INFECTION AND IMMUNITY, June 1982, p. 1013–1018
0019-9567/82/061013-06$02.00/0

Vol. 36, No. 3

Blastogenic Responses of Lymphocytes from Mice Immunized by Sublethal Infection with Yeast Cells of *Histoplasma capsulatum*

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Blastogenic responses of spleen cells to histoplasmin and ribosomal antigens and to the mitogens concanavalin A, phytohemagglutinin, and lipopolysaccharide were studied in normal and immunized mice (10^5 live yeast cells of *Histoplasma capsulatum* given by the subcutaneous route). Cells (10^6 per well) were cultured with and without antigens and mitogens in microtiter plates with RPMI 1640–5% heat-inactivated normal mouse serum for 72 h at 37°C. Cells were harvested after a 16- to 18-h pulse with 1 μCi of [^{3}H]thymidine (6.7 Ci/mol), and thymidine incorporation was measured by scintillation counting. The initial blastogenic response to concanavalin A (54×10^3 cpm) was suppressed ($P < 0.001$) from 4 to 14 days post-immunization and returned to control levels on day 21. The response to phytohemagglutinin was suppressed up to 21 days. Lipopolysaccharide responses, however, were affected to a lesser degree. Blastogenic responses to histoplasmin and *H. capsulatum* ribosomes were similar on day 0 in normal and immune lymphocytes, but by day 4 cells from immunized mice were more responsive ($P < 0.01$). The maximum response to *H. capsulatum* antigens was detected on day 42 and was 9- to 16-fold higher than in controls. These results demonstrate in vitro responses of primed lymphocytes on exposure to *H. capsulatum* antigens and suppressed responses to mitogens during early stages of the immune response.

The cell-mediated immune defenses are of primary importance in protective immunity to histoplasmosis. The cellular changes at different stages of the disease are affected by the immune status of the host, the virulence and dose of the strain of *Histoplasma capsulatum*, and the route of infection. There is mounting evidence to suggest that disseminated infection with *H. capsulatum* induces complex disturbances in the immunoregulatory control mechanisms in humans (2, 5, 20).

Artz and Bullock (3, 4), while studying immunoregulatory responses in experimental disseminated histoplasmosis, demonstrated suppression in cellular immune responses of mice in association with the generation of potent immunosuppressor activity by spleen cells after intravenous injection with 5×10^5 to 10×10^5 yeast cells of *H. capsulatum*. Maximum suppressor activity of delayed hypersensitivity responses to sheep erythrocytes and histoplasmin and impairment of concanavalin A (ConA)- and histoplasmin-induced blastogenic responses were observed during 1- to 3-week periods (3, 16). The suppressive activity disappeared after 8 weeks.

We have previously described an experimental model in which stable immunity is elicited by subcutaneous (s.c.) immunization with a sublethal dose of live yeast cells of *H. capsulatum* (24–26). Immunization with live cells provided 90 to 100% protection during 1 to 12 weeks post-immunization against a lethal intravenous challenge with yeast cells of *H. capsulatum*. The immunity was transferred to syngeneic recipients by immune lymphoid cells (25). The immune spleen and peritoneal cells which confer immunity to histoplasmosis are thymus-dependent T lymphocytes, and their active proliferation in the recipient is necessary for the expression of the protective immunity. Lymphoproliferative responses of these cells to mitogens or specific antigens, however, were not studied.

In this investigation we have studied the blastogenic responses of spleen cells to histoplasmin and *H. capsulatum* ribosomal antigens and to the mitogens ConA, phytohemagglutinin (PHA), and lipopolysaccharide (LPS) in normal and immunized mice (10^5 live yeast cells of *H. capsulatum*, s.c.). Lymphoproliferative response to histoplasmin in vitro was compared with that induced by purified *H. capsulatum*

ribosomes, a better-defined subcellular antigen.

MATERIALS AND METHODS

Animals. Female C_3H/He mice weighing 14 to 16 g were obtained from Lab Supply Co., Indianapolis, Ind. The mice were divided randomly into groups of five, housed in plastic cages, and given food and water ad libitum.

Organism. *H. capsulatum*, brown type (G-217B), was obtained from a subculture of a primary human isolate. The organism in the yeast phase was maintained on brain heart infusion agar slants (Difco Laboratories, Detroit, Mich.) supplemented with 1% glucose and 0.1% L-cysteine hydrochloride. Cultures were stored at 4°C and transferred every 4 to 6 weeks, and the morphological type was periodically monitored. Yeast cells of *H. capsulatum* were grown in brain heart infusion broth (Difco) supplemented with 1% glucose, and the cell suspension for inoculation was standardized as described before (26).

Immunization of mice. Mice were immunized s.c. with 10^5 live yeast cells of *H. capsulatum* (sublethal infection). Control mice received 0.5 ml of phosphate-buffered saline.

Mitogens. ConA (jackbean PHA) lyophilized in NaCl A grade (M.I. 9:2462) and PHA-M B grade (M.I. 9:7195) were obtained from Calbiochem, La Jolla, Calif., and lipopolysaccharide (*Salmonella typhimurium*) lyophilized powder was obtained from Sigma Chemical Co., St. Louis, Mo. The mitogens were reconstituted in RPMI 1640 to appropriate concentrations and stored at −70°C.

Antigens. Histoplasmin was prepared from mycelial phase cultures of *H. capsulatum* G-217B as described by Ehrhard and Pine (9, 10). The preparation was concentrated by ultrafiltration through a Diaflo membrane at 10,000-molecular-weight cutoff, dialyzed extensively against phosphate-buffered saline, (pH 7.2), and sterilized by membrane (Millipore Corp., Bedford, Mass.) filtration. The protein content of the dialysate as determined by the method of Lowry et al. (15) was 3 mg/ml. This antigen, after standardization on the basis of protein content, has been subjected to potency testing in sensitized animals, with histoplasmin (National Communicable Disease Center, lot 76-0121) as the standard antigen. The histoplasmin produced both H and M bands against a standard antihistoplasma serum. The histoplasmin (National Communicable Disease Center, lot 76-0121) was kindly supplied by Leo Kaufman, National Communicable Disease Center, Atlanta, Ga. Gradient purified ribosomes were prepared by the procedure of VanEtten (28). The chemical analysis of the ribosomal preparation was performed as described previously (11). The ribosomes contained 47% protein and 53% RNA. The absorbance ratio at 260 and 280 nm was 1.9. The ribosomal preparations were stored at −70°C.

Preparation of spleen cells. At different intervals from 0 to 63 days post-immunization, immunized and control mice were anesthetized and bled by cardiac puncture; serum was separated and stored at −70°C.

Spleen cell suspensions were prepared in RPMI 1640 with HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (GIBCO Laboratories, Grand Island, N.Y.) by using a cell collector with a 60-mesh stainless steel sieve (Bellco Glass, Inc., Vineland, N.J.). Erythrocytes in spleen cell suspension were lysed by treatment with a hypotonic solution of Tris-buffered NH_4Cl. Spleen cells were filtered through a 4-cm column of acid-washed cotton wool to remove cell aggregates and cellular debris. The resulting cell suspension contained 75 to 80% lymphocytes and 20 to 25% macrophages. At least 90% of the cells were viable as detemined by trypan blue dye exclusion. The cells were finally suspended in RPMI 1640 with HEPES buffer supplemented with 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 5% heat-inactivated normal mouse serum (Colorado Serum Co., Denver, Colo.). The cells were adjusted to a concentration of 10^7 cells per ml.

Lymphocyte transformation assay. Spleen cells from normal and immunized animals (five mice per group) were prepared in RPMI 1640 with 5% heat-inactivated normal mouse serum at 10^7 cells per ml, and the lymphocyte transformation assay was performed by a modification of the procedure of Stobo et al. (21). Briefly, 0.1 ml of the cell suspension was dispensed into 96-well flat bottom microtiter plates (Flow Laboratories, McLean, Va.). Different concentrations of mitogens and antigens (0.1 ml) were added to triplicate wells. Cells were cultured for 72 h at 37°C in a humidified atmosphere of 5% CO_2. After 72 h 1 μCi of [^{3}H]thymidine (specific activity, 6.7 Ci/mol; New England Nuclear Corp., Boston, Mass.) in 20 μl of RPMI 1640 was added to each well. Cells were harvested after a further incubation of 18 h by an automatic cell harvester (Flow Laboratories), and uptake of radioactivity was measured by liquid scintillation counting. Results were expressed as counts per minute in control cultures subtracted from counts per minute in experimental cultures.

Statistical methods. Analysis of variance with a randomized blocks design was used for making specific planned comparisons between means at different times. The Student *t* test was used to determine levels of significance between means of paired and unpaired samples.

RESULTS

Dose response of spleen cells to mitogens and antigens of *H. capsulatum*. The optimal concentrations of mitogens and antigens determined in preliminary experiments were 1 μg for ConA and histoplasmin and 5 μg for PHA, LPS, and ribosomal antigen. However, more than one dose of mitogens and antigens was used to ascertain that the effects observed were not the apparent consequence of a displacement of the titration curves of the immunized mouse spleen cells with respect to those from unimmunized controls. In all of our experiments, the background counts with the medium alone ranged from 1.5×10^3 to 3.7×10^3 cpm. The blastogenic responses of spleen cells from control animals were 14×10^3 and 43×10^3 cpm in the presence of 5 and 1 μg of ConA, respectively (Table 1). The response of immune splenocytes (30×10^3 cpm) was not significantly different from that in controls on day 21. In response to PHA, immune cells at 21 days showed a significantly suppressed response, 10×10^3 cpm compared with

TABLE 1. Blastogenic response of spleen cells from immunized and control mice to mitogens and *H. capsulatum* antigens[a]

Mitogen or antigen (μg/well)	[^{3}H]thymidine incorporation by spleen cells[b]	
	Immune	Control
Medium alone	2,387 ± 95	2,042 ± 83
ConA (5)	14,682 ± 330	14,732 ± 1,774
ConA (1)	30,274 ± 1,236	43,656 ± 3,851
PHA (5)	10,435 ± 1,875	31,149 ± 2,338
PHA (1)	7,647 ± 681	12,812 ± 475
LPS (10)	7,785 ± 1,953	19,172 ± 111
LPS (5)	11,977 ± 2,638	26,314 ± 2,018
LPS (1)	6,778 ± 849	12,558 ± 479
Histoplasmin (10)	32,397 ± 2,013	4,978 ± 236
Histoplasmin (5)	30,145 ± 686	7,264 ± 540
Histoplasmin (1)	37,546 ± 2,432	4,898 ± 312
Ribosomes (10)	33,258 ± 3,874	4,411 ± 742
Ribosomes (5)	44,777 ± 2,577	8,265 ± 601
Ribosomes (1)	22,373 ± 1,102	4,478 ± 312

[a] Mice were immunized s.c. with 10^5 live yeast cells of *H. capsulatum*, and blastogenic response was determined 3 weeks later. Pooled spleen cells from five immune and five control mice were used for the transformation assay.

[b] Each value represents the mean (triplicate determinations) of the experimental minus control counts per minute ± the standard error of the mean.

31×10^3 cpm in controls, ($P < 0.001$). The blastogenic response to LPS was also significantly suppressed in immune mice ($P < 0.001$). The blastogenic responses of immune cells to both histoplasmin and ribosomes, on the contrary, were higher than those of controls ($P < 0.001$).

Blastogenic response to ConA, PHA, and LPS of spleen cells from normal and immunized mice at different time intervals after immunization. Four days after immunization, the mean response to ConA by splenocytes from immunized mice was significantly depressed ($P < 0.001$) as compared with the response of control mice (Fig. 1). The response remained depressed at days 7 and 14; at day 21, however, it was not significantly different from that in controls. The suppressor effect was observed at both doses of ConA. The blastogenic response of immune cells to PHA at 4 days after immunization (8.4×10^3 cpm) was significantly less than that of spleen cells from control mice (Fig. 2). Significant suppression was observed at days 7, 14, and 21 after immunization ($P < 0.001$). The response was similar to control values at days 42 and 63. The response of spleen cells from immune mice to stimulation by LPS was also suppressed, but to a lesser extent (Fig. 1). The counts were lower than those in controls at days 4, 7, and 42 ($P < 0.01$) and at days 14 and 21 ($P < 0.001$) post-immunization.

Blastogenic response of spleen cells from normal and immunized mice to histoplasmin and histoplasma ribosomes. The blastogenic responses to histoplasmin were similar in immune and control mice at day 0 (Fig. 2). At day 4 after immunization the response of immune cells was twice that of control cells (11.5×10^3 versus 6.5×10^3 cpm). At days 7, 14, 21, 42, and 63 the responses of spleen cells from immunized mice were significantly higher than those in controls ($P < 0.001$). In immunized mice the response to histoplasmin increased steadily from day 4 onward, peaked at day 14 and at day 42, and showed a decline at day 63. The response and the pattern at different time intervals was similar with all three concentrations of histoplasmin tested, 1 μg per well being the optimal concentration. The responses by spleen cells from control mice varied from 1.1×10^3 to 5.2×10^3 at different time intervals tested. The response of spleen cells from immune mice to *H. capsulatum* ribosomes was not different from that of controls on day 0 (Fig. 2). Immune cells showed a higher response than did controls at day 4. The responses were significantly higher in immune cell cultures at days 7, 14, 21, 42, and 63 compared with those from controls ($P < 0.001$). The cells from immunized mice showed steadily increasing counts beginning day 4 after immunization, peaked at day 42, and showed a slight decline at day 63. The cells from immunized mice were responsive at all three doses of ribosomal antigen tested.

DISCUSSION

Suppression and recovery of proliferative responses to mitogens were observed in mice immunized subcutaneously with live yeast cells of *H. capsulatum*. The suppressed responses were detected 4 days after immunization and continued to 14 days for ConA and 21 days for PHA and LPS. Concurrently, blastogenic responses to histoplasmin and *H. capsulatum* ribosomes were first observed at 4 days after immunization and steadily increased, reaching a peak on day 42, and then showed a slight decline on day 63. Both histoplasmin and ribosomes were equally efficient in detecting blastogenic responses of lymphocytes in vitro. Nonspecific depression to mitogens was a transient event, and the response was completely restored at 42 days post-immunization.

We have shown previously (25, 26) that the s.c. immunization of C_3H/He mice with yeast cells of *H. capsulatum* provides a highly reproducible model for the study of immunity to experimental histoplasmosis. High levels of delayed hypersensitivity reaction to histoplasmin and ribosomes and production of macrophage migration inhibition factor by immune peritoneal exudate cells in the presence of *H. capsulatum*

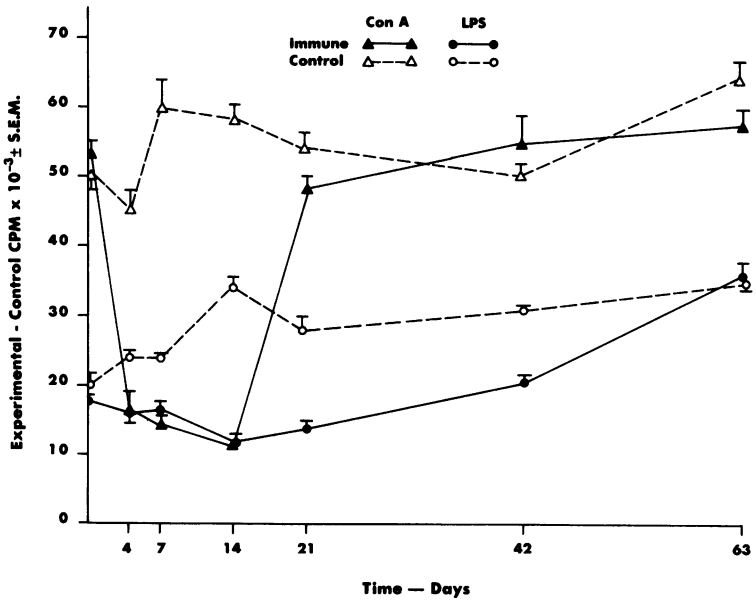

FIG. 1. Blastogenic responses of spleen cells to ConA and LPS from normal mice and mice immunized by sublethal infection with yeast cells of *H. capsulatum* (10^5 cells, s.c.). Each value represents the mean of five observations.

antigens were also observed early in the course of immunization (19). These immunological reactivities were correlated with a high degree of protective immunity to a homologous challenge (24). Thus in the self-limiting infection of mice with *H. capsulatum*, there seems to be an inverse relationship between nonspecific depression of blastogenic responses of spleen cells and the host capability to develop a successful immune response to the organism. Specific responses to *H. capsulatum* antigens were demonstrated in the immunized mice during the period

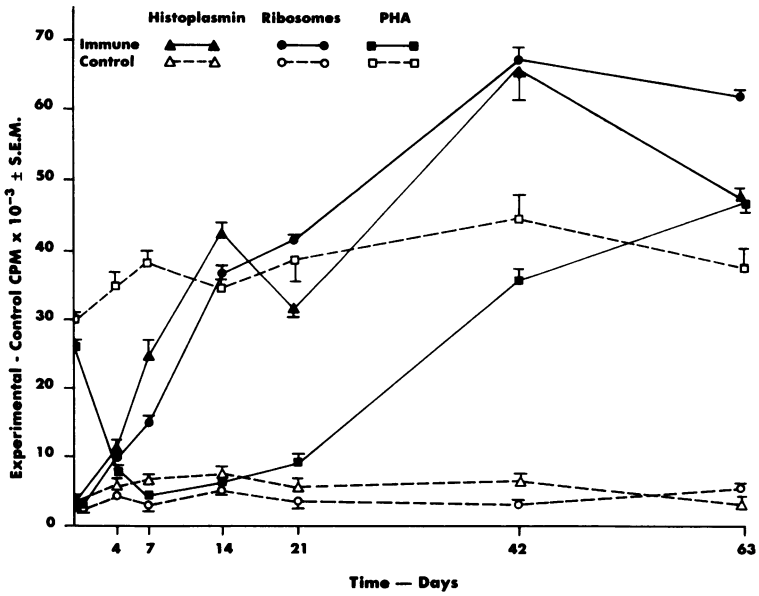

FIG. 2. Blastogenic responses of spleen cells to histoplasmin, *H. capsulatum* ribosomes, and PHA from normal mice and mice immunized by sublethal infection with yeast cells of *H. capsulatum* (10^5 cells, s.c.). Each value represents the mean of five observations.

when blastogenic responses to mitogens were severely depressed.

A similar paradoxical situation has also been described, in which the presence of antiparasitic antibodies and protective immunity are accompanied by nonspecific suppression of the antibody response to T-dependent and T-independent antigens in mice infected with *Trypanosoma brucei* (8, 12), *Leishmania mexicana* (18), and *Toxoplasma gondii* (22, 23). Furthermore, Strickland et al. (22) showed that the in vitro proliferative responses of spleen cells to both T- and B-cell mitogens were suppressed during the first 2 to 3 weeks of infection of mice with toxoplasma which is similar to our findings. In addition, a marked depression of PHA and ConA reactivity in spleen cell cultures from mice bearing primary Moloney scarcoma virus induced tumors has been described previously (14).

Artz and Bullock (3, 4), while studying the immunoregulatory responses in experimental disseminated histoplasmosis, demonstrated diminished cellular immune responses of mice in association with the generation of potent immunosuppressor activity by spleen cells. The suppressor activity was at a peak during most active infection from 1 to 3 weeks after intravenous inoculation of 5×10^5 to 10×10^5 yeast cells. No suppressor activity was demonstrated, however, in spleens of mice inoculated with heat-killed organisms. In their experimental model the blastogenic response to histoplasmin was demonstrated at 3 weeks and 8 weeks postinoculation with heat-killed and live yeast cells of *H. capsulatum*, respectively. The differences in the antigenic response between their study and our findings may be due to differences in (i) the route of inoculation of the animals and the infecting dose, (ii) assay conditions used for blastogenic responses, and (iii) the type of antigens used.

The depressed mitogenic responses during early stages of immunization with *H. capsulatum* could be attributed to (i) dilution of lymphocytes in the spleen with cells unreactive to mitogens, (ii) modification of T cells in such a way that they were less responsive to mitogens, (iii) induction of nonspecific suppressor T cells, (iv) activation of macrophages which depressed T-cell function non-specifically, or (v) competition at the receptor level prior to the development of memory cells.

In our studies the suppression of mitogenic response coincided with splenomegaly, the increased cellularity and spontaneous [^{3}H]thymidine uptake (data not shown) which corresponded to proliferative responses to specific antigens. A diluting-out effect thus provides at least a partial explanation for the loss of reactivity to mitogens. *H. capsulatum* infection stimulates the migration of T cells from the thymus to the thymus-dependent areas of the peripheral lymphoid tissues (4). Since these cells have already been activated and transformed into blastoid cells, they may be unable to take up any further [^{3}H]thymidine on exposure to T cell mitogens (17). Nonspecific T-cell suppressor activity has been described in mice infected with *T. brucei* (13), *Mycobacterium bovis* (27), and also with *H. capsulatum* (3, 4). Nickerson et al. (16) demonstrated two populations of spleen cells capable of suppressor function in their model of disseminated histoplasmosis. One population was identified as T cells, and the other suppressor population had macrophage-like properties. Alternatively, soluble products released by infected macrophages or the organisms themselves could inhibit the function of other cells participating in proliferative responses. In terms of antigenic competition, initial stimulation of the immune system by fungal antigens may stimulate suppressor cells or inhibitors which regulate the response to a second stimulus. As the antigenic load reduces, the cells recover their capability to respond to other stimulants. The continued specific blastogenic response to *H. capsulatum* antigens seems to be the result of initiation and persistence of memory cells.

The studies on lymphocyte functions in vitro have been conducted with histoplasmin, whole cells, autolysates from yeast cells, or cell wall fractions (1, 6, 7). To our knowledge, a defined subcellular antigen has not been used before to study the lymphoproliferative responses in histoplasmosis. The gradient-purified ribosomal antigens used in this study were very effective in detecting a blastogenic response of primed cells. This observation correlates with our earlier findings (19) on the use of ribosomal antigens in demonstrating delayed hypersensitivity and macrophage migration inhibition factor production in immunized mice.

A study of the relationship between this nonspecific suppression to mitogenic responses and the development of protective immunity to histoplasmosis warrants further characterization of the lymphocyte subpopulations at different time intervals after immunization.

ACKNOWLEDGMENTS

This investigation was supported in part by Public Health Service research grant AI-16158 from the National Institute of Allergy and Infectious Diseases.

We thank Barbara Reichert for excellent secretarial assistance and Tom Menten for statistical analysis of the data.

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