INFECTION AND IMMUNITY, Mar. 1979, p. 893-902
0019-9567/79/03-0893/10$02.00/0

Vol. 23, No. 3

Immunoregulatory Responses in Experimental Disseminated Histoplasmosis: Depression of T-Cell-Dependent and T-Effector Responses by Activation of Splenic Suppressor Cells

RONALD P. ARTZ AND WARD E. BULLOCK*

The Division of Infectious Diseases, Department of Medicine, University of Kentucky College of Medicine, Lexington, Kentucky 40506

Received for publication 27 October 1978

The cellular immune responses of mice with disseminated histoplasmosis are markedly diminished in association with the generation of potent immunosuppressor activity by spleen cells. The zenith of suppressor activity was observed during most active infection, from 1 to 3 weeks after inoculation. During this time there was: (i) depression of the delayed-type hypersensitivity response to sheep erythrocytes and histoplasmin, (ii) impairment of concanavalin A- and histoplasmin-induced blastogenic transformations by splenocytes in vitro, (iii) depressed cytotoxic activity of spleen cells from infected mice, and (iv) marked suppression by splenocytes from infected mice of the primary antibody response to sheep erythrocytes by normal spleen cell cultures. With resolution of the infection by week 8, there was a shift of immunoregulatory function from dominant suppressor activity to expression of helper activity. At this time, delayed-type hypersensitivity responses to the above antigens were vigorous; furthermore, the cytotoxic activity and plaque-forming cell response of splenocytes from 8-week-infected mice were equal to or greater than normal control values. The shift in the immunoregulatory response from a suppressor to a helper mode indicated that the net amount of help or suppression measured at any given time during infection represented the algebraic sum of both helper and suppressor activities mediated by different populations or subpopulations of cells within the splenic microenvironment of infected mice.

Disseminated infection with the biphasic yeast *Histoplasma capsulatum* is a life-threatening disease in which the mortality rate exceeds 80% among untreated cases (28). The prognosis of patients with systemic histoplasmosis has improved considerably since the advent of amphotericin B therapy; however, mortality rates remain disturbingly high, ranging from 7 to 20% in published clinical studies (20, 22). Furthermore, relapses after completion of "adequate" therapy are not uncommon.

There is clear evidence that compromise of the cell-mediated immune defenses, as in individuals receiving immunosuppressive drugs, can predispose to dissemination of *H. capsulatum*, presumably from foci of latent infection within the pulmonary parenchyma or thoracic lymph nodes (8, 17). On the other hand, very little is known of the immunoregulatory perturbations which may be induced by disseminated histoplasmosis itself in individuals without obvious defects of cell-mediated immunity before the onset of infection. That complex disturbances of immunoregulation do occur as a consequence of systemic histoplasmosis is suggested by the high prevalence of skin test anergy to histoplasmin among patients with this form of infection (20, 22, 28) and poor lymphocyte blastogenic responses to stimulation by *Histoplasma* antigens and mitogens (1, 19, 24). In addition, there is preliminary evidence to suggest that the poor response of blood T cells to antigens or mitogens in some cases may be caused, at least in part, by the presence of a T cell subpopulation capable of suppressing other potentially reactive T lymphocytes (24).

In an effort to better understand the complexities of disordered immune function in humans with disseminated histoplasmosis, our laboratory has used a murine model to study the immunoregulatory disturbances associated with this type of infection. The serological responses and histopathology of primary and secondary lymphoid organs in mice with disseminated histoplasmosis are detailed in a companion report (2). In this communication, we demonstrate the evolution of potent immunosuppressor cell activity within the spleens of infected mice. During

the period of most intense suppressor cell activity, from 1 to 3 weeks after inoculation, the delayed-type hypersensitivity (DTH) response to *Histoplasma* antigens was depressed, as was the response to intradermal (i.d.) challenge with sheep erythrocytes (SRBC) 5 days after sensitization of infected mice with SRBC. As animals recovered from infection by week 8, the suppressor activity of spleen cells from infected mice shifted to a helper mode, and previously weak skin reactivity to *Histoplasma* antigens and SRBC became vigorous.

MATERIALS AND METHODS

Animals. Male C3H/Anf and B6D2F$_1$ (C57BL/6 × DBA/2) mice were obtained from Cumberland View Farms, Clinton, Tenn. Male C3D2F$_1$ (C3H/HeJ × DBA/2) mice were purchased from Jackson Laboratories, Bar Harbor, Maine. All mice were housed under controlled conditions of humidity, temperature, and light (standardized 13-h cycle). Food and acidified and chlorinated water were provided ad libitum.

Infection with *H. capsulatum*. C3H/Anf mice, 6 to 8 weeks of age, were injected intravenously with 0.2 ml of an inoculum containing from 5×10^5 to 1×10^6 yeast cells of *H. capsulatum* strain G-217B as detailed elsewhere (2). A control group of littermates was inoculated with 1×10^6 heat-killed yeast cells of *H. capsulatum*. Yeast suspensions were killed by immersion in a 60°C water bath for 60 min; nonviability was confirmed by absence of conversion to the mycelial phase on Emmons modified Sabouraud 2% dextrose agar (Difco Laboratories, Detroit, Mich.). Another group of littermates served as uninoculated controls.

Antigens and mitogen. SRBC were purchased from the Colorado Serum Company, Denver, Col. The histoplasmin preparation, HKC-43, was generously provided by Coy Smith of the University of Kentucky College of Medicine, Lexington, Ky. It was prepared from seven mycelial strains of *H. capsulatum* grown in Smith asparagine medium. Before use, HKC-43 was dialyzed against cold phosphate-buffered saline, pH 6.9, for 48 h. Protein content of the dialysate, as determined by the method of Waddell (25), was 585 μg/ml. Salt- and carbohydrate-free lyophilized concanavalin A (ConA), grade IV (Sigma Chemical Co., St. Louis, Mo.), was used in lymphocyte transformation studies.

Measurement of DTH responses. To measure DTH response to SRBC, paired groups of infected and control mice were sensitized intravenously with 0.2 ml of washed SRBC in a 0.01% suspension. Preliminary studies demonstrated that peak DTH responses to SRBC in normal mice occurred 5 days after sensitization. Therefore, 5 days later mice were challenged i.d. with 0.05 ml of a 20% SRBC suspension in the footpad of one hind paw. Footpad swelling was measured with a digital micrometer (Brown and Sharpe Co., North Kingstown, R.I.) 24 h after challenge. The DTH response was expressed as the percent increase in footpad size over that measured immediately before SRBC challenge. Mean values were determined from groups of at least six mice. The degree of banal inflammatory response to injection of the SRBC challenge dose only was measured in the footpads of nonimmunized controls from both the normal and the infected groups.

The specific DTH response to histoplasmin in paired control and infected groups also was expressed as percent increase in footpad swelling 24 h after i.d. challenge with 0.05 ml of histoplasmin, HKC-43, at a dilution of 1:10. This dilution was selected as optimal for i.d. challenge based upon a series of dose response experiments.

Cell preparation techniques. Spleen cell suspensions were prepared from pools of at least three spleens by gentle teasing between two ground glass slides in Hanks balanced salt solution. After removal of tissue debris by sedimentation for 5 min, cells were washed twice and brought to a final concentration of 10^8/ml in Hanks balanced salt solution supplemented with 5% fetal calf serum (Grand Island Biological Co. [GIBCO], Grand Island, N.Y.). A population of splenocytes enriched for T lymphocytes was prepared by the nylon fiber column method of Julius et al. (16). From 13 to 20% of total cells added to the columns were recovered, and at least 95% of eluted cells were viable as determined by trypan blue dye exclusion. Nonspecific esterase stains (27) of the effluent cells revealed less than 0.2% monocyte-macrophage contamination. B lymphocytes comprised from 3 to 5% of the effluent cells as determined by methods described previously (5). All cells were washed three times before culture and resuspended in medium containing 1% penicillin–streptomycin, 1% L-glutamine, and 100 U of nystatin (GIBCO) per ml. For studies of lymphocyte transformation, RPMI 1640 medium containing 10 mM *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid buffer (GIBCO) plus 10% fresh human serum was used. In mixed lymphocyte cultures (MLC), the RPMI medium was supplemented with 10 mM *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid, 5×10^{-5} M 2-mercaptoethanol (Eastman Kodak Co., Rochester, N.Y.) and 13% fetal calf serum. Lymphocyte cytotoxicity assays were performed in *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid-buffered RPMI 1640 medium containing 5% fetal calf serum. Eagle minimal essential medium supplemented with 1% pyruvate and 1% nonessential amino acids (Microbiological Associates, Walkersville, Md.) was used for plating of cell cultures by the method of Mishell and Dutton (18). To this medium were added 10% fetal calf serum and 5×10^{-5} M 2-mercaptoethanol.

Lymphocyte blastogenic transformation. Unfractionated spleen cells from paired groups of normal and infected mice were suspended in plating medium. To 5×10^5 cells in 0.1 ml was added 0.1 ml of a 1:25 dilution of histoplasmin (HKC-43) or 1.5 μg of ConA in Microtest II culture plates (Falcon Plastics, Oxnard, Calif). Cells were cultured in triplicate for 5 days at 37°C in humidified atmosphere of 5% CO_2. The concentrations of antigen or mitogen and the duration of culture had been established as optimal by extensive preliminary testing. Sixteen hours before cell harvest, 0.5 μCi of [^{3}H]thymidine (specific activity, 6.7 Ci/mmol) (New England Nuclear Corp. Boston, Mass.) was added to each culture. Cells were collected by an automatic sample harvester (Mash II; Microbiological Associates, Walkersville, Md.), and uptake of radio-

activity was measured in a liquid scintillation counter; results are expressed as counts per minute.

Cell-mediated lymphocytotoxicity (CML). In vitro cytotoxicity by splenocytes from infected and control mice was measured by the method of Simpson et al. (21). There are two components to the assay: (i) T effector cells are generated in a one-way MLC culture, and (ii) the cytotoxic activity of these T cells is determined by the extent to which they lyse ^{51}Cr-labeled target cells. Procedural details are as follows.

(i) MLC. Stimulator spleen cells from C3D2F$_1$ (H-$2^{k,d}$) mice were incubated with 30 μg of mitomycin C (Sigma) per ml for 45 min at 37°C. After four washes in Hanks balanced salt solution 1×10^6 stimulator cells were cultured with 3×10^6 to 6×10^6 responder splenocytes from either infected or control C3H/Anf (H-^{2k}) mice in 16-mm tissue culture plates (Linbro Scientific, Inc., Hamden, Conn.). The total volume per well was 2.3 ml. Triplicate cultures were incubated for 5 days at 37°C in humidified atmosphere of 5% CO_2.

(ii) Cytotoxic activity assay. Target cells were P815 mastocytoma cells (H-2^d) maintained by weekly intraperitoneal passage in B6D2F$_1$ mice. P815 cells thus shared H-2 histocompatibility determinants with the sensitizing cell population used in the MLC. Target cells were incubated with 100 μCi of ^{51}Cr (Specific activity, 248 to 350 μCi/mg) (New England Nuclear) per ml for 90 min at 37°C. After three washes, radiolabeled cells were resuspended at a concentration of 10^6/ml and used within 30 min. Effector cells generated during the MLC were harvested, washed once, and resuspended at three different dilutions. A 0.1-ml amount of each dilution was incubated in triplicate in microtiter wells with 10^5 ^{51}Cr-labeled target cells for 3 h at 37°C. These dilutions of harvested cells provided effector cell/target cell ratios ranging from 3:1 to 8:1. After centrifugation at $327 \times g$ for 6 min, 0.1 ml of each culture supernatant was carefully removed for counting of radioactivity in a gamma scintillation spectrometer. Lytic activity was expressed as the percent corrected lysis measured by ^{51}Cr release from target cells. The radioactivity released from target cells in medium alone represented the spontaneous (minimal) lysis; maximal lysis of target cells was induced by incubation with 10% Triton X-100 (Sigma). Results were calculated according to the formula:

$$\text{percent corrected lysis} = \frac{\text{cpm}_{\text{experimental}} - \text{cpm}_{\text{spontaneous}}}{\text{cpm}_{\text{maximal}} - \text{cpm}_{\text{spontaneous}}} \times 100$$

Primary in vitro antibody response to SRBC. Spleen cell suspensions from infected mice and age-matched controls were cultured by the method of Mishell and Dutton (18). Viable spleen cells (10^7) were placed in 35-mm tissue culture dishes (Falcon Plastics) and immunized with 3.5×10^6 SRBC. Cultures were incubated at 37°C for 4 days with gentle rocking in an atmosphere containing 7% O_2, 10% CO_2, and 83% N_2. Cultures were fed daily with a nutritional "cocktail" containing essential and nonessential amino acids, glutamine (Microbiological Associates, Walkersville, Md.), and dextrose. The immunoglobulin M antibody-forming cells per culture were quantitated by the Cunningham-Szenberg modification of the Jerne plaque assay (7). The mean number of plaque-forming cells (PFC) per culture was calculated from the results of quadruplicate cultures. In experiments to measure the effect of adding splenocytes (5×10^6) from infected mice to normal splenocytes (1×10^7), the percent suppression or augmentation was calculated according to the formula:

$$\text{percent suppression or augmentation} = 1 - \frac{\text{mean PFC per culture infected}}{\text{mean PFC per culture normal}} \times 100$$

Statistical methods. Student's t test for unpaired samples and the Mann-Whitney test for two independent samples were used to determine levels of significance within and among groups (23).

RESULTS

DTH responses to SRBC. The DTH responses of normal and infected mice to i.d. challenge with SRBC, 5 days after immunization with SRBC, are summarized in Fig. 1. In infected mice at weeks 1 and 3, the increases in footpad thickness 24 h after SRBC challenge were only 14 ± 1.5 and 17 ± 1.6%, respectively. By contrast, age-matched controls responded with 39 ± 2.5 and 34 ± 1.8% increases in swelling at these respective times ($P < 0.001$). By week 8, the DTH response of infected mice (35 ± 2.5%) had increased twofold and did not differ significantly from that of normal controls (38 ± 1.8%). After resolution of infection at 18 weeks, the responses of normal and infected groups were equal. The banal inflammatory responses to injection of the SRBC challenge dose alone into the footpads of nonimmunized controls from both the normal

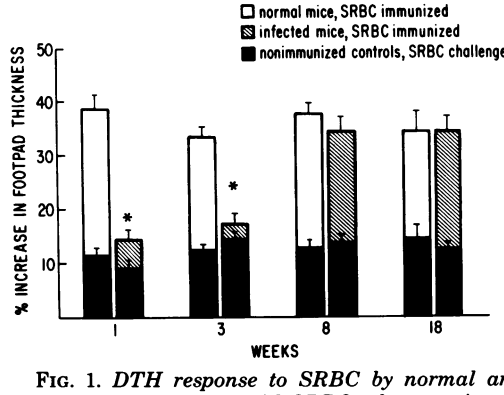

FIG. 1. *DTH response to SRBC by normal and infected mice sensitized with SRBC 5 days previously. Black bars indicate banal footpad responses in separate groups of nonimmunized controls (normal or infected) to challenge with SRBC only. Mean responses are shown by the tops of the bars and the standard errors are shown by brackets. Asterisks indicate significant differences in response between normal and infected mice sensitized to SRBC (see text).*

and the infected groups are also shown in Fig. 1. At each time interval, the banal response did not differ significantly between the normal and the infected groups. Thus, the very poor DTH responses to SRBC by infected mice at 1 and 3 weeks could not be accounted for by a generalized impairment of the inflammatory response to a nonspecific stimulus.

Additional studies of the DTH response to SRBC were performed on mice that had been inoculated with 10^6 heat-killed cells of *H. capsulatum*. The mean increases in footpad thickness within this group at 1, 3, and 8 weeks after inoculation ranged from 23 ± 1.4 to 26 ± 1.1%, as compared with increases ranging from 24 ± 1.6 to 28 ± 1.9% in age-matched controls. Thus, in the numbers used, only viable organisms were capable of inducing depression of the DTH response to SRBC.

DTH response to histoplasmin. In other experiments, infected and normal mice were challenged i.d. with histoplasmin (HKC-43). Infected mice were unresponsive to histoplasmin at 1 week of infection (Fig. 2, top). At 3 weeks, the DTH response by infected mice (13.7 ± 1.4%) was greater than that of controls (7.5 ± 0.8%) ($P < 0.025$). However, it was not until week 18 that the response to histoplasmin reached a maximum of 25.5 ± 2.1%, as compared with a response of 9.5 ± 1.5% by the control group ($P < 0.005$). In contrast to these findings, the footpad response to histoplasmin by mice given 10^6 heat-killed cells of *H. capsulatum* was significantly greater ($P < 0.005$) than that of controls at 1 week post-inoculation (Fig. 2, bottom). Moreover, the DTH response was greatest at 3 weeks (15.2 ± 0.7%), after which time it diminished. As a result, the percent increase in footpad thickness at week 18 was significantly less ($P < 0.001$) than that of mice injected with viable organisms, as can be seen by comparing the upper and lower sets of 18-week bar graphs in Fig. 2. These results suggest that the poor footpad responses during early phases of infection resulted from a true depression of specific reactivity to histoplasmin and did not reflect an inertial phase in the development of sensitivity to *Histoplasma* antigens.

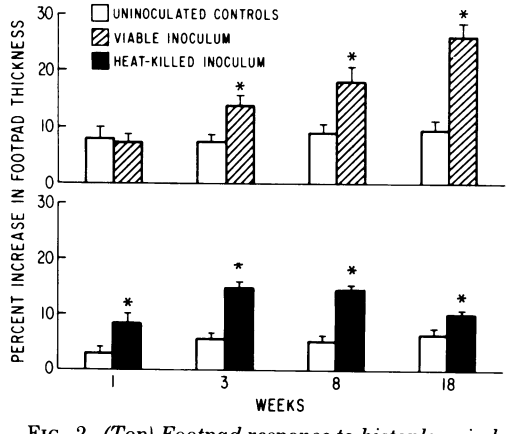

FIG. 2. *(Top) Footpad response to histoplasmin by normal control mice and mice infected with viable H. capsulatum. (Bottom) Footpad response to histoplasmin by normal control mice and mice given heat-killed H. capsulatum. Mean ± standard error and significant differences between groups are indicated as in Fig. 1.*

Lymphocyte transformation studies. The in vitro blastogenic responses to ConA by spleen cells from infected and control mice are summarized in Table 1. One week after inoculation, the mean response to ConA by splenocytes from infected mice was profoundly depressed as compared with the response of normal controls ($P < 0.001$). By week 3, the response to ConA by splenocytes from infected mice had increased 10-fold but remained significantly below the mean response of control spleen cells ($P < 0.001$). On the other hand, the blastogenic responses of infected and normal mice did not differ significantly at 8 and 18 weeks. Clearly, then, ConA-induced blastogenesis was depressed severely during the first 3 weeks of infection. However, as the mice recovered, blastogenic responses to this mitogen returned to normal levels.

In contradistinction to the depressed responses to ConA by spleen cells from mice given viable yeasts, the responses of cells from groups given dead organisms did not differ significantly from those of uninoculated controls at 1, 3, and 8 weeks (Table 1). Thus, stimulation of the immunoregulatory mechanism by an inoculum of viable organisms resulted in a marked suppression of ConA-induced blastogenesis, whereas a heat-killed inoculum failed to evoke measurable suppression.

The blastogenic responses to histoplasmin by spleen cells from normal and infected groups are listed in Table 2. At 1 and 3 weeks, splenocytes from infected mice failed to respond to histoplasmin, as indicated by a stimulation index of 1.0 at each time. However, at 8 and 18 weeks, the mean response of cells from infected mice was significantly greater than that of normal controls ($P < 0.01$). The response of spleen cells from mice inoculated with dead *H. capsulatum* did not differ from controls at 1 week, but it was significantly greater ($P < 0.001$) at 3 weeks (Table 2).

The absence of a detectable response to histoplasmin by splenocytes from mice inoculated with either viable or dead organisms at 1 week

TABLE 1. *Blastogenic response to ConA by spleen cells from normal and infected mice*

Week	Viable inoculum				Heat-killed inoculum			
	Group	cpm[a]			Group	cpm[a]		
		Unstimulated	ConA stimulated[b]			Unstimulated	ConA stimulated[b]	
1	Normal	3,306 ± 395	47,059 ± 2,969	(14.2)	Normal	5,019 ± 594	38,199 ± 1,685	(7.6)
	Infected	2,726 ± 566	3,450 ± 648	(1.3)[c]	Inoculated	3,811 ± 380	36,839 ± 1,483	(9.7)
3	Normal	6,654 ± 797	59,795 ± 3,364	(9.0)	Normal	4,358 ± 620	43,120 ± 4,746	(9.9)
	Infected	7,007 ± 673	33,171 ± 5,156	(4.7)[c]	Inoculated	4,608 ± 598	54,652 ± 1,839	(11.9)
8	Normal	9,790 ± 858	72,482 ± 7,963	(7.4)	Normal	10,196 ± 1,182	39,895 ± 1,576	(3.9)
	Infected	7,991 ± 836	58,808 ± 4,110	(7.4)	Inoculated	11,103 ± 981	35,851 ± 1,937	(3.2)
18	Normal	5,643 ± 1,050	77,249 ± 3,051	(12.8)		ND[d]	ND	
	Infected	5,495 ± 1,177	58,191 ± 3,656	(10.6)		ND	ND	

[a] Mean counts per minute ± standard error of the mean, calculated from a minimum of three separate experiments, each with cultures in triplicate.

[b] Figures in parentheses indicate stimulation indexes (counts per minute of ConA-stimulated cultures ÷ counts per minute of unstimulated cultures).

[c] Significant difference between infected and normal groups ($P < 0.001$).

[d] ND, Not done.

TABLE 2. *Blastogenic response to histoplasmin by spleen cells from normal and infected mice*

Week	Viable inoculum				Heat-killed inoculum			
	Group	cpm[a]			Group	cpm[a]		
		Unstimulated	Histoplasmin stimulated[b]			Unstimulated	Histoplasmin stimulated[b]	
1	Normal	6,525 ± 686	6,302 ± 528	(1.0)	Normal	7,499 ± 1,771	7,193 ± 828	(0.9)
	Infected	5,397 ± 549	5,438 ± 426	(1.0)	Inoculated	7,231 ± 1,942	8,681 ± 1,572	(1.2)
3	Normal	6,654 ± 797	6,733 ± 640	(1.0)	Normal	4,358 ± 620	3,729 ± 191	(0.9)
	Infected	7,815 ± 432	7,755 ± 490	(1.0)	Inoculated	4,608 ± 598	10,119 ± 609	(2.2)[c]
8	Normal	8,156 ± 388	7,190 ± 445	(0.9)	Normal	10,196 ± 1,182	7,013 ± 970	(0.8)
	Infected	7,991 ± 836	14,614 ± 1,643	(1.8)[d]	Inoculated	11,103 ± 981	27,220 ± 595	(2.4)[c]
18	Normal	5,643 ± 1,050	4,193 ± 580	(0.9)		ND[e]	ND	
	Infected	6,470 ± 1,436	13,265 ± 3,055	(2.1)[d]		ND	ND	

[a] Mean counts per minute ± standard error of the mean, calculated from a minimum of three separate experiments, each with cultures in triplicate.

[b] Figures in parentheses indicate stimulation indexes (counts per minute of histoplasmin-stimulated cultures ÷ counts per minute of unstimulated cultures).

[c] Significant difference between inoculated and normal groups ($P < 0.001$).

[d] Significant difference between infected and normal groups ($P < 0.01$).

[e] ND, Not done.

indicates that a longer time period must elapse before sensitization to *Histoplasma* antigens can be detected by this in vitro method. However, the finding that splenocytes from mice receiving dead yeasts did respond to histoplasmin at 3 weeks whereas spleen cells from infected mice did not suggests strongly that the poor response by the latter cells was consequent to immunosuppression.

To control for the possibility that the poor responses to ConA or histoplasmin may have been caused by the presence of *H. capsulatum* in spleen cell cultures prepared from infected mice, 2×10^5 viable yeasts were added to normal spleen cell cultures stimulated by ConA. Adding this number of yeasts resulted in a yeast/spleen cell ratio approximately 1,000-fold greater than the ratio calculated to be present in the spleens of infected mice at week 1 (4). Despite this high ratio, the blastogenic transformation of normal

splenocytes was not reduced as compared with cultures to which no *Histoplasma* was added (data not shown).

CML by spleen cells from infected mice. Since active infection appeared to trigger immunosuppressor activity in the spleen, we studied the cytotoxic activity of splenocytes from infected and control mice after sensitization in a MLC system (see Materials and Methods for details). At 1 week, the cytotoxic activity of unfractionated spleen cells from infected mice was severely depressed (Table 3). On the other hand, the capacity of a T-cell-enriched population of spleen cells from infected animals to lyse P815 target cells was less impaired. In two separate experiments, the ^{51}Cr releases induced by T cells from 1-week-infected mice were 69 and 85%, respectively, of those induced by splenic T cells from normal mice. Results were similar at week 3, when cytolyses by unfractionated spleen cells from infected mice were only 5 and 30% of the respective control values. As before, lysis of target cells by T cells was greater than lysis by unfractionated splenocytes. At week 8, the cytolytic activities of both unfractionated spleen cells and splenic T cells from infected mice were supernormal, as shown by experiments in which the average lyses of target cells were 118 and 132%, respectively, above controls.

In other experiments, the lysis of target cells by splenocytes from mice given heat-killed *H. capsulatum* was consistently within normal range when assayed at 1, 3, and 8 weeks. Addition of 2×10^4 viable yeasts to MLC systems containing normal splenocytes or T cells neither suppressed nor augmented the generation of a lytic response (data not shown). Last, the viability of cells was determined at the termination of MLC by trypan blue dye exclusion. The mean viability of spleen cells from mice infected for 1 week (53.2 ± 2.0%) was slightly less than that of normal splenocytes (61.5 ± 2.0%). Clearly, this very small difference could not explain the profound depression of CML at 1 week of infection. Median viabilities of "infected" splenocytes at weeks 3 and 8 were slightly higher than normal.

Primary antibody response to SRBC in vitro. Another series of experiments were performed to measure the T-cell-dependent primary antibody response to SRBC in vitro. One week after inoculation with viable organisms, the immunoglobulin M PFC responses to SRBC by spleen cells from infected mice were decreased to less than 2% of the response by normal splenocytes cultured simultaneously (Table 4). At 3 weeks, the PFC responses by "infected" spleen cells in two experiments were depressed to 29 and 25%, respectively, of the control values. Conversely, at weeks 8 and 18, the responses of splenocytes from infected mice were equal to or greater than the responses of normal spleen cells. In harmony with results previously obtained, antibody formation by splenocytes from mice given heat-killed *Histoplasma* was not suppressed at any time subsequently (Table 4).

The possibility that *H. capsulatum* in cell cultures from infected mice may have lowered the PFC response was examined by adding up to 2×10^5 yeasts to normal spleen cell cultures. In no case was antibody formation suppressed. Of the spleen cells added to culture dishes on day 0, the percent viable at harvest on day 4 was reduced in cultures from mice infected for 1 week as compared with control cultures. The mean percentage of viable cells recovered was 26.9 ± 2.6%, whereas the viable recovery from normal splenocyte cultures was 37.1 ± 2.6%. This difference in viability, however, could not explain the virtual absence of a PFC response by cultures from infected mice. Differences in viability were small at week 3, with 31.6 ± 2.0% mean viable cell recovery from splenocyte cultures of infected mice and 37.5 ± 4.4% viable recovery from normal cultures. Nevertheless,

TABLE 3. *CML by spleen cells from normal and infected mice*

Week	Group	% Lysis[a]			
		Unfractionated spleen cells		Splenic T cells	
		Expt 1	Expt 2	Expt 1	Expt 2
1	Normal	29.1 ± 0.6	32.8 ± 0.7	47.7 ± 0.2	68.7 ± 1.7
	Infected	2.4 ± 0.5 (8)	2.7 ± 0.2 (8)	32.8 ± 1.0 (69)	58.2 ± 3.0 (85)
3	Normal	37.3 ± 1.7	28.5 ± 0.6	50.5 ± 0.2	43.3 ± 0.7
	Infected	1.8 ± 0.4 (5)	8.6 ± 0.3 (30)	19.7 ± 0.7 (39)	20.8 ± 0.5 (48)
8	Normal	39.0 ± 1.5	35.3 ± 0.3	46.0 ± 0.6	48.3 ± 0.9
	Infected	46.0 ± 1.2 (118)	46.7 ± 0.9 (132)	71.3 ± 0.3 (155)	78.7 ± 0.3 (163)

[a] Figures in parentheses are the percentages of corresponding normal control lytic activities.

TABLE 4. *Primary antibody response to SRBC by splenocytes from normal mice and mice inoculated with viable or heat-killed H. capsulatum*

Week	Expt	PFC/culture[a]			
		Viable inoculum		Heat-killed inoculum	
		Normal mice	Infected mice	Normal mice	Inoculated mice[b]
1	1	4,640 ± 108	40 ± 23 (0.8)	2,320 ± 160	3,140 ± 171 (135)
	2	2,240 ± 229	40 ± 23 (1.7)	1,147 ± 27	1,040 ± 160 (91)
3	1	2,400 ± 212	600 ± 250 (25)	3,402 ± 365	3,360 ± 231 (99)
	2	2,440 ± 116	700 ± 192 (29)	2,440 ± 116	2,320 ± 142 (95)
8	1	5,260 ± 342	4,800 ± 432 (91)	2,360 ± 237	2,907 ± 133 (123)
	2	1,680 ± 240	2,080 ± 240 (124)	2,107 ± 141	2,000 ± 231 (95)
18	1	2,640 ± 185	2,933 ± 133 (111)	ND[c]	ND
	2	960 ± 46	940 ± 38 (98)	ND	ND

[a] Mean PFC per culture ± standard error of the mean, calculated from quadruplicate cultures.
[b] Figures in parentheses represent percentages of the corresponding PFC responses by normal splenocytes.
[c] ND, Not done.

the PFC response in cultures from infected mice was reduced greatly.

Effect of splenocytes from infected mice on primary antibody formation by normal splenocytes. A final experiment was performed to determine if splenocytes from infected mice were capable of suppressing the primary antibody response of normal spleen cells. Concentrations of cells ranging from 5×10^4 to 5×10^6 were added to 1×10^7 normal splenocytes at 1, 3, 8, and 18 weeks after inoculation. At week 1, as few as 5×10^4 splenocytes from infected mice markedly inhibited the PFC response of normal spleen cells (Fig. 3). Even greater suppression was achieved by adding 5×10^5 cells or 5×10^6 splenocytes, the latter reducing the response by 98% from control values. At 3 weeks, suppression was induced by the higher cell concentrations from infected mice; however, 5×10^4 cells were ineffective. A dramatic shift from dominant suppressor activity to distinct helper activity was observed by week 8. At this time, 5×10^5 spleen cells from infected mice augmented the response of normal cells by 25%, and 5×10^6 cells increased it by 50% (Fig. 3). At week 18, splenocytes from infected mice neither increased nor decreased the PFC response of normal spleen cells by more than 10%.

Addition of 5×10^6 spleen cells from mice inoculated with dead yeasts to 1×10^7 normal spleen cells did not suppress primary antibody formation at 1, 3, and 8 weeks (data not shown).

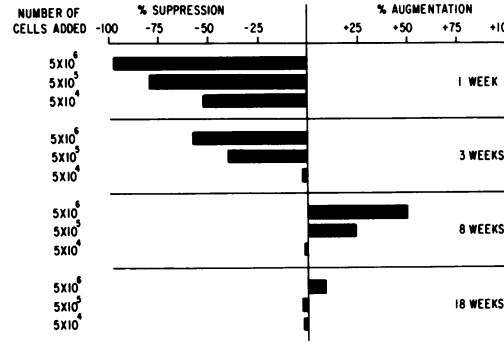

FIG. 3. *Effects of adding varying numbers of splenocytes from mice infected for 1, 3, 8, or 18 weeks upon the primary antibody response to SRBC by 10^7 normal spleen cells in vitro.*

DISCUSSION

These studies have demonstrated that the cellular immune responses of mice with disseminated histoplasmosis are markedly diminished in association with the generation of potent immunosuppressor activity by spleen cells. Suppressor activity reached its zenith in the most active phase of infection, from 1 to 3 weeks after inoculation. During this time, there was (i) depression of the DTH response to SRBC and histoplasmin, (ii) impairment of ConA- and histoplasmin-induced blastogenic transformation by splenocytes in vitro, (iii) depressed cytotoxic activity of spleen cells in infected mice, and (iv) marked suppression by splenocytes from infected mice of the primary antibody response to SRBC by normal spleen cell cultures.

Coincident with resolution of the infection by week 8, there was a striking shift of immunoregulatory function from dominant suppressor activity to expression of helper activity. This was indicated by an improvement in the DTH responses as well as by CML and PFC responses

of splenocytes from 8-week-infected mice that were equal to or greater than normal control values. Furthermore, addition of splenocytes from these mice to cultures of normal spleen cells greatly augmented the primary antibody response to SRBC, whereas 4 weeks previously the response of normal cells clearly had been suppressed (Fig. 3). Indeed, this shift in the immune response from a suppressor to helper mode suggests that the degree of help or suppression measurable at any given point in time during infection represents the algebraic sum of the helper and suppressor activities which are mediated by a complex and dynamic series of interactions between the regulatory and "regulatee" cell populations.

A factor that may be critical in driving the immunoregulatory mechanism to function in a suppressor mode is the size of the antigen load, as suggested by the severity of depression observed during the phase of most active yeast cell multiplication within the lymphoreticular system. This assumption is supported by the fact that inoculation of mice with equal numbers of *H. capsulatum* heat killed to prevent multiplication failed to evoke detectable suppressor activity by any of the measurements used. Why this infection and others, such as murine leprosy (5) and experimental trypanosomiasis (15), in which antigen loads are high, should preferentially stimulate the suppressor arm of the immune response is unknown at present. Teleologically, it can be argued that such inhibitory activity may serve to protect against the widespread necrosis of tissues that might ensue in disseminated infections if the positive cellular immune response to large numbers of microbes were uncontrolled. As the antigen load falls consequent to destruction of infecting organisms by cell-mediated immune mechanisms, the need for feedback control over these responses presumably would diminish. However, should large quantities of antigen persist either through failure by cells of the reticuloendothelial system to kill microorganisms or through failure to metabolize the antigenic residua of microbial carcasses, then a chronic state of suppressor activity may be perpetuated, as has been demonstrated in infections caused by *Mycobacterium lepraemurium* (5).

In mice, it is known that large numbers of immunoregulatory cells with the capacity to function in a bidirectional manner are present within the thymus and spleen (13, 26). Cells belonging to this subpopulation are relatively immature, are short-lived, and bear the surface phenotype $Ly1^+2^+3^+$ as determined by reaction with specific alloantisera (6). These cells tend to boost T-cell-mediated immune responses under conditions of minimal antigenic stimulation by augmenting the activity of T-helper cells of phenotye $Ly1^+23^-$ (12). Conversely, under conditions of intense antigenic stimulation, $Ly1^+2^+3^+$ cells act to suppress the immune response by augmenting development of suppressor T cells of phenotype $Ly1^-23^+$ or possibly by direct feedback suppression of $Ly1^+23^-$ cells (6, 10).

Recently, we have obtained preliminary evidence suggesting that during *Histoplasma* infection a subpopulation of T cells is generated which may depress the CML response when added to cultures of normal spleen cells (R. P. Artz and W. E. Bullock, unpublished observations). The Ly phenotype of these cells currently is unknown; however, experiments are planned to resolve this question by means of Ly alloantisera, using C57BL/6 mice infected with histoplasmosis. Of significance is the fact that far greater suppression of the CML response of normal splenocytes is induced by adding unfractionated spleen cells from infected mice. Thus, more than one type of spleen cell appears to manifest suppressor activity in this model. Currently, we are attempting to determine if macrophage-like cells function as suppressor cells either primarily or secondarily to the acquistion of suppressor properties by interaction with a subpopulation of suppressor T cells. Aside from the probable suppressor activity of macrophages, it is also possible that the capacity of those cells "infected" with *Histoplasma* yeasts to process antigen, such as SRBC, and/or to present antigen to T cells for activation of DTH and cell-mediated immune responses may be impaired.

As reported in a companion paper, the spleen enlarges greatly during the first 3 weeks of infection, and thereafter it returns gradually to normal size by week 18 (2). Concurrently, there is a massive loss of cells from cortical lobules of the thymus during active infection followed by a gradual repopulation of these areas during recovery. Although the possibility of thymic cell loss consequent to stress-induced elevations of serum corticosteroid levels has yet to be excluded rigorously, reversible thymic involution of a very similar type has been demonstrated in adrenalectomized mice infected with *Toxoplasma gondii*, a species that also can induce depression of the immune response in disseminated forms of infection (14).

At this juncture, we are exploring the hypothesis that cortical thymocytes may migrate rapidly to the spleen in response to the stimulus of yeast cell deposition and multiplication within this organ. Of interest in this regard are the histopathological findings in infected spleens which reveal multiple granulomata plus many

yeast cells directly within the marginal zones and periarteriolar sheaths of the splenic white pulp (2). It is to these same areas, notably the marginal zones, that antigen-stimulated cortical thymocytes have been shown to migrate following intravenous administration to neutral syngeneic hosts (9). Furthermore, it is known that cortical lobules of the thymus are composed predominantly of spleen-seeking cells that bear the $Ly1^+2^+3^+$ phenotype (6). Thus, in experimental *Histoplasma* and *Toxoplasma* infections, an immunoregulatory cell population may be delivered rapidly to anatomic regions containing high antigen concentrations and there be stimulated to function in a suppressor mode during early phases of infection.

The splenic white pulp is an area uniquely suited for transmittal of suppressor cell signals, since it is through this same area that an extensive recirculation of immunocompetent T cells takes place (11). Moreover, there is good evidence from studies of mycobacterial infection that the presence of granulomata within the splenic white pulp induces extensive trapping of recirculating T cells (3, 4). Therefore, involvement of the splenic white pulp by granulomatous infection appears to create a microenvironment which is most favorable to intimate contact between antigen, macrophages, a short-lived subpopulation of immunoregulatory cells, and a recirculating population of T cells that may be modulated to suppressor or helper function during a prolonged sojourn within the spleen. Future studies designed to ablate selected populations or subpopulations of immunoregulatory cells, as, for example, by adult thymectomy or by giving specific cytotoxic agents, should provide further insight into the intricacies of immunoregulatory disturbances associated with chronic granulomatous infections in general and histoplasmosis in particular.

ACKNOWLEDGMENTS

We thank Mona Havens and Carol Hubbell for skilled technical assistance and Kathy Bultema for typing the manuscript.

This work was supported by U. S. Public Health Service grant AI-10094 from the National Institutes of Health.

LITERATURE CITED

1. **Alford, R. H., and R. A. Goodwin.** 1972. Patterns of immune response in chronic pulmonary histoplasmosis. J. Infect. Dis. **125**:269-275.
2. **Artz, R. P., and W. E. Bullock.** 1979. Immunoregulatory responses in experimental disseminated histoplasmosis: lymphoid organ histopathology and serological studies. Infect. Immun. **23**:884-892.
3. **Bullock, W. E.** 1976. Perturbation of lymphocyte circulation in experimental murine leprosy. I. Description of the defect. J. Immunol. **117**:1164-1170.
4. **Bullock, W. E.** 1976. Perturbation of lymphocyte circulation in experimental murine leprosy. II. Nature of the defect. J. Immunol. **117**:1171-1178.
5. **Bullock, W. E., E. M. Carlson, and R. K. Gershon.** 1978. The evolution of immunosuppressive cell populations in experimental mycobacterial infection. J. Immunol. **120**:1709-1716.
6. **Cantor, H., and E. A. Boyse.** 1977. Regulation of cellular and humoral immune response by T cell subclasses. Cold Spring Harbor Symp. Quant. Biol. **41**:23-32.
7. **Cunningham, A. J., and A. Szenberg.** 1968. Further improvements in the plaque forming technique for detecting single antibody-forming cells. Immunology **14**: 599-600.
8. **Davies, S. F., M. Khan, G. A. Sarosi.** 1978. Disseminated histoplasmosis in immunologically suppressed patients. Am. J. Med. **64**:94-100.
9. **Durkin, H. G., J. M. Carboni, and B. H. Waksman.** 1978. Antigen-induced increase in migration of large cortical thymocytes (regulatory cells?) to the marginal zone and red pulp of the spleen. J. Immunol. **121**:1075-1081.
10. **Eardley, D. D., J. Hugenberger, L. McVay-Boudreau, F. W. Shen, R. K. Gershon, and H. Cantor.** 1978. Immunoregulatory circuits among T-cell sets. I. T-helper cells induce other T-cell sets to exert feedback inhibition. J. Exp. Med. **147**:1106-1115.
11. **Ford, W. L.** 1975. Lymphocyte migration and immune responses. Prog. Allergy **19**:1-59.
12. **Gershon, R. K., D. D. Eardley, K. F. Naidorf, and W. Ptak.** 1977. The hermaphrocyte: a suppressor-helper T cell. Cold Spring Harbor Symp. Quant. Biol. **41**:85-91.
13. **Gershon, R. K., E. M. Lance, and K. Kondo.** 1974. Immunoregulatory role of spleen localizing thymocytes. J. Immunol. **112**:546-554.
14. **Huldt, G., S. Gard, and S. G. Olovson.** 1973. Effect of *Toxoplasma gondii* on the thymus. Nature (London) **244**:301-303.
15. **Jayawardena, A. N., and B. H. Waksman.** 1977. Suppressor cells in experimental trypanosomiasis. Nature (London) **265**:539-541.
16. **Julius, M. H., E. Simpson, and L. A. Herzenberg.** 1973. A rapid method for the isolation of functional thymus-derived murine lymphocytes. Eur. J. Immunol. **3**:645-649.
17. **Kaufman, C. A., K. S. Israel, J. W. Smith, A. C. White, J. Schwarz, and G. F. Brooks.** 1978. Histoplasmosis in immunosuppressed patients. Am. J. Med. **64**:923-932.
18. **Mishell, R. I., and R. W. Dutton.** 1967. Immunization of dissociated spleen cell cultures from normal mice. J. Exp. Med. **126**:423-442.
19. **Newberry, W. M., Jr., J. W. Chandler, T. D. Y. Chin, and C. H. Kirkpatrick.** 1968. Immunology of the mycoses. I. Depressed lympohcyte transformation in chronic histoplasmosis. J. Immunol. **100**:436-443.
20. **Reddy, P., D. F. Gorelick, C. A. Brasher, and H. Larsh.** 1976. Progressive disseminated histoplasmosis as seen in adults. Am. J. Med. **48**:629-636.
21. **Simpson, E., R. Gordon, M. Taylor, J. Mertin, and P. Chandler.** 1975. Micromethods for induction and assay of mouse mixed lymphocyte reactions and cytotoxicity. Eur. J. Immunol. **5**:451-455.
22. **Smith, J. W., and J. P. Utz.** 1972. Progressive disseminated histoplasmosis. A prospective study of 26 patients. Ann. Intern. Med. **76**:557-565.
23. **Snedecor, G. W., and W. G. Cochran.** 1967. Statistical methods, 6th ed., p. 91-135. Iowa State University Press, Ames.
24. **Stobo, J. D., P. Sigrun, R. E. VanScoy, and P. E. Hermans.** 1976. Suppressor thymus-derived lymphocytes in fungal infection. J. Clin. Invest. **57**:319-328.
25. **Waddell, W. J.** 1956. A simple ultraviolet spectrophotometric method for the determination of protein. J. Lab.

Clin. Med. **48**:311-314.
26. **Wu, C.-Y., and E. M. Lance.** 1974. Immunoregulation by spleen-seeking thymocytes. II. Role in the response to sheep erythrocytes. Cell. Immunol. **13**:1-11.
27. **Yam, L. T., C. Y. Li, and W. H. Crosby.** 1971. Cytochemical identification of monocytes and granulocytes. Am. J. Clin. Pathol. **55**:283-290.
28. **Zarafonetis, C. J. D., and R. J. Parsons.** 1945. Histoplasmosis in man. Report of seven cases and a review of seventy-one cases. Arch. Intern. Med. **75**:1-23.