ cells. An imbalance in T cell population that would lead to production and peripheralization of immature T cells or altered lymphoid subpopulations, or a low expression of T3 and normal expression of T11 could be responsible for this phenomena. The role of CD2+CD3- cells in schistosomiasis is still unclear. Although this cell population presents phenotype indicative of natural killer cells, there are studies demonstrating that CD2+CD3- cells could represent an activated T cell population that lost CD3 marker upon activation (Jahn et al. 1987).

The parallel study of different cell populations in the peripheral blood and in the spleen of HS in comparison to NOR have shown that some alterations can be observed only in the peripheral blood. Our results demonstrated a reduced mean percentage of T lymphocytes in the peripheral blood of HS with consequent reduction of both CD4+ and CD8+ cells. On the other hand, no significant alterations in the T cell compartment was observed in the spleen of HS in comparison to NOR. Analysis of activation markers on T cells showed a similar situation in the peripheral blood and the spleen. HS presented an increased mean percentage of activated CD4+ cells and CD8+ cells in both compartments, spleen and peripheral blood. These data suggested an interchange of cells between these two compartments. However, it is interesting to observe that during hepatosplenic schistosomiasis although the increase of activated CD8+ cells can be observed in both compartments, in the peripheral blood there is a decrease of CD8+CD28+ T cells that is not detected in the spleen. It has been proposed that the effective activation of T cells requires the participation of at least one costimulatory event besides TCR/MHC engagement. The absence of these costimulatory events can lead to anergy or programmed cell death by apoptosis. Recent studies have shown that apoptosis, regulating the immune response can occur in specific organs as well as in the periphery. Taken together these data support the hypoth-

esis that the lost of CD28 by CD8+ cells, an important mechanism regulating the immune response in HS is an event that takes place outside the spleen during schistosomiasis.

In conclusion, the spleen seems to be a potential site for the activation and storage of activated T cells and B1 cells during schistosomiasis. The similarities of the phenotypic alterations in T cell subpopulations observed in the spleen and in the peripheral blood suggest the occurrence of a cellular interchange between these two compartments during the infection by *S. mansoni*. Consistent with this hypothesis, we have also demonstrated here that after splenectomy there is a significant decrease in the mean percentage of activated T cells as well as CD5+ B cells. Despite the studies suggesting the main role of the omentum membrane as a major source of CD5+ cells (Lenzi pers. comm.), our data demonstrated that splenectomy, with the preservation of the omentum membrane, also leads to a decrease in the mean percentage of B1 cells in the peripheral blood. These data are also important to support the splenectomy as an immunological intervention to ameliorate the patient immune profile in cases of hepatosplenic disease. The data presented here is an initial attempt to define a standard phenotypic profile of spleen cells from NOR and are used as a parameter to address the questions of the effect of a *S. mansoni* infection on spleen cell populations and subpopulations. Since the *S. mansoni* infection is endemic in many Brazilian states, these results are also important as a basic reference parameter since they describe for the first time the phenotypic profile of spleen cells for the Brazilian population that is racially highly heterogeneous. For future directions we are interested in addressing the question of cell migration into the organ by investigating the expression of different adhesion molecules by lymphoid cells using two color flow cytometric analysis as well as the identification of the preferential expression of adhesion molecules on different organ domains. Once elucidated these features we will be able to compare them with those observed in another splenic diseases and better understand the role of lymphocyte's homing in the development of splenic dysfunction during human diseases.

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