Failure of Immunosuppressive Mechanisms in Human *Schistosoma mansoni* Infection with Hepatosplenomegaly

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The basis for development of hepatosplenic disease and attendant morbidity in *Schistosoma mansoni*-infected individuals is uncertain but may relate to defective modulation of immunopathology. Individuals 14 to 30 years of age from a village in the Nile Delta in Egypt were selected for study: 32 were infected with *S. mansoni* but lacked hepatosplenomegaly (mean fecal egg excretion $\pm$ standard error of the mean, $1,142 \pm 79$ eggs per g), 9 had *S. mansoni* infection and hepatosplenomegaly ($1,267 \pm 197$ eggs per g), and 12 were uninfected. The ratio of OKT4 helper/OKT8 suppressor cells in peripheral blood mononuclear cells was reduced in infected subjects without hepatosplenomegaly to 1.4 ± 0.1 compared with a ratio of 1.7 ± 0.1 ($P < 0.05$) in uninfected subjects. In contrast, this ratio was increased in the group with hepatosplenomegaly to 2.7 ± 0.3 ($P < 0.01$). Schistosome antigen-induced [³H]thymidine incorporation in peripheral blood mononuclear cells was comparable in infected subjects without ($5,837 \pm 1,009$ cpm) and with ($3,329 \pm 738$ cpm; $P > 0.1$) hepatosplenomegaly. Depletion of adherent suppressor cells significantly increased the responses in the group lacking organomegaly ($14,028 \pm 1,683$ cpm; $P < 0.001$) but not in the hepatosplenomegaly group ($5,046 \pm 1,830$ cpm; $P > 0.5$); this difference in response of nonadherent cells to soluble worm antigenic preparation was statistically significant ($P < 0.02$) and not explained by quantitative shifts in OKT8 suppressor cells. Thus, in *S. mansoni* infection, subjects with hepatosplenomegaly are distinctive in their lack of an immunosuppressive balance of T-lymphocyte subpopulations and in the absence of functional adherent suppressor cells. Defective immunoregulatory mechanisms could be important in the genesis of hepatosplenic disease and its morbid sequelae.

In areas endemic for *Schistosoma mansoni*, only a small fraction of the infected population develops hepatosplenomegaly (18, 20, 23, 33, 34). Whereas epidemiologic data indicate a close relationship between intensity of infection and development of hepatosplenomegaly (18, 23, 33, 34), this correlation is not exact. Several lines of evidence point to a genetically determined diathesis to hepatosplenic disease: the relative resistance of Brazilian blacks (2), altered susceptibility of individuals with specific blood groups (4, 28), the demonstration of household aggregation of hepatosplenomegaly in some endemic areas (A. A. F. Mahmoud et al., unpublished data), and the association between schistosomal hepatosplenomegaly and the histocompatibility antigens HLA-A1 and B5 (1).

The immune response may be an important determinant of host susceptibility to *S. mansoni* infection and disease. In chronic human infection with *S. mansoni*, lymphocyte blastogenic responses to schistosomal antigens are depressed (7, 8, 25, 26, 36) and correlate inversely with intensity of infection (13). Several suppressor mechanisms specific for schistosomal antigens contribute to depressed T-lymphocyte blastogenesis: serum factors (8, 27, 31), adherent suppressor cells (24, 37), and inducible suppressor T lymphocytes (10, 32). Regulation of the immune response also may influence the expression of disease. During the course of *S. mansoni* infection in mice, modulation of disease occurs and parallels diminution of host granulomatous hypersensitivity mediated by suppressor T lymphocytes (5, 6, 11). Failure to modulate granulomatous hypersensitivity, as in some inbred strains of

animals, may result in more severe disease (15). Suppressor splenic T lymphocytes, in fact, have been defined in some patients with far-advanced human hepatosplenic disease (12).

The current study was designed to explore the potential contribution of aberrant regulation of the immune response to the genesis of hepatosplenomegaly. In a community-based study of *S. mansoni* infection in an Egyptian village, subjects with *S. mansoni* infection and hepatosplenomegaly did not have an immunosuppressive balance of T-lymphocyte subpopulations and lacked functional adherent suppressor cells, both features of immunoregulation in subjects with matched intensity of infection but no organomegaly.

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MATERIALS AND METHODS

Study population. We obtained clinical and parasitologic data on 342 individuals between the ages of 14 and 30 years in Kafr Hakeem, an agricultural village 20 kilometers outside of Cairo in the Nile Delta. *S. mansoni* egg excretion was quantified by the Kato technique (33). The presence or absence of hepatosplenomegaly was clinically determined by two observers independently and defined as a palpable spleen or a liver 6 or more cm below the right costal margin. The possibility of concurrent *Schistosoma haematobium* infection was evaluated in all subjects by examination of filtrates of 10 ml of urine for eggs (29). Other illnesses were excluded by history and physical examination. The population selected for study consisted of an uninfected control group and two groups with *S. mansoni* infection: one with hepatosplenomegaly and the other without hepatospleno-

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megaly but with similar intensity of infection. Hepatosplenomegaly was not observed in subjects without *S. mansoni* infection. Ten percent of the serum samples contained hepatitis B surface antigen; the frequency of antigenemia was similar in each of the three groups.

Cell separation and characterization and source of antigens. Peripheral blood mononuclear cells (PBMC) were obtained from heparinized blood by Ficoll-Hypaque sedimentation. T-lymphocyte-enriched populations (T cells) were prepared by two-step adherence depletion on plastic plates and nylon-wool columns. PBMC (10^7 /ml) in RPMI 1640 medium (KC Biologicals, Lenexa, Kans.) were incubated with heat-inactivated 10% fetal calf serum (KC Biologicals) in plastic petri dishes for 1 h at 37°C. The plastic nonadherent cells were eluted and incubated on 600-mg acid-washed nylon-wool columns (Fenwall Laboratories, Grove Park, Ill.) for 30 min at 37°C (16). A T-lymphocyte-enriched population was collected by dropwise elution and was 70 to 90% E-rosette producing, <1% surface immunoglobulin bearing (39), and <0.5% peroxidase positive (17).

PBMC were depleted of OKT8⁺ cells by antibody-dependent complement lysis. PBMC (4×10^6 to 5×10^6 /ml) were incubated with a 1:40 dilution of OKT8 antibody (Ortho Diagnostics, Inc., Raritan, N.J.) in RPMI 1640 for 30 min at room temperature. Reconstituted lyophilized rabbit complement (Cedarlane, Hornby, Ontario, Canada) was added to the cell-antibody mixture to achieve a final complement dilution of 1:3, and incubation continued for 60 min at room temperature. The cells were washed three times, resuspended in RPMI 1640–10% fetal calf serum, and incubated 60 min at 37°C. The resulting cells were >95% viable by trypan blue staining and depleted $40 \pm 5\%$ of OKT8⁺ cells as assayed by indirect immunofluorescence microscopy.

S. mansoni soluble worm antigenic preparation (SWAP) was prepared as described previously (7, 8, 26). Streptokinase-streptodornase (SKSD; Lederle Laboratories, Pearl River, N.Y.) was purchased, dialyzed to remove preservatives, and diluted in RPMI 1640. Antigens were used at concentrations inducing optimal [³H]thymidine incorporation: SWAP at 50 to 100 µg/ml and SKSD at 100 U of streptokinase per ml.

Quantification of T-lymphocyte subpopulations in PBMC. T-lymphocyte subpopulations in PBMC were quantified by indirect immunofluorescence microscopy with monospecific mouse anti-human antibodies to all peripheral, helper-inducer, and suppressor-cytotoxic T lymphocytes (OKT3, OKT4, and OKT8, respectively; Ortho Diagnostics). Plastic-adherent cells were analyzed with monoclonal antibodies OKIa1 and OKM1 (Ortho Diagnostics). Cells (5×10^5) were incubated with 50 µl of a 1:40 dilution of reconstituted OKT3, OKT4, OKT8, or OKM1 or a 1:150 dilution of OKIa1 antibody for 30 min at 4°C. After being washed, the cells were suspended in 50 µl of a 1:20 dilution of fluorescein-labeled rabbit anti-mouse immunoglobulin [F(ab')₂ fragment; Cooper Biomedical, Inc., West Chester, Pa.] and incubated

for 30 min at 4°C. The cells then were washed in Hanks balanced salt solution (KC Biologicals), fixed in 2% glutaraldehyde (Sigma Chemical Co., St. Louis, Mo.) for 15 min, and washed twice. A sample of labeled cells was coded and examined microscopically in a blind manner by phase-contrast and epi-illumination fluorescence (Dialux; Leitz/Opto-Metric Div. of E. Leitz Inc., Rockleigh, N.J.). Two hundred cells were scored per sample. The absolute numbers of lymphocyte marker-positive subsets were derived from the erythrocyte and differential counts of a Wright-stained blood film. The same lots of reagents were used throughout the investigation, and all dilutions had been standardized previously for optimal specificity and sensitivity.

Assay of [³H]thymidine incorporation. PBMC and T cells were cultured at 10^5 cells per well in 0.1 ml of RPMI 1640 supplemented with gentamicin (5 µg/ml)–penicillin (50 U/ml)–L-glutamine (2 mM; Flow Laboratories, Inc., McLean, Va.)–heat-inactivated 10% human serum (pooled from healthy subjects in Cleveland) in round-bottom Falcon microtiter plate wells (Becton Dickinson Labware, Oxnard, Calif.). Quadruplicate wells were prepared for each variable. The tissue culture plates were incubated at 37°C in 5% CO₂-humidified air for 5 days. These culture intervals and conditions yielded optimal [³H]thymidine incorporation in this assay. One microcurie of methyl-tritiated thymidine (specific activity, 5 Ci/mM; Amersham Corp., Arlington Heights, Ill.) was added to each well 18 h before harvesting. Cells were collected onto glass filter paper disks and washed with a semiautomated microharvesting device. The disks were placed in ACS scintillation fluid (Amersham), and ³H content was determined by liquid scintillation spectrophotometry. Data were expressed as changes in counts per minute (antigen-stimulated counts minus unstimulated counts).

Statistical analysis. Data are presented as means $\pm$ standard errors of the mean. Unless otherwise stated, the significance of differences among three or more means was determined by one-way analysis of variance and Fisher's least significant difference test; differences between two means were determined by Student's *t* test.

RESULTS

Clinical and parasitologic data in study populations. Because hepatosplenomegaly and heavy egg excretion occur in only a small proportion of *S. mansoni*-infected individuals, we surveyed 342 villagers to ensure a sufficient pool of subjects in each group for study. Of the individuals surveyed, 18% had concomitant *S. mansoni* and *S. haematobium* infections, 63.5% had active *S. mansoni* infections alone, and 1.5% had active *S. haematobium* infections alone; 17% were not infected. Within the *S. mansoni*-infected group, 74% had <600 eggs per g of stool (light to moderate infection), 26% had ≥ 600 eggs per g of stool

TABLE 1. Demographic and parasitologic description of the study population

Group	No. and sex (male/female) of subjects	Age (yr)		No. of eggs/g of stool	
		Mean	Range	Mean	Range
Uninfected (control)	12 (7/5)	18.1	14–25	0	0
<i>S. mansoni</i> infected					
Without hepatosplenomegaly	32 (27/5)	17.9	14–30	1,142	600–2,640
With hepatosplenomegaly	9 (6/3)	19.9	14–30	1,267	300–2,060

TABLE 2. Leukocyte and lymphocyte counts and subclasses in study populations

Group (n)	Mean no. (%) $\pm$ SEM of cells/ μ l					OKT4 ⁺ /OKT8 ⁺ ratio $\pm$ SEM
	Leukocyte	Lymphocyte	OKT3 ⁺	OKT4 ⁺	OKT8 ⁺	
Uninfected (12)	7,044 $\pm$ 835	2,796 $\pm$ 274	1,854 $\pm$ 197 (59 $\pm$ 3)	1,174 $\pm$ 121 (39 $\pm$ 1)	694 $\pm$ 95 (23 $\pm$ 2) ^a	1.7 $\pm$ 0.1 ^{b,c}
<i>S. mansoni</i> infected						
Without hepatosplenomegaly (22)	7,810 $\pm$ 802	3,057 $\pm$ 612	2,048 $\pm$ 188 (58 $\pm$ 2)	1,261 $\pm$ 128 (35 $\pm$ 2) ^a	924 $\pm$ 88 (26 $\pm$ 1) ^b	1.4 $\pm$ 0.1 ^c
With hepatosplenomegaly (6)	8,787 $\pm$ 1,213	2,544 $\pm$ 282	2,269 $\pm$ 300 (67 $\pm$ 6)	1,478 $\pm$ 153 (45 $\pm$ 6) ^a	599 $\pm$ 87 (18 $\pm$ 2) ^{a,b}	2.7 $\pm$ 0.3 ^b

^a Significantly different, $P < 0.05$.^b Significantly different by Wilcoxon rank sum test, $P < 0.05$.^c Significantly different, $P < 0.01$.

(heavy infection), and 12% had hepatosplenomegaly. Criteria used for selection of the study populations were ages between 14 and 30 years and good general health; infected subjects without hepatosplenomegaly were selected to match the intensity of *S. mansoni* infection with the hepatosplenomegaly group. The demographic and parasitologic description of the 53 patients chosen for study (Table 1) did not reveal significant differences between uninfected and infected subjects in age and male-to-female ratio. Among the infected subjects, these characteristics, as well as intensity of infection, were similar. Within the infected group with hepatosplenomegaly, the mean liver enlargement extended to 4 cm below the right costal margin (range, 0 to 10 cm) and the mean spleen enlargement extended to 2 cm below the left costal margin (range, 0 to 6 cm). In all cases, the livers and spleens were smooth and nontender.

Lymphocyte subclasses were determined in all 12 uninfected subjects. We did not evaluate 10 of the 32 infected subjects without hepatosplenomegaly and 3 of the 9 infected subjects with hepatosplenomegaly. However, functional studies of [³H]thymidine incorporation were performed on all 41 subjects.

Quantification of T-cell subclasses in *S. mansoni* infection. In infected subjects without hepatosplenomegaly, no significant differences from control subjects were observed in the total leukocyte and lymphocyte count and in the number of OKT3⁺, OKT4⁺, and OKT8⁺ cells (Table 2). However, a significant decrease to 1.4 was apparent in the ratio of OKT4 helper to OKT8 suppressor cells. In infected subjects with hepatosplenomegaly, the percentage of OKT8⁺ cells was significantly reduced compared with that of controls. The ratio of OKT4 helper to OKT8 suppressor cells was significantly increased to twice the ratio of infected subjects without hepatosplenomegaly ($P < 0.001$). Contributing to this difference in T-cell ratio between the two groups of infected subjects were the differences in percentages of OKT4⁺ and OKT8⁺ positive cells ($P < 0.05$ and $P < 0.01$, respectively). Five of six subjects with hepatosplenomegaly had a ratio of OKT4 helper to OKT8 suppressor cells that fell outside the 95% confidence interval for control subjects (ratios = 2.27, 3.33, 3.03, 3.23, and 2.56), whereas the T-cell ratio for one subject (1.45) fell within the limits of the control group.

Functional studies of [³H]thymidine incorporation. SWAP- and SKSD-stimulated DNA synthesis by cultured PBMC was similar in infected subjects without and with hepatosplenomegaly (Fig. 1). In infected subjects without hepatosplenomegaly, adherent-cell depletion resulted in a 2.5-fold increment in both SWAP- and SKSD-induced blastogenesis. Immunofluorescence studies before and after nylon-wool column elution did not show a significant alteration in T-cell

ratios (data not shown). In contrast, in subjects with hepatosplenomegaly, removal of adherent cells did not change the T-lymphocyte responses. This indicated adherent suppressor cell activity in infected subjects without hepatosplenomegaly that was absent in subjects with hepatosplenomegaly.

The absolute T-cell blastogenic responses to SWAP and SKSD were significantly less in subjects with than without hepatosplenomegaly. The differences in T-cell response between these groups cannot be explained by the different number of OKT8 suppressor cells, because subjects without hepatosplenomegaly had greater T-cell responses and a greater number of OKT8 cells than did subjects with hepatosplenomegaly; rather, OKT8 suppressor phenotype and T-cell blastogenic responses were dissociable in *S. mansoni* infection.

OKT8 depletion studies were performed to further examine the role of OKT8 cells in the regulation of blastogenesis. Depletion of OKT8⁺ cells from the PBMC of eight *S. mansoni*-infected subjects without hepatosplenomegaly produced numbers of OKT8 approximating those in the hepatosplenomegaly group but did not significantly change the blastogenic responses to either SWAP or SKSD (Fig. 2).

Evaluation of adherent-cell populations. The percentages of monocytes in PBMC determined by the criterion of esterase

FIG. 1. Antigen-induced [³H]thymidine incorporation in PBMC and adherent depleted T cells from *S. mansoni*-infected patients with ($n = 9$) and without ($n = 32$) hepatosplenomegaly. Data are expressed as means $\pm$ standard errors of the mean. The increment in [³H]thymidine incorporation with adherent cell depletion is significant only in individuals without hepatosplenomegaly for both SWAP and SKSD ($P < 0.001$ by paired t test). The T-cell responses to both SWAP and SKSD in individuals with hepatosplenomegaly were less than the responses in subjects without hepatosplenomegaly ($P < 0.02$ and $P < 0.05$, respectively).

staining (19) were $15 \pm 2\%$ in subjects without and $12 \pm 3\%$ with hepatosplenomegaly ($P =$ not significant). Plastic-adherent monocytes were $66 \pm 5\%$ monocytes by the staining criterion and were similar in both groups. Indirect immunofluorescence microscopy of the plastic-adherent cells did not reveal differences in percent HLA-DR positivity ($59 \pm 3\%$ without versus $67 \pm 8\%$ with hepatosplenomegaly; P not significant) or in the percent of cells staining with OKM1 (mean, 88% in both groups).

DISCUSSION

The basis for development of hepatosplenomegaly in only a small proportion of individuals infected with *S. mansoni* is not understood. Epidemiologic studies suggest that host factors which may be genetically determined predispose subjects to hepatosplenomegaly in *S. mansoni* infection (2, 4, 28). Experimental models support the notion of genetic differences in the morbidity of chronic schistosomiasis mansoni (15). Moreover, in chronic murine infections, suppressor T cells ($\text{Lyt}2^+ \text{Ia}^+$) modulate granulomatous hypersensitivity (5, 11), possibly through a soluble mediator (14), and limit morbidity (6). These considerations suggest that abnormalities in the regulation of cellular immunity may be important in the immunopathogenesis of human hepatosplenic disease.

Most previous studies, including one by our group (13), of the immune response in hepatosplenomegaly patients with *S. mansoni* infection have included hospitalized patients with advanced disease complicated by esophageal varices, gastrointestinal bleeding, malnutrition, and anemia. Because the effect of complications of advanced disease on the immune response is unpredictable, interpreting these studies is difficult. Furthermore, patients with advanced hepatosplenic disease are few, and despite advanced disease, the etiology of the hepatosplenomegaly is difficult to establish securely. In an effort to avoid some of these potential pitfalls, we embarked on a community-based study of asymptomatic subjects with uncomplicated hepatosplenomegaly. Hepatosplenomegaly in our subjects was attributed to *S. mansoni* infection on the basis of the presence of *S. mansoni* infection, the absence of hepatosplenomegaly in uninfected village controls, and, when possible by noninvasive means (history, physical exam, complete blood count, and hepatitis B surface antigen screening), exclusion of other known causes of hepatosplenomegaly in Egypt. These other factors include hepatitis B infection, malaria, visceral leishmaniasis, malnutrition, malignancies, and alcoholic liver disease (extremely rare in this Muslim population). We felt that wedge liver biopsy for more definitive diagnosis of the etiology of hepatosplenomegaly was not justifiable.

We examined immunoregulatory T-cell subsets and the effect of adherent-cell depletion on T-lymphocyte blastogenic responses in two groups with severe *S. mansoni* infection, one without and the other with hepatosplenomegaly. *S. mansoni* infection without hepatosplenomegaly was associated with a decreased ratio of OKT4 helper to OKT8 suppressor cells, adherent suppressor cell activity, and increased T-cell blastogenic responses after adherent cell depletion. In a study of *S. mansoni* infection in Brazil, Colley et al. (9) could not demonstrate quantitative shifts in T-cell subpopulation ratios in a group of *S. mansoni*-infected individuals without hepatosplenomegaly. The difference in findings may represent differences in intensity of infection between the two populations studied, differences between Egypt and Brazil in host response to infection, or geographic

FIG. 2. Antigen-induced [^3H]thymidine incorporation by PBMC and PBMC depleted of OKT8 $^+$ cells (PBMC-T8) from *S. mansoni*-infected subjects. Lines connect responses in PBMC before and after antibody- and complement-dependent lysis of OKT8 $^+$ cells in eight infected subjects without hepatosplenomegaly. The horizontal line indicates the mean of each group.

differences in the pathogen itself. Perturbations in the helper-to-suppressor ratio similar to that found in *S. mansoni* infection without hepatosplenomegaly have been demonstrated in other chronic infections such as lymphatic filariasis (30) and leprosy (21, 38). In our study, OKT8 $^+$ cell depletion did not significantly affect PBMC responses in subjects with severe *S. mansoni* infection. Rather, the sole contributor to depressed blastogenic responses identified in serious *S. mansoni* infection was the adherent suppressor cell. The identity of the adherent suppressor cell is uncertain. It could be either an adherent T lymphocyte or, more likely, a monocyte, as in the case of the adherent suppressor cell in tuberculosis (M. E. Kleinhenz and J. J. Ellner, Clin. Res. 31:368A, 1983).

In a previous study of blastogenic responses to SWAP (22), we found that individuals with severe infection but no hepatosplenomegaly fell into one of two groups on the basis of monocyte effector studies. One group had adherent suppressor monocytes that were activated by the criteria of increased killing of schistosomula and adherence to plastic. A second group lacked adherent suppressor cells and did not demonstrate evidence of monocyte activation. In the current study, we did not examine monocyte effector functions and did not attempt to stratify subjects by the presence or absence of activated monocytes and adherent suppressor cells; presumably, most subjects expressed this regulatory mechanism. In view of the current finding, longitudinal studies would be of interest to determine whether the absence of adherent cell suppression in a subject with severe infection, in fact, constitutes a risk factor for the development of hepatosplenomegaly.

S. mansoni infection with hepatosplenomegaly was characterized by an increased ratio of OKT4 helper to OKT8 suppressor cells, the absence of adherent suppressor cell activity, and diminished T-cell blastogenic responses. Colley et al. (9) examined T-cell subpopulations in the blood of patients with advanced hepatosplenic disease and found a decreased ratio of helper to suppressor cells. The major factor contributing to the difference in T-cell ratios between their study and our own may be the difference in evolutionary stage of hepatosplenomegaly. Early in the development of hepatosplenic disease, OKT8 suppressor cells may be

decreased; the increase apparent by end stage disease could reflect the course of the parasitic infection or associated complications.

The numbers in the circulating OKT8⁺ lymphocyte subpopulation contrasted with the depressed lymphocyte blastogenic responses observed in our subjects with hepatosplenomegaly. Depressed T-cell responses in hepatosplenic subjects may be due to a dearth of, or defect in, circulating antigen-responsive cells. Another possibility is a nonadherent OKT8⁻ suppressor cell, perhaps the circulatory counterpart to the suppressor T lymphocyte identified in the spleens of patients with hepatosplenic schistosomiasis (12). Recent studies of human T-cell subsets have identified a suppressor cell within the activated OKT4⁺ population that is radiosensitive and reactive with the monoclonal antibody OKT17 (22). Furthermore, cells from patients with virus-induced T-cell leukemia and lymphoma express OKT4 surface antigen yet function as suppressor cells (3, 35). An abnormality in monocyte accessory function is yet another possible explanation for decreased lymphocyte blastogenic responses in hepatosplenic subjects, and one which requires more study.

This is the first demonstration of the absence of immunosuppressive mechanisms in a severely infected group with *S. mansoni* infection and hepatosplenomegaly. Unlike the situation in human lymphatic filariasis and leprosy, in which depressed immune responses may predispose subjects to more severe disease, immunosuppression in *S. mansoni* infection may play a beneficial role by decreasing the host response to parasite antigens, thereby minimizing host tissue destruction and its sequelae. In schistosomiasis mansoni, therefore, lack of an immunosuppressive T-cell profile and adherent suppressor cells may be intrinsic to the immunopathogenesis of hepatosplenomegaly.

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