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ALTERED LYMPHOCYTE PROLIFERATION AND CYTOKINE RELEASE IN HEPATOSPLENIC SCHISTOSOMIASIS MANSONI IN BRAZIL

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Alterations of lymphocyte proliferative response upon *in vitro* stimulation with mitogens and schistosome antigen preparations are most accentuated in patients with the hepatosplenic form of schistosomiasis (D. G. Colley et al., 1986, *Am. J. Trop. Med. Hyg., 35:* 793-802). The phenotype distribution of circulating lymphocytes is characterized by an increase of B-cells at the expense of T-cells, and, within the T-cell compartment, a subgroup of E. rosette forming T-cells failing to express the CD3 antigen (H. Feldmeier et al., 1985, *Clin. Exp. Immunol., 60:* 225-233).

Mitogens employed in previous studies mostly act upon T as well as B lymphocytes. In order to discriminate more precisely between T- and B-cell blastogenesis, we used a T- cell-receptor specific monoclonal antibody capable of triggering polyclonal expansion and pokeweed mitogen, respectively, in a lymphocyte proliferation assay.

CD3 expressing circulating lymphocytes susceptible to the mitogenic effect of the monoclonal antibody were quantified.

Cytokine-containing supernatants were generated and assayed for Interleukin-2 (IL-2) and Tumor Necrosis Factor-alpha (TNF-α). The effect of a schistosomicidal drug regimen upon these parameters was evaluated 4-6 months after treatment with praziquantel.

PATIENTS

Clinical investigations and primary cell cultures were carried out in Maceió, Alagoas, NE-Brazil. Patients with hepatosplenic (n = 15) and intestinal (n = 15) schistosomiasis were stringently matched for age, sex and the intensity of faecal egg excretion. An age and sex matched control group was formed in a low-income neighborhood of Maceió, were socio-economic conditions equivalent to those of major endemic zones prevail.

The median age was 28 years in hepatosplenic patients and controls, and 29 years in intestinal schistosomiasis (ranges 8-71). Median egg excretion per gram of faeces was 55 in hepatosplenic and 26 in intestinal schistosomiasis, maxima were 222 and 240 ova/g, respectively.

The hepatosplenic patients were examined after hospitalization for consequences of portal hypertension (8) or active case detection by screening of a large group of *Schistosoma mansoni* infected individuals (7). Current and previous complications among the whole of hepatosplenic patients included upper intestinal tract bleeding (6), ascites (6) and infantilism (1).

The liver was enlarged and hardened in all patients. The spleen was palpable in all but three hepatosplenic patients; they had been splenectomized two or more years previously (2) or had ascites per magna impeding palpation (1).

Intestinal schistosomiasis patients matching with hepatosplenic cases were selected from a large group of individuals screened parasitologically by SUCAM. Faecal egg excretion was calculated from five Kato-Katz slides each from three separate stool samples. Fifteen Kato-Katz slides and MIF-concentration examination of three separate stool samples was required from controls.

While all hepatosplenic patients had proof of viable schistosome ova, eggs were detected in a rectal biopsy specimen, but not in stools, in one patient.

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Patients with hepatosplenomegaly received 30 mg/kg praziquantel on each of six consecutive days. Intestinal schistosomiasis was treated with 40 mg/kg praziquantel in equally divided doses four hours apart.

IMMUNOLOGICAL METHODS

Peripheral blood mononuclear cells (pbmnc) were prepared on a Ficoll density gradient, washed twice in RPMI 1640 medium and handled in complete RPMI 1640 containing 10% FCS from then on. 50.000 pbmnc were applied per well of teflon coated microscopy slides (Dunn, Asbach FRG), incubated with anti-CD3 monoclonal antibody (Behring BMA 030, Marburg FRG), labelled with peroxidase-conjugated anti-mouse Ig and stained with diaminobenzidine. Relative frequency (%) was calculated from 200 cells with lymphocyte morphology counted per well.

For $_3$H-thymidine incorporation assays, 2 x 10^5 pbmnc were cultured in round bottom microtiter plates either in the presence of 2 or 0.5 μg/ml pokeweed mitogen (Biochrom, Berlin FRG) or anti-CD3 monoclonal (Behring BMA 030, Marburg FRG) 1:5000 final dilution in complete RPMI 1640. 1 μCi ^{3}H-thymidine was added after 48 h of culture and cells harvested after a further 18 h. Results are presented as stimulation indices (experimental cpm/background cpm) calculated from the median values of triplicates.

For supernatant generation, 2 x 10^6 pbmnc were cultured in 24-well plates. Mitogen concentrations were as stated above, in a final volume of 1650 μl/well. 1000 μl of supernatant was collected after 48 h and frozen at -20°C until lymphokine determination was carried out. IL-2 was assessed by ^{3}H-thymidine incorporation of an IL-2 dependent CTLL cell line. TNF activity was assessed by lysis of L-929 target cells.

Data are presented as medians and 95% confidence intervals (CI) of the median.

RESULTS

Table I shows stimulation indices after culture in the presence of anti-CD3 monoclonal antibody, and optimal (2 μg/ml) as well as suboptimal (0,5 μg/ml) concentrations of pokeweed mitogen. Post-therapeutic increase of the stimulation index was observed in hepatosplenic schistosomiasis patients with low anti-CD3 responses before chemotherapy.

Phenotypically, patients with hepatosplenic schistosomiasis had fewer lymphocytes expressing the CD3 antigen than controls, both in absolute terms (95% CI 815-1248 CD3+/μl peripheral blood, median 1081/μl, vs. 1424-1620, median 1534/μl in controls) and in relative frequencies (40-54%, median 46% in hepatosplenic schistosomiasis vs.59-77%, median 65.5% in controls). Intestinal schistosomiasis patients had intermediate values (1152-1512 CD3 + /μl, median 1225 /μl; relative frequency 46-64%, median 56%). After therapy, CD3 + counts in the hepatosplenic schistosomiasis patients were 1025-2116 cells /μl, median 1404, or 47-72%, median 56,5%. In intestinal schistosomiasis patients, 1098-2010 CD3 + /μl, median 1610 /μl, were counted after treatment.

TABLE I
Lymphocyte proliferation

Group	Control (N = 15)	Hepatosplenic Before therapy (N = 14)	Hepatosplenic After therapy (N = 13)	Intestinal Before therapy (N = 15)	Intestinal After therapy (N = 12)
Anti-CD3	27.7 (15.5-38.6)	10.8 (3.8-33.8)	31.3 (20.8-37.1)	30.2 (19.0-48.7)	29.6 (19.8-39.8)
PWM 2μg/ml	30.7 (25.3-31.9)	17.8 (6.6-28.7)	20.7 (17.0-26.4)	27.8 (20.0-46.3)	26.0 (18.4-38.9)
PWM 0,5 μg/ml	30.5 (12.4-35.9)	16.5 (5.8-22.0)	19.5 (17.0-29.0)	16.3 (11.4-35.9)	29.0 (18.3-33.2)

Stimulation indices (experimental cpm/background cpm) upon stimulation with monoclonal anti-T-cell-receptor antibody, and pokeweed mitogen, before and after treatment with praziquantel. Medians (95% confidence interval).

TABLE II
Cytokines in pokeweed stimulated culture supernatants

Group	Control	Hepatosplenic		Intestinal	
	(N = 10)	Before therapy (N = 12)	After therapy (N = 7)	Before therapy (N = 9)	After therapy (N = 8)
Interleukin 2 (U/ml)	7.3 (6.6- 8.6)	2.9 (1.6- 5.0)	8.1 (2.8- 8.6)	3.7 (2.8- 4.6)	7.8 (6.6- 8.6)
Tumor Necrosis Factor (U/ml)	23.2 (11.6-53.9)	0.6 (0.0- 7.8)	4.8 (0.0-36.3)	18.6 (9.8-48.3)	38.0 (28.7-44.1)

Concentrations of IL-2 and TNF-α (U/ml) in supernatant fluid of peripheral blood mononuclear cell cultures before, and four to six months after treatment with praziquantel. Data are presented as medians (95% confidence intervals).

IL-2 was detected in low quantities only in supernatants from untreated schistosomiasis patients, intestinal or hepatosplenic (Table II). Normalization occurred within four months after treatment with praziquantel.

The macrophage/monocyte product TNF-alpha was severely decreased in supernatants of hepatosplenic schistosomiasis patients (Table II). In both patient groups, TNF-alpha production tended to rise after chemotherapy; however, it failed to attain control levels within the time of observation in the hepatosplenic schistosomiasis group.

DISCUSSION

The dichotomy, in schistosomiasis, between impaired T-cell mediated responses on one hand, and polyclonal activation of B lymphocytes on the other, is well known. Previous studies of mitogen-induced proliferation have relied primarily on lectins such as phytohaemagglutinin or concanavalin A, which stimulate T and B cells, although in variable proportions. We have measured T-cell receptor-induced mitogenesis using a monoclonal antigen binding to the CD3 antigen. Whereas patients with intestinal schistosomiasis responded well to this stimulus, low responders were encountered in the group with hepatosplenic schistosomiasis. This does not surprise when CD3 antigen expression within the circulating lymphocyte population is taken into account. Since the relative frequency of CD3 + cells in hepatosplenic patients was significantly lower than in controls, fewer cells possessing the prerequisite for T-cell receptor induced proliferation, that is, fewer cells expressing the T-cell receptor on their surface, were present in the assay in which overall cell numbers are kept constant.

Four months after therapy with praziquantel, CD3 + cells counts in these patients approached normal values and, concordantly, CD3-induced proliferation attained control levels. This finding bears relevance beyond the mitogenic stimulation situation we used, since T-cells can recognize antigens presented by macrophages only in the context of the T-cell receptor, that is, the complex of which CD3 forms part.

Pokeweed mitogen is considered a B-cell mitogen, although it requires the presence of T-cells for the induction of mitogenesis. Alterations in proliferative responses were less pronounced upon stimulation with pokeweed than upon CD3 induction. A tendency was noted for improved response to suboptimal pokeweed mitogen after therapy in intestinal schistosomiasis, but less so in hepatosplenic cases. Pokeweed mitogen was used for the generation of cytokine containing supernatant in a concentration yielding optimal responses in proliferation assays (2 μg/ml) on account of its capacity to stimulate the release of alpha class lymphotoxin (R. S. Yamamoto, 1985. In: *Investigation of cellular immunity*. T. Yoshida (ed.). Churchill Livingstone, Edinburgh, p. 127), now called tumor necrosis factor. TNF-α was released in normal quantity by intestinal schistosomiasis patients but was undetectable in almost half of the hepatosplenic cases before therapy. TNF-α production in hepatosplenic schistosomiasis remained defective four months after chamotherapy. Since

TNF-α modifies the activity of many cell types capable of collagen production, it may play a role in the down-regulation of fibrogenesis.

IL-2 activity was significantly decreased before therapy in both intestinal and hepatosplenic schistosomiasis, and attained control values in both groups four months after chemotherapy. It has to be emphasized, however, that the bioassay used measures an activity of unbound IL-2 molecules.

In an apparent contradiction, the low-affinity receptor for IL-2 as recognized by anti-TAC monoclonal was expressed in increased quantity by circulating lymphocytes of our patients (data not shown). Since this receptor molecule is to some extent shedded from the cell surface into surrounding medium, its presence in culture supernatant liquid may have interfered with our assay. If so, IL-2 binding by low-affinity receptor may be a factor hampering clonal expansion of T-cells *in vivo,* too. Soluble IL-2 receptor has been detected in significantly increased amounts in the sera of patients with intestinal and, even more so, hepatosplenic schistosomiasis (Josimovic-Alasevic et al., 1987, *Clin. Exp. Immunol., 72:* 241-254). Studies on the Kinetics of IL-2 receptor and the way it is affected by antischistosomal therapy are under way to complement our data.

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