INFECTION AND IMMUNITY, Apr. 1976, p. 1176-1183

Vol. 13, No. 4
Printed in U.S.A.

Suppressive Effect of a Chronic Helminth Infection, Schistosomiasis Mansoni, on the In Vitro Responses of Spleen and Lymph Node Cells to the T Cell Mitogens Phytohemagglutinin and Concanavalin A

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Received for publication December 1975

Chronic murine schistosomiasis mansoni is associated with depressed cell-mediated immune responses to *Schistosoma mansoni* egg antigens. The present study has examined the possibility that factors develop during infection that are capable of altering the response of lymphocytes to stimuli other than specific schistosomal antigens. Egg production begins at 5 weeks, and 1 to 3 weeks later there is a moderate degree of unresponsiveness of lymph node and spleen cells to the mitogens concanavalin A and phytohemagglutinin. This was associated with an altered dose response curve to the mitogens similar to that observed in antigenic systems. Seven weeks after the initiation of antigenic stimulation (egg production) lymph node and spleen cells from chronically infected animals were profoundly unresponsive to all concentrations of concanavalin A and phytohemagglutinin tested. These investigations suggest that, in addition to possible blockade by serum antibody, other suppressive factors may be involved in the spontaneous modulation of immunopathology in chronic schistosomiasis. These are detectable 1 to 3 weeks after the onset of egg production and are prominent at 12 weeks. Such findings are consistent with, but do not prove, the existence of suppressor T cells in chronic schistosomiasis.

Mice with chronic light *Schistosoma mansoni* infections undergo a spontaneous diminution of cell-mediated granulomatous lesions around *S. mansoni* eggs and amelioration of hepatosplenic disease (1, 8, 16). Along with this decrease in granulomatous hypersensitivity, on stimulation with soluble egg antigens there is a decrease in delayed footpad swelling and production of the lymphokines, macrophage migration inhibitory factor, and eosinophil stimulation promoter (5, 6). Furthermore, rising antibody titers occur concomitantly with the declining cell-mediated immunity (CMI) and granulomatous hypersensitivity (5, 6). The suppression of CMI in the presence of an increasing humoral immunological response reveals that central immunological tolerance (unresponsiveness of both CMI and humoral immunological response) is not responsible for the modulation of this cell-mediated disease; this phenomenon is, therefore, similar to split tolerance or immune deviation (2). Whether the suppression of CMI is due to antibody and immune complexes (11) or to suppressor T cell function (14) is not as yet clear. Antibody may act peripherally in a form of immunological blockade to prevent the reaction of antigen with effector cells for CMI, resulting in lack of reponse to a specific antigenic stimulus. Alternatively, suppressor T cells may act centrally to prevent the production or function of cell-mediated immune effector cells, causing a generalized lack of response to both antigens and mitogens. Since the inhibition of the cellular immune reponse to specific stimulation by soluble egg antigen has already been demonstrated (5, 6), the present study investigates the in vitro responses of lymph node and spleen cells to nonspecific mitogens (concanavalin A [Con A] and phytohemagglutinin [PHA]) at various time periods after infection.

MATERIALS AND METHODS

Swiss albino female mice, 18 to 20 g in weight (Flow Laboratories, Dublin, Va.), were infected with 10 cercariae of *S. mansoni* by subcutaneous injection (5). At various time intervals thereafter, the mesenteric lymph node and spleen were removed aseptically from each animal and placed individually in 43-mm culture dishes containing 5 ml of Hanks balanced salt solution with 100 U of penicillin per ml and 100 μg of streptomycin per ml (Flow Laboratories, Rockville, Md.). The organs were each

teased apart, aspirated 25 times into a 1-ml syringe through a 19-gauge needle, and passed through one layer of 4-ply gauze. The volume was adjusted to 10 ml with Hanks balanced salt solution, and the suspension was centrifuged at 1,000 rpm for 10 min at room temperature. The supernatants were discarded and the cells were washed twice with 10 ml of Hanks balanced salt solution. The pellet was resuspended in 5 ml of RPMI 1640 with 100 U of penicillin and 100 μg streptomycin per ml, 0.01 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer, and 5% heat-inactivated fetal bovine serum (complete RPMI). Cell suspension (10 μl) was diluted in 10 ml of Isoton solution with 3 drops of Zap-Isoton and counted in a model Z Coulter counter (Coulter Diagnostics, Inc., Hialeah, Fla.) to give the total number of leukocytes per milliliter. The cell suspension was diluted in complete RPMI 1640 to give a final cell concentration of 2.5×10^6 cells/ml. Spleen cell cultures were also prepared from uninfected control mice age-matched to the 8-week infected mice.

Con A was obtained from Pharmacia Fine Chemicals, Piscataway, N.J., and PHA was obtained from Difco Laboratories, Detroit, Mich. Various concentrations of Con A (0.1 to 30 μg/well) or PHA (0.01 to 1.0 μl of undiluted stock solution per well) were added in triplicate in 10-μl amounts to individual wells in tissue culture plates (MicroTest II, Falcon Plastics, Division of Becton-Dickinson & Co., Oxnard, Calif.). A 0.2-ml portion of 2.5×10^6 cells/ml (0.5×10^6 cells) was then added to each well, and the plate was sealed with pressure-sensitive film (Falcon Plastics) and incubated at 37 C for 60 h. Then 0.5 μCi of [^{3}H]thymidine (10 μl of 50 μCi of [methyl-^{3}H]thymidine per ml; Searle Analytics, Inc., Des Plaines, Ill.) was added to each well. Twelve hours later (at 72 h) the cultures were harvested on glass-fiber filter paper (Reeve Angel, Clifton, N.J.) and the cell pellets were washed 20 times with normal saline by a MASH cell harvester (Micro Harvester, Otto Hiller Co., Madison, Wis.). The filter papers were dried at 65 C for 2 h, and each was placed in a 5-ml glass scintillation vial and 2 ml of complete counting cocktail (no. 3a40, Research Products International Corp., Elk Grove Village, Ill.) was added. Radioactivity was determined by counting in an Isocap/300 counter (Searle Analytics, Inc., Des Plaines, Ill.). Data were analyzed on a Wang 600 programmable calculator with a built-in statistical module (ROM).

RESULTS

A major antigenic stimulus in schistosomiasis mansoni is the large number of eggs produced daily by the adult worm pairs, beginning 5 weeks after infection and continuing for many years in humans and for the complete life span of mice. Soon after the onset of egg laying, immunological stimulation is manifested by increased synthesis of deoxyribonucleic acid (DNA) in the draining lymphoid organs (Fig. 1). Table 1 demonstrates that 7 weeks after

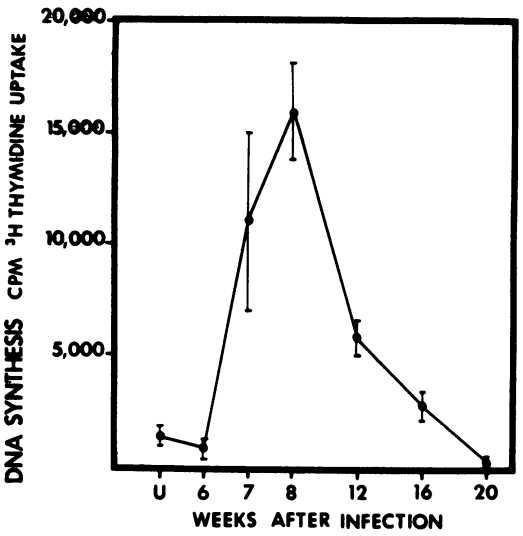

FIG. 1. *Elevation of baseline DNA synthesis of lymph node cells at various times after infection with 10 cercariae of S. mansoni. Results expressed are the mean counts per minute (CPM) ± standard error of the mean of cultures from 7 to 18 animals incubated in the absence of mitogens or any exogenous antigen. This figure is a graphic representation of the data in the upper part of Table 1.*

infection cells from the mesenteric lymph node maintained in in vitro culture in the absence of exogenous stimuli synthesized eight times as much DNA ("baseline" DNA) as did those from uninfected control animals. Figure 1 shows that this elevation of baseline DNA synthesis reached a maximum 8 weeks after infection and subsequently declined. The elevation observed in spleen cells was not as pronounced as that in lymph node cells. By 20 weeks of infection, however, both spleen and lymph node cells synthesized significantly less DNA in the absence of exogenous stimulation than did normal cells (Tables 1 and 2). Thus studies of baseline DNA synthesis with these organs appear to reflect initial stimulation of the immune system followed by subsequent suppression.

The suppression was studied by determining the capacity of T lymphocytes to respond to stimuli other than specific antigen. At periodic intervals after infection, mesenteric lymph node and spleen cells were cultured with varying doses of the T cell mitogens PHA and Con A and DNA synthesis was determined. In Tables 1 and 2 the results of these studies are expressed both as stimulation index and as counts per minute at the optimal mitogen concentrations. Six weeks after infection there was no significant change in the total DNA synthetic response of lymph node cells to Con A or of

TABLE 1. *Suppression of response of mesenteric lymph node cells to optimal concentrations of mitogens during chronic S. mansoni infection*

Determination	Uninfected controls	Infected cells					
		6 weeks	7 weeks	8 weeks	12 weeks	16 weeks	20 weeks
Baseline DNA synthesis							
CPM (mean ± SEM[a]) cultures with no mitogen	1,389 ± 424	717 ± 395	11,008 ± 3,928	16,033 ± 2,248	5,790 ± 812	2,769 ± 692	280 ± 43
Number of animals	18	8	7	8	8	7	15
Infected/control ratio (significance)[b]		0.51 (NS)	7.9 ($P < 0.005$)	11.5 ($P < 0.001$)	4.17 ($P < 0.01$)	2.00 (NS)	0.20 (decrease significant $P < 0.05$)
Response to optimal doses of Con A							
Counts/min (mean ± SEM)							
0.3 μg of Con A	41,069 ± 7,481	33,921 ± 16,200	15,811 ± 2,562	21,015 ± 7,551	1,658 ± 1,254	9,625 ± 1,618	1,223 ± 414
1.0 μg of Con A	38,610 ± 4,927	36,591 ± 13,366	8,588 ± 2,511	33,532 ± 4,654	2,629 ± 1,017	1,888 ± 784	2,082 ± 892
Peak stimulation index (significance)[c]	30.00 ($P < 0.001$)	55.00 ($P < 0.05$)	1.43 (NS)	2.09 ($P < 0.01$)	0.45 (NS)	3.47 ($P < 0.01$)	7.43 ($P < 0.05$)
Peak suppression compared to uninfected control (significance)[d]		17% (NS)	78% ($P < 0.01$)	49% (NS)	96% ($P < 0.005$)	95% ($P < 0.001$)	97% ($P < 0.001$)
Response to optimal doses of PHA							
Counts/min (mean ± SEM)							
0.3 μl of PHA	36,233 ± 6,875	Not determined	28,452 ± 9,628	15,996 ± 2,414	5,108 ± 1,830	5,766 ± 1,130	1,457 ± 655
0.10 μl of PHA	34,932 ± 6,830		24,050 ± 8,815	9,937 ± 1,539	6,038 ± 2,625	7,043 ± 1,634	1,365 ± 479
Peak stimulation index (significance)	26.00 ($P < 0.001$)		2.57 (NS)	1.59 (NS)	1.04 (NS)	2.52 (NS)	5.2 ($P < 0.05$)
Peak suppression compared to uninfected control (significance)			31% (NS)	71% ($P < 0.01$)	86% ($P < 0.005$)	84% ($P < 0.025$)	96% ($P < 0.001$)

[a] SEM, Standard error of the mean.

[b] Infected/control ratio comparing infected with control uninfected animals was calculated as: ratio = (counts/min in cultures without mitogen of cells from infected mice)/(counts/min in cultures without mitogen of cells from uninfected control mice). Significance was determined by comparing cultures from infected and control animals by Student's t test; P values greater than 0.05 were considered not significant (NS).

[c] Stimulation index at each time period was calculated as: index = (counts/min of cultures at optimum mitogen dose)/(counts/min of cultures without mitogen). Significance was determined by comparing at each time period the baseline DNA synthesis with DNA at the optimal mitogen dose by Student's t test.

[d] Peak suppression at a given mitogen concentration was calculated as: percent suppression = {1 − [(counts/min in cultures of infected mice)/(counts/min in cultures of uninfected control mice)]} × 100. In this table the percent suppression expressed is for the greater of the two mitogen concentrations. 0% suppression indicates no suppression and 100% suppression indicates complete suppression.

TABLE 2. *Suppression of response of spleen cells to optimal concentrations of mitogens during chronic S. mansoni infection*[a]

Determination	Uninfected controls	Infected cells					
		6 weeks	7 weeks	8 weeks	12 weeks	16 weeks	20 weeks
Baseline DNA synthesis							
Counts/min (mean ± SEM) cultures with no mitogen	7,778 ± 1,493	10,857 ± 3,323	7,639 ± 1,899	11,944 ± 1,576	3,425 ± 284	6,182 ± 611	1,836 ± 349
Number of animals	12	8	7	12	8	7	14
Infected/control ratio (significance)		1.39 (NS)	1.00 (NS)	1.53 (NS)	0.44 (decrease significant $P < 0.05$)	0.80 (NS)	0.24 (decrease significant $P < 0.001$)
Response to optimal doses of Con A							
Counts/min (mean ± SEM)							
0.3 μg of Con A	48,168 ± 6,280	39,952 ± 11,338	11,157 ± 2,122	20,863 ± 3,522	3,703 ± 493	7,964 ± 1,557	2,290 ± 430
1.0 μg of Con A	49,755 ± 6,517	31,902 ± 9,101	9,729 ± 1,832	33,726 ± 2,487	4,656 ± 490	9,706 ± 2,635	3,261 ± 656
Peak stimulation index (significance)	6.40 ($P < 0.001$)	3.67 ($P < 0.02$)	1.55 (NS)	2.82 ($P < 0.001$)	1.36 (NS)	1.57 (NS)	1.77 (NS)
Peak suppression compared to uninfected control (significance)		25% (NS)	80% ($P < 0.001$)	57% ($P < 0.005$)	92% ($P < 0.001$)	83% ($P < 0.001$)	97% ($P < 0.001$)
Response to optimal doses of PHA							
Counts/min (mean ± SEM)							
0.03 μl of PHA	29,829 ± 5,336	25,269 ± 7,855	16,404 ± 3,535	17,700 ± 4,092	6,423 ± 956	7,207 ± 1,087	3,483 ± 649
0.10 μl of PHA	38,091 ± 6,117	10,644 ± 3,815	15,113 ± 4,670	20,167 ± 6,449	6,789 ± 1,464	7,364 ± 997	2,972 ± 460
Peak stimulation index (significance)	3.83 ($P < 0.001$)	2.32 (NS)	2.12 (NS)	1.68 (NS)	1.87 ($P < 0.025$)	1.19 (NS)	1.89 ($P < 0.05$)
Peak suppression compared to uninfected control (significance)		72% (NS)	60% ($P < 0.025$)	48% (NS)	82% ($P < 0.005$)	81% ($P < 0.01$)	92% ($P < 0.001$)

[a] The infected/control ratios, stimualtion index, and peak suppression were calculated in the same manner as in Table 1. Abbreviations are given in Table 1.

spleen cells to optimal doses of PHA or Con A. (Note: experiments with PHA and lymph node cells were not conducted at 6 weeks.)

Inhibition of DNA synthesis at optimal mitogen doses was first observed 7 weeks after infection (Fig. 2). At this time the responses of lymph node cells to Con A (Table 1) and that of spleen cells to PHA and Con A (Table 2) were 50% lower than control responses. A similar picture was also observed 8 weeks after infection. The progressive rise in baseline DNA synthesis resulted, however, in a spurious decline in the stimulation index. For example, 8 weeks after infection the stimulation index of Con A-incubated lymph node cells was 2.09 (Table 1), a suppression of 93%. Actually these cultures synthesized 33,500 counts/min of DNA (Table 1), which was only 14% less than mitogen-stimulated synthesis in control cultures. Because of a marked increase in baseline DNA synthesis, therefore, there was an apparent but not real decrease in mitogen response when stimulation index alone was used to measure lymphocyte reactivity.

Twelve weeks after infection, all parameters studied (baseline DNA synthesis as well as mitogen stimulation) were profoundly suppressed (Tables 1 and 2). Total DNA synthesis in cultures incubated with optimal concentrations of mitogens was only 12% of that observed in control cultures (Tables 1 and 2). This degree of suppression continued as long as the study persisted (20 weeks). Constantly declining baseline DNA synthesis resulted in a paradoxical rise in stimulation index in the face of increasing suppression (Tables 1 and 2). Thus, at 20 weeks, when the DNA synthetic response to T cell mitogens was suppressed 88%, the average stimulation index to all mitogens of all organs studied was 4.07.

Early after infection (6 to 8 weeks) there was only partial (40%) suppression of response to optimal mitogen dose in comparison with the profound (89%) unresponsiveness that exists 12 weeks after infection (Fig. 2). At 6 to 8 weeks, however, there was significant (77%) and consistent inhibition of the response to concentrations of mitogen (3.0 μg of Con A and 0.3 μl of PHA) just beyond the optimal dose (Fig. 3), but the net response to suboptimal doses (0.1 μg of Con A or 0.01 μl of PHA) was minimally (20%) affected. Eleven parameters were studied at four mitogen concentrations: lymph node and Con A (1. 6 weeks, 2. 7 weeks, 3. 8 weeks); spleen and Con A (4. 6 weeks, 5. 7 weeks, 6. 8 weeks); lymph node and PHA (7. 6 weeks, 8. 7 weeks, 9. 8 weeks); spleen and PHA (10. 7 weeks, 11. 8 weeks). Although some of the parameters were occasionally inhibited at low mitogen dose (e.g., lymph node-Con A at 6 weeks and spleen-Con A and PHA at 7 weeks), there was no consistent pattern of inhibition: only 3 of these 11 parameters were significantly suppressed. At supraoptimal mitogen concentrations, however, 10 out of the 11 parameters studied showed significant suppression when compared with the control values. Figure 3 graphically represents this effect for four representative parameters. This shift in dose response could not be studied later than 8 weeks after infection because DNA synthesis was too low to allow appropriate comparison (Fig. 2). Thus, the generalized suppression of lymphocyte responsiveness in chronic schistosomiasis, which begins 12 weeks after infection, is preceded by a period of qualitatively altered mitogen responsiveness.

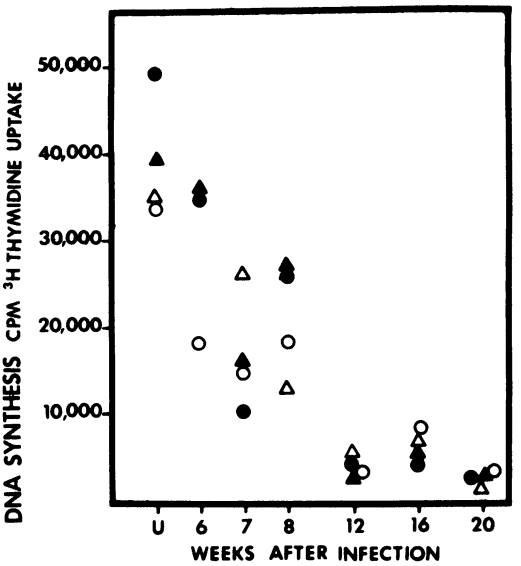

FIG. 2. *Progressive decline in total DNA synthesis in mitogen-stimulated cultures with increasing time after S. mansoni infection. This figure is a graphic summation of the data from the lower portions of Tables 1 and 2. Each point represents the mean DNA synthesis at the optimal mitogen concentration of an experiment on at least seven mice. Open points are the results of cultures with PHA; closed points are those of cultures with Con A. Circles are the results of cultures of lymph node cells; triangles are those of cultures of spleen cells.*

DISCUSSION

Mice respond to the onset of egg laying in *S. mansoni* infections by a wave of immunological stimulation manifested as increased DNA synthesis in the lymph nodes draining the site of

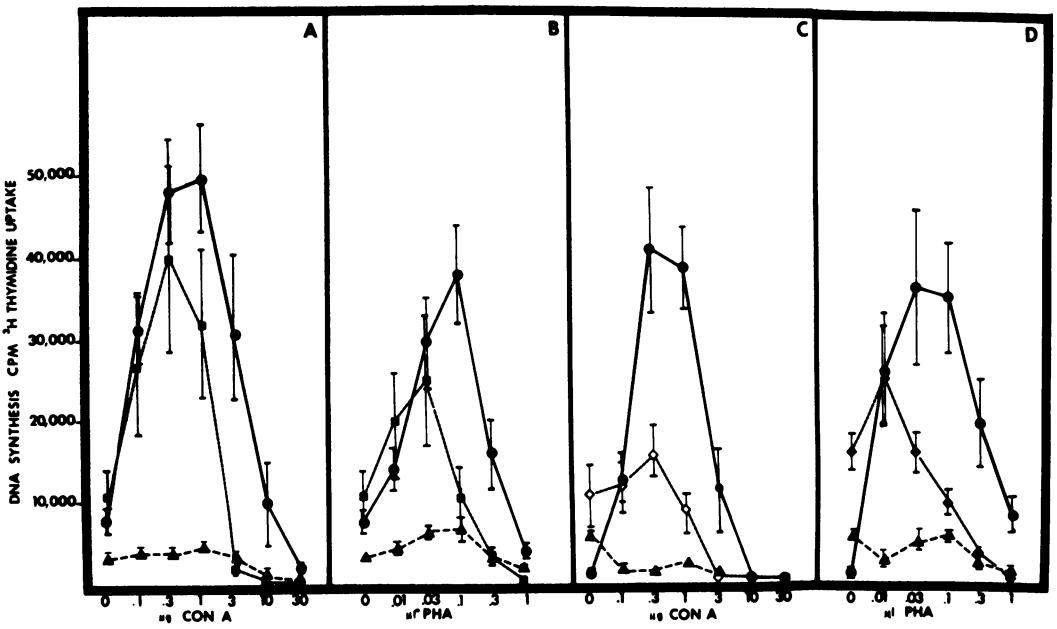

FIG. 3. *Representative alterations in dose response curves to Con A and PHA during chronic schistosomiasis. Data shown are the mean counts per minute (CPM) ± standard error of the mean for cultures of spleen cells (A and B) and lymph node cells (C and D). Results of cultures from control animals are indicated (●: A, B, C, and D), cultures 6 weeks after infection (■: A and B), 7 weeks after infection (◇: C), 8 weeks after infection (◆: D), and 12 weeks after infection (▲: A, B, C, and D). Results are the means of the same cultures on 7 to 18 animals from which the data in Tables 1 and 2 were selected.*

infection (Fig. 1). During this period granulomatous hypersensitivity toward schistosome egg antigens (a form of CMI) appears, which results in immunopathology (17). Concomitant with the waning of the increased DNA synthesis, granulomatous hypersensitivity decreases (5, 6) and the hepatosplenic disease undergoes amelioration (1, 8, 16). This is accompanied by decreased cellular reactivity to schistosome egg antigens and increased amounts of specific circulating antibody (5, 7).

The present study suggests that negative immunoregulatory factors appear early during the course of schistosome infection and become stronger as infection progresses. Immunoregulatory effects are first noted within 1 to 2 weeks after the onset of antigenic stimulation by schistosome eggs 6 to 8 weeks after infection. They are manifested as a moderate but inconsistent inhibition of the DNA synthetic response to optimal concentrations of the T cell mitogens Con A and PHA. In addition, there is a profound qualitative alteration of T cell function manifested by an altered mitogen dose-response curve. Thus, at the time when CMI is maximal and before the appearance of large amounts of anti-egg antibody (5, 6), modulating influences were detected. Our data on lymphocyte activity 6 to 8 weeks after infection are remarkably similar to the data of Gershon et al. (10) and Bash and Waksman (4) on murine suppressor cell activity after the single injection of various antigens. These investigators found an equivalent degree of suppression of the response of spleen cells to optimal doses of PHA after injection of mice or rats with ovalbumin, bovine serum albumin, bovine gamma globulin, and sheep erythrocytes. Particularly striking was the finding in both of these studies that the strongest suppression was seen when doses of mitogen just beyond the optimum were used. Further characterization of this phenomenon demonstrated the role of suppressor T cells in mediating this inhibition of mitogen responsiveness (4). Thus the present study has shown that 1 to 3 weeks after the onset of egg laying (antigen production) by the schistosomes there was a moderate degree of suppression of lymphocyte reactivity comparable to that observed 1 to 7 days after injection of a single dose of antigen (4, 10).

In mice with chronic schistosomiasis continued antigenic stimulation of the immune system resulted in the development of profound unresponsiveness. Thus, 7 weeks after the onset of egg laying, lymph node cells and spleen

cells became completely unresponsive to all concentrations of PHA and Con A (Tables 1 and 2). This is the time at which alteration of other immunological parameters was first seen (Table 3). Specific granulomatous hypersensitivity and delayed cutaneous reactivity were both specifically depressed in comparison with the peak responses occurring 3 weeks after the onset of egg laying (5, 6). At 12 to 16 weeks after infection, antigen-stimulated lymphokine production by spleen cells displayed an altered dose-response curve (5) similar to that described with mitogen in this paper. That is, the peak in vitro cellular immunological response occurred with constantly decreasing doses of antigen and supraoptimal doses of antigen were increasingly suppressive. The data in this study demonstrate that at 12 to 16 weeks after infection the response to agents other than specific antigen is inhibited. Twenty weeks after infection all parameters of CMI (granulomatous hypersensitivity, delayed footpad swelling, and migration inhibitory factor production) were suppressed (5, 6) and mitogen-incubated lymphocytes produced only 4% of the DNA of normal cells (Tables 1 and 2).

Altered reactivity to immunologically unrelated antigens has been observed in a wide variety of viral, bacterial (for review, see 15), and parasitic infections (9, 12, 13). The antigen most frequently used in these studies has been the sheep erythrocyte. In previous studies we did not observe diminished reactivity to challenge injections of purified protein derivative of *Mycobacterium tuberculosis* in mice concomitantly immunized with complete Freund adjuvant. Our failure to observe differences in that system may be due to (i) the ability of a strong immunogen-like *Mycobacteria* to overcome suppression and (ii) the lack of precise methodology to quantitate responses to this stimulus in vitro. In the present study using sensitive in vitro techniques, we are able to document generalized hyporeactivity of the lymphoid system.

Suppressor T cell activity is the most likely explanation for this depression of mitogen reactivity that develops during chronic schistosomiasis. Our findings are consistent with the effects observed in the classical studies on suppressor T cell activation after immunization, that is, a rise in baseline DNA synthesis followed by partial suppression of mitogen reactivity and a shift in the dose-response curve to mitogens. The mitogen suppressive activity began just prior to the spontaneous modulation of CMI in chronic infection. Although this study does not prove the existence of schistosome egg antigen-specific suppressor T cells, the absence of in vitro cell-mediated reactivity to specific antigen (5, 6) coupled with the loss of mitogen reactivity in the face of rising antibody titers is not a characteristic of antibody-mediated immunological blockade but is consistent with the newer interpretation of the mechanism of immune deviation (3), which envisions control of CMI by suppressor T cells. Further investigations will obviously be needed to resolve the relative roles of suppressor T cells and antibody in the spontaneous modulation of hepatosplenic schistosomiasis mansoni.

ACKNOWLEDGMENTS

This project was supported by a grant from the Edna McConnell Clark Foundation and by Public Health Service grant AI08163 from the National Institute of Allergy and Infectious Diseases.

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TABLE 3. *Immune reactivity during chronic schistosomiasis*

Activity	Unifected	Infected		
		8 weeks	12–16 weeks	20 weeks
CMI				
In vivo[a]	Absent	Maximal	Diminished	Suppressed
In vitro[b]	Absent	Maximal	Diminished[c]	Absent
Humoral immunity	Absent	Low	Rising	Maximal
Mitogen reactivity	Maximal	Diminished[d]	Suppressed	Suppressed

[a] Delayed cutaneous hypersensitivity and enhanced granulomatous hypersensitivity.
[b] Antigen-stimulated migration inhibitory factor production by spleen cells.
[c] Altered dose-response curve (increasing avidity, increasing supraoptimal inhibition).
[d] Altered dose-response curve.

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