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Modification of Macrophage Phagocytosis in Murine Cryptococcosis

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Normal C57BL/6J peritoneal cells exhibited a decreased phagocytosis when cultured with a cell wall antigen of *Cryptococcus* sp. and lymphocytes from mice infected with *C. neoformans*. Direct cell-to-cell interaction was not required in that supernatant fluids from cultures of lymphocytes from infected animals could be incubated with normal macrophage monolayers to give comparable suppression. The induction of the suppressor factor required specific cryptococcal antigen; however, suppression at the macrophage level was nonspecific in that the phagocytoses of *C. neoformans* and *Saccaromyces cerevisiae* were both decreased. Suppression appeared with lymphocyte supernatants taken from mice infected for 14 days and thereafter. The factor was heat stable, trypsin sensitive, and allospecific.

Macrophages play a critical role in immune induction. This is an accessory function, including uptake, catabolism, and presentation of antigen to responding lymphocytes, and argues for a fundamental role for phagocytes in immune stimulation and avoidance of tolerance. The helper role of macrophages includes their ability to release regulatory molecules that can modify lymphocyte proliferation and differentiation. Back regulation by distinct subpopulations of T lymphocytes is also observed where by-products of stimulated lymphocytes can regulate macrophage tumoricidal activity (17).

It has been shown in our laboratory that mice injected with *Cryptococcus neoformans* demonstrate decreased immune responsiveness. During the disease process, mice infected with *C. neoformans* exhibited depressed humoral immunity and depressed cell-mediated responses to mitogens, to specific antigen, and to a T-lymphocyte-dependent antigen (sheep erythrocytes). Both adherent and nonadherent suppressor cells were capable of inhibiting lymphocyte transformation responses to both specific and nonspecific antigens (17) (R. A. Blackstock and N. K. Hall, submitted for publication).

The participation of the macrophage in altered immune states induced by mycotic disease has been investigated, as has the potential influence of lymphocytes on the modulation of macrophage functions (1). This paper describes a third suppressor cell which we have detected in our model of murine cryptococcosis. We have detected the suppression of macrophage phagocytosis in systems where lymphocyte-macrophage interactions were evaluated. The suppression observed is attributed to the release of a lymphokine from T lymphocytes taken from the spleens of infected animals.

MATERIALS AND METHODS

Reagents. Tissue culture medium (minimum essential medium with Eagle salts [MEM], no. 320-1090), 7.5% sodium bicarbonate (no. 670-5080), antibiotic-antimycotic (no. 600-5240), 200 mM glutamine (no. 320-5039), penicillin and streptomycin (100 U/ml and 100 μg/ml; no. 600-5145), Dulbecco phosphate-buffered saline (PBS; no. 450-1300), Hanks balanced salt solution (HBSS; no. 310-4170), incomplete Freund adjuvant (no. 660-5720), and newborn calf serum (no. 617H1) were purchased from GIBCO Laboratories, Grand Island, N.Y. Complete MEM contained MEM, 0.75% sodium bicarbonate, 20 mM glutamine, 1% antibiotic-antimycotic or 100 U of penicillin per ml and 100 μg of streptomycin per ml, and 10% heat-inactivated newborn calf serum. Difco Laboratories (Detroit, Mich.) was the supplier of mycological agar (no. 0405-17), and thimerosol (E3, 5237) was supplied by Aldrich Chemical Co., Milwaukee, Wis.

Organisms. Three strains of *C. neoformans* were used in this study. Nonencapsulated strain M7 (1) was used in phagocytosis assays to circumvent the inhibition of phagocytosis by capsular polysaccharide. Pseudohyphal strain NU-2P, produced during the coculture of *C. neoformans* with *Acanthamoeba polyphaga* (14), was used to immunize mice since we have previously reported that this organism can induce prolonged survival to challenge with a virulent, encapsulated

strain of *C. neoformans* (5). Encapsulated virulent strain NU-2, originally obtained from the University of Nebraska Medical Center, Department of Microbiology stock cultures, was used to establish infection. *Saccharomyces cerevisiae* was used as a yeast control. This yeast cell was chosen because it was unlikely to share antigenic cross-reactivity with *C. neoformans*. Organisms were maintained in the University of Oklahoma Health Sciences Center stock culture collection, Department of Microbiology and Immunology.

Animals. Male C57BL/6J, BALB/cJ, A/J, CB6F1/J (BALB/c × C57BL/6), and B6AF1/J (C57BL/6J × A/J) were obtained from Jackson Laboratories, Bar Harbor, Maine at 6 to 8 weeks of age and were fed Purina mouse chow and water ad libitum.

Infection. *C. neoformans* NU-2 was grown for 3 days on mycological agar and then washed from the plates with PBS. The yeast cells were adjusted to a concentration of 1×10^4 cells per ml by direct hemacytometer count. A 0.5-ml amount of this suspension was inoculated into the lateral tail vein. Mice were used 7, 14, and 21 days post-inoculation. This dose of cryptococci was determined to kill all animals, with a mean survival of 26 days (Blackstock, unpublished data).

Antigen preparation. *S. cerevisiae* and *C. neoformans* NU-2P were grown on mycological agar for 72 h and washed with sterile distilled water three times. Cells were suspended to a heavy slurry in distilled water and placed with an equal volume of sterile glass beads (Glasperlen, 0.45 to 0.50 mm; no. 54170, VWR Scientific, Houston, Tex.) in a homogenizing flask. The slurry was submitted to three 5 min bursts in a Bronwell MSK homogenizer (Braun) under a constant stream of CO_2. Disrupted cells were decanted and centrifuged at 800 × *g* for 15 min in an RC2B centrifuge (Dupont-Sorvall). The pellet was suspended and lyophilized. This crude cell wall preparation was stored at −20°C and used at a concentration of 100 μg (dry weight) per ml of complete MEM.

Preparation of spleen cells and soluble factors. Spleens were removed aseptically from mice and passed through sterile screens (60 gauge), followed by sequential passage through 20- and 22-gauge needles. Usually, spleen cells were used as a pool of cells taken from five mice per experimental group. When cellular subpopulations were examined, the number of animals per group was increased to 10. The cells were washed three times in cold sterile Hanks balanced salt solution. They were then diluted to a final concentration of 1×10^6/ml for suppressor factor assays and 5×10^5/ml for cell-to-cell interaction assays. For the production of soluble factor(s), spleen cells were suspended at 1×10^6 cells per ml in complete MEM. A 0.5-ml amount of this suspension was incubated with either 0.5 ml of a 200-μg/ml cell wall antigen suspension or 0.5 ml of MEM. Cultures were incubated at 5% CO_2 at 37°C for 72 h. The culture supernatants were harvested after two successive centrifugations at 2,500 rpm and stored at −20°C for no longer than 1 month. Control supernatants consisted of infected spleen cells incubated in the absence of cryptococcal antigen and normal spleen cells incubated with or without cryptococcal antigen.

PC. Peritoneal cells (PC) were obtained from the unstimulated cavities of C57BL/6J mice by a modification of the procedure described by Cohn and Benson (2). Briefly, mice were killed by cervical dislocation, and the abdominal skin was reflected. A 3-ml amount of MEM containing 5 U of heparin per ml and antibiotics (100 U of penicillin per ml and 100 μg of streptomycin per ml) was injected intraperitoneally. Fluid was withdrawn with a Pasteur pipette. Cells were sedimented at 400 × *g* for 10 min, washed once with MEM, and counted by hemacytometer. The cell concentration was adjusted to 2.5×10^5 macrophages per ml. The viability of the macrophages was determined to be greater than 95% by trypan blue exclusion. The viability of adherent macrophages after 3 days of incubation in the presence of supernatants was consistently 96 to 97%.

Macrophage monolayers were prepared in eight-chamber tissue culture slides (Lab-Tek Products, Div. Miles Laboratories Inc., Westmont, Ill.). A 0.5-ml amount of PC was added to each chamber, incubated at 37°C at 5% CO_2 for 90 min, and washed once with prewarmed MEM.

Phagocytosis. Adherent monolayers were overlaid with 0.5 ml of yeast (either *Saccharomyces* sp. or *Cryptococcus* sp.) containing 1.25×10^6 cells per ml for a final adherent cell/yeast ratio of 5:1. Phagocytosis occurred over a 2-h period in 5% CO_2 at 37°C. The monolayers were washed five times with warm MEM and stained with a 1:10 dilution of Giemsa. One hundred cells were assessed to determine the percent phagocytosis (percentage of macrophages which had engulfed one or more yeast cells). Each sample was run in triplicate.

For the detection of the possible modification of macrophage function by lymphocytes, adherent monolayers were overlaid with 2.5×10^5 spleen cells and incubated for 72 h in 5% CO_2 at 37°C. Cell cultures were assessed for the percent phagocytosis by adding *C. neoformans* M7 or *S. cerevisiae* for 2 h of incubation at 37°C in a 5% CO_2 atmosphere. The supernatants (0.3 ml) were pipetted onto macrophage monolayers and incubated for 72 h. Monolayers were washed once with prewarmed MEM, yeast was added for a period of 2 h, and phagocytosis was measured by the procedure outlined above.

Separation of cells by nylon-wool columns. Splenic lymphocytes were separated into nonadherent and adherent subpopulations by a nylon-wool column technique (8). After equilibration of the nylon-wool (Fenwol Lab) column with warmed RPM1 1640 medium (GIBCO) containing 5% heat-inactivated fetal calf serum (GIBCO), 1×10^8 spleen cells in 2 ml of medium were added to the column. After the incubation of the column for 45 min at 37°C, the nonadherent cells were eluted with 10 to 20 ml of warm RPMI 1640 containing 5% fetal calf serum. Adherent cells were removed by vigorously washing the nylon wool in 10 ml of cold RPMI 1640. The two subpopulations recovered were diluted to a concentration of 1×10^6 cells per ml in complete MEM and cultured with and without cryptococcal antigen. Normal peritoneal macrophages in a lymphocyte/macrophage ratio of 10:1 were added to the nonadherent subpopulation cultures to replace accessory cell function. Culture supernatants were obtained by centrifugation and added to individual macrophage monolayers. Monolayers were then assessed for the percent macrophages participating in phagocytosis as previously described.

The adherent and nonadherent subpopulations were

TABLE 1. Phagocytosis after addition of lymphocytes to macrophage monolayer with and without cryptococcal antigen

Macrophage plus	Antigen	% Phagocytosis of:	
		C. neoformans	*S. cerevisiae*
No lymphocytes[a]	−	74 ± 4	50 ± 3
Normal lymphocytes[b]	−	71 ± 2	50 ± 1
Normal lymphocytes	+	73 ± 3	49 ± 2
Infected lymphocytes[c]	−	70 ± 3	51 ± 2
Infected lymphocytes	+	48 ± 3[d]	34 ± 3[d]

[a] Monolayers of C57BL/6J macrophages (1×10^5 cells per ml).

[b] Monolayers of C57BL/6J macrophages (1×10^5 cells per ml) tested with the addition of 2.5×10^5 lymphocytes from normal C57BL/6J mice.

[c] Monolayers of C57BL/6J macrophages (1×10^5 cells per ml) tested with the addition of 2.5×10^5 lymphocytes from C57BL/6J mice infected 21 days previously with 5×10^3 *C. neoformans* NU-2.

[d] $P < 0.005$ when lymphocytes from infected animals incubated without antigen were compared with cultures incubated with antigen (70 ± 3% versus 48 ± 3% and 49 ± 2% versus 34 ± 3%).

analyzed by lymphocyte transformation (6) to confirm the efficiency of the separation procedure. Lymphocytes were cultured with 1:2,000 and 1:4,000 dilutions of phytohemagglutinin (PHA; Difco) and 50 μg of lipopolysaccharide (LPS; Difco), and responses were recorded. The nonadherent subpopulation responded to PHA (typically 20,000 cpm), but had reduced responses to LPS (20,000 cpm reduced to 1,800 cpm). The adherent cells were LPS responsive (13,500 cpm), but had reduced responses to PHA (20,000 cpm reduced to 3,700 cpm). These controls were included each time the cell separation procedure was performed.

Separation of cells by mass cytolosis. A 5-ml amount (2×10^7 cells) of a single-cell suspension of spleen cells obtained from infected or normal mice was mixed with 0.25 ml of rabbit anti-mouse T cell serum (Litton Bionetics, Kensington, Md.) or goat anti-mouse immunoglobulin (IgG) and IgM (Meloy Laboratories, Inc., Springfield, Va.). The cells were incubated for 45 min at 4°C. The cells were washed two times ($200 \times g$ for 5 min) in PBS and suspended in 5 ml of a 1:10 dilution of rabbit complement in RPMI 1640 and incubated for 30 min at 37°C. After an additional three washes in 10 ml of PBS, the cells were suspended in complete MEM. The number of viable cells present in the suspension was determined by trypan blue exclusion. Cells were diluted to a final concentration of 5×10^6 viable cells per ml in complete MEM. Cell subpopulations remaining after this treatment were designated anti-T cells and anti-immunoglobulin-treated cells. The purity of cellular subpopulations was determined as described above. Anti-T cell serum treatment typically reduced PHA responses from 21,000 to 2,100 cpm. Anti-immunoglobulin treatment reduced LPS responses from 19,000 to 1,800 cpm.

Trypsin digestion of suppressor factor. Supernatants prepared from both normal and infected mice were incubated with purified trypsin (Calbiochem-Behring Corp., La Jolla, Calif.) by the technique developed by Nakamura et al. (13). The trypsin solution was prepared by dissolving 100 mg in 100 ml of sterile PBS; then 50 μl was pipetted into a tube containing 1 ml of supernatant prepared from infected or normal spleen cultures. This preparation was incubated for 30, 60, and 120 min in a 37°C water bath. The heated solutions were pipetted onto prepared macrophage monolayers and rinsed once with MEM, and a suspension of *C. neoformans* was added to the monolayers as previously described. The macrophages were assessed for phagocytic ability.

Temperature sensitivity of suppressor factor. Supernatants obtained from infected spleen cell homogenates cultured with and without cryptococcal cell wall antigen were incubated for 15 and 30 min in a 56°C water bath. Macrophage monolayers were then overlaid with unheated supernatant and the three preheated supernatants. These cultures were incubated for 24 h at 37°C in a 5% CO_2 atmosphere; monolayers were washed once with prewarmed MEM and then incubated with a suspension of *C. neoformans*. The percentage of *C. neoformans* phagocytosis was determined from Giemsa-stained slides.

Allospecificity. The allospecificity of the suppressor factor was determined by methods developed by Eisenthal et al. (4). Spleens were aseptically removed from infected C57BL/6J, BALB/cJ, CB6F1/J, and B6AF1/J mice. Individual spleen cell suspensions were prepared as previously discussed. The four culture supernatants were collected and then pipetted onto individual macrophage monolayers prepared from PC obtained from C56BL/6J, BALB/cJ, CB6F1/J, and A/J mouse strains. Phagocytic activity was determined as previously described.

Statistical analyses. The differences between two means of experimental groups were compared with the Student *t* test for unpaired data. When the *P* value was less than 0.05, differences were considered significant. The percent phagocytosis was expressed as the means of triplicate samples plus or minus the standard error of the mean.

TABLE 2. Modification of macrophage phagocytosis by supernatants of lymphoid cells in the presence or absence of cryptococcal antigen

Lymphocytes	Antigen	% Phagocytosis of:	
		C. neoformans	*S. cerevisiae*
Normal[a]	−	77 ± 2	69 ± 4
Normal	+	74 ± 3	76 ± 2
Infected[b]	−	67 ± 2	73 ± 2
Infected	+	38 ± 4[c]	37 ± 3[c]

[a] Supernatants of spleen cells taken from normal C57BL/6J mice after culture for 72 h in the presence or absence of cryptococcal antigen were added to macrophage monolayers.

[b] Supernatants of spleen cells taken from C57BL/6J mice infected 21 days previously with 5×10^3 *C. neoformans* NU-2.

[c] $P < 0.05$ as compared with lymphocytes from infected animals incubated without antigen.

TABLE 3. Kinetics of the appearance of a suppressor factor for macrophage phagocytosis in supernatants of lymphocyte cultures

Supernatant	Antigen	% Phagocytosis of *C. neoformans* at (wk):		
		1	2	3
Normal[a]	−	74 ± 3	65 ± 3	56 ± 2
Normal	+	69 ± 2	68 ± 4	55 ± 2
Infected[b]	−	74 ± 2	73 ± 2	64 ± 3
Infected	+	71 ± 3	39 ± 3[c]	38 ± 2[c]

[a] Lymphocytes from normal C57BL/6J mice.
[b] Lymphocytes from C57BL/6J mice infected 7, 14, or 21 days previously with 5×10^3 *C. neoformans* NU-2.
[c] Statistically different from phagocytosis in the presence of lymphocytes from infected animals incubated without antigen.

RESULTS

Effect of addition of lymphocytes upon phagocytosis by normal macrophages. Peritoneal exudate cells from normal C57BL/6J mice were used to prepare macrophage monolayers. *C. neoformans* and *S. cerevisiae* at a concentration of 1.25×10^6 yeast cells per ml were added to individual monolayers, and phagocytosis was measured. The percent phagocytosis with *C. neoformans* and *S. cerevisiae* was 74 and 50%, respectively. The addition of normal or infected lymphocytes did not alter phagocytosis when they were added to the macrophage monolayers (Table 1).

Lymphocyte-macrophage cultures were incubated with 100 μg of cryptococcal cell wall antigen per ml. The addition of antigen to cultures containing only normal macrophages or normal macrophage and normal spleen cells did not alter phagocytosis. However, the addition of antigen to cultures of macrophages and lymphocytes taken from *C. neoformans*-infected mice decreased the phagocytosis of *C. neoformans* and *S. cerevisiae* significantly ($P < 0.005$). Results similar to these were observed consistently in five similar experiments. Significant differences were found in each experiment with P values of 0.01 or less.

From the preceding data, it was concluded that a lymphocyte-macrophage interaction alone was not sufficient for the suppression of phagocytosis. Suppression was produced only when lymphocyte-macrophage cultures were stimulated with cryptococcal antigen.

Suppression of macrophage phagocytosis by supernatants of antigen-stimulated lymphocytes. Normal or infected lymphocytes were cultured in vitro with cryptococcal antigen. Seventy-two hours later, the supernatants were collected and added to normal macrophage monolayers. The effect of these supernatants on normal macrophage phagocytosis was measured. The results from this experiment (Table 2) suggested that a soluble suppressor factor was present in the supernatants from antigen-stimulated, infected lymphocytes as phagocytosis was significantly decreased ($P < 0.05$) by this supernatant preparation. The decrease in phagocytosis did not occur with lymphocyte culture supernatants from the spleen cells of normal mice or with supernatants collected from infected spleens incubated without added cryptococcal antigen. This latter observation ruled out the possibility that capsular polysaccharide which may be present in supernatants of cultured lymphocytes was suppressive.

When a cell wall antigen of *S. cerevisiae* was used to stimulate lymphocytes from normal and infected animals, the supernatants collected from these cultures did not alter phagocytosis (data not shown).

Time course for production of soluble suppressor factor. Animals were examined over the first 3 weeks of cryptococcal infection to determine when the soluble suppressor could first be detected. The appearance of the factor was measured by placing antigen-stimulated lymphocyte culture supernatants from 7-, 14-, and 21-day-infected mice on normal macrophage monolayers and measuring phagocytosis with *C. neoformans* (Table 3). The soluble factor was not detected until 14 days into the infection and remained at 21 days of infection.

Characterization of the subpopulation of spleen cells responsible for production of suppressor

TABLE 4. Macrophage phagocytosis in the presence of supernatants from nylon-wool-column-separated lymphoid cells

Lymphocytes	Antigen	% Phagocytosis of *C. neoformans*
Unseparated[a]	−[b]	79 ± 5
Unseparated	+	52 ± 4[c]
Adherent[d]	−	76 ± 4
Adherent	+	72 ± 5
Nonadherent[e]	−	77 ± 2
Nonadherent	+	53 ± 1[c]

[a] Supernatants of spleen cells from C57BL/6J mice infected 21 days previously with 5×10^3 *C. neoformans* NU-2.
[b] Indicates presence or absence of cryptococcal cell wall antigen in lymphoid cell cultures.
[c] $P < 0.01$ when compared with identical cultures incubated without antigen.
[d] Supernatants of nylon-wool-adherent spleen cells from C57BL/6J mice infected 21 days previously with 5×10^3 *C. neoformans* NU-2.
[e] Supernatants of nylon-wool-nonadherent spleen cells taken from C57BL/6J mice infected 21 days previously with 5×10^3 *C. neoformans* NU-2.

TABLE 5. Macrophage phagocytosis in the presence of supernatants from lymphoid cells separated by mass cytolysis

Lymphocytes	Antigen	% Phagocytosis of *C. neoformans*
Untreated[a]	−	69 ± 3
Untreated	+	46 ± 4[b]
Anti-T[c]	−	70 ± 2
Anti-T	+	65 ± 2
Anti-immunoglobulin[d]	−	71 ± 3
Anti-immunoglobulin	+	47 ± 1[b]

[a] Supernatants of spleen cells from C57BL/6J mice infected 21 days previously with 5×10^3 *C. neoformans* NU-2.

[b] Significant suppression ($P < 0.005$) when compared with cultures incubated without antigen.

[c] Supernatants of anti-T-cell antisera-treated spleen cells from C57BL/6J mice infected 21 days previously with 5×10^3 *C. neoformans* NU-2.

[d] Supernatants of anti-immunoglobulin-treated spleen cells from C57BL/6J mice infected 21 days previously with 5×10^3 *C. neoformans* NU-2.

factor. (i) Separation by nylon-wool columns. The ability of spleen cells to adhere to nylon-wool columns was used as one means of subpopulating lymphocytes from cryptococcus-infected mice. Results (Table 4) showed that the factor was produced by a nonadherent spleen cell subpopulation, as phagocytosis was reduced from 77 to 53% ($P < 0.01$) by supernatants from the nonadherent fraction. Decreased phagocytosis did not occur with supernatants prepared from the antigen-stimulated adherent population. Similarly treated cells and supernatants from normal animals showed no differences in ability to alter phagocytosis (data not shown).

(ii) Separation by mass cytolysis. The lysis of T cells with anti-T cells or that of B cells with anti-immunoglobulin and complement was used to separate the lymphocyte subpopulations on the basis of their membrane characteristics. The results of this investigation are contained in Table 5. A decrease in phagocytosis occurred with macrophages treated with supernatants prepared from the untreated ($P < 0.005$) and anti-immunoglobulin-treated ($P < 0.005$) cell populations. The supernatant prepared from the anti-T-treated cell population did not influence the phagocytic capability of normal macrophages (Table 5). Parallel studies of cellular subpopulations and supernatants from normal animals showed no influence on the phagocytic ability of normal macrophages (data not shown).

Characterization of the suppressor factor. The temperature sensitivity and trypsin digestion of the suppressor factor were studied. The suppressor factor was found not to be sensitive to 30 min of heating at 56°C because it remained as active as the control supernatant. The soluble factor was sensitive to 30 min of treatment with trypsin (Table 6). Trypsin-treated supernatants from nonantigen-induced infected spleen cells did not alter the macrophage's ability to respond to the lymphokine. Thus, the presence of trypsin in the supernatant did not alter the phagocytosis assay.

Allospecificity of suppressor factor. Evidence of allospecificity was obtained when lymphocyte culture supernatants prepared from infected mouse strains which were genetically different from C57BL/6J were investigated for the production of the suppressor factor. Suppressor factor produced by the C57BL/6J strain was able to suppress macrophage phagocytosis of C57BL/6J mice, B6AF1/J, and CB6F1/J ($P < 0.005$, $P < 0.005$, and $P < 0.01$, respectively), but did not suppress the phagocytosis of macrophages from genetically unrelated mice. In a similar manner, antigen-induced factors from B6AF1/J suppressed C57BL/6J, B6AF1/J, and CB6F1/J target cells ($P < 0.005$, $P < 0.005$, and $P < 0.01$, respectively), and the CB6F1/J factor suppressed C57BL/6J, B6AF1/J, and CB6F1/J macrophages ($P < 0.01$, $P < 0.01$, and $P < 0.005$, respectively). These studies showed a need for genetic identity between the lymphocytes used as the source of the suppressive lymphokine and the macrophage which was the target for its activity. BALB/cJ mice did not produce a soluble suppressor factor for macrophage phagocytosis. In addition, the macrophages of this mouse strain were unresponsive to the suppressor factor produced by the (BALB/c × C57BL/6)F1 hybrid (Table 7).

DISCUSSION

Generalized immunosuppression is a major characteristic of experimental and clinical cryptococcosis. In previous reports from our laboratory, we have demonstrated depressed immune responsiveness (both humoral and cellular). In vitro lymphocyte transformation assays revealed the presence of both adherent and nonadherent suppressor cell populations (B. E. Robinson, N. K. Hall, G. S. Bulmer, and R. Blackstock, *Mycopathologia*, in press). Both of these suppressor cells were antigen nonspecific (Robinson et al., in press).

The complex interaction between lymphocytes and macrophages may serve a synergistic function in primary immunity against cryptococcosis. Since phagocytosis alone is not sufficient to prevent infection by the yeast (3, 9), the reactivity of the T cells is an important host defense mechanism. The suppression of this T-cell activity and T cell-macrophage interaction is an important consideration in the immune response to cryptococcosis.

TABLE 6. Characterization of soluble suppressor to macrophage phagocytosis

Lymphocyte source	Antigen	% Phagocytosis of M7						
		56°C at (min):			With trypsin (50 μg/ml) at 37°C at (min):			
		0	15	30	0	30	60	120
Infected[a]	−	64 ± 2	64 ± 3	62 ± 3	70 ± 2	72 ± 3	71 ± 3	71 ± 3
Infected	+	35 ± 1[b]	35 ± 3[b]	33 ± 1[b]	49 ± 3[b]	69 ± 1	67 ± 2	67 ± 3

[a] Supernatants of spleen cells (C57BL/6J) infected 21 days previously with 5×10^3 *C. neoformans*.
[b] Significant suppression, $P < 0.001$.

Normal macrophage phagocytosis was measured in the C57BL/6J mouse strain. Although the percentage of cryptococcal yeast cells phagocytized varied slightly from experiment to experiment, the established level of 65 to 77% was easily reproducible. *Saccharomyces* sp. was phagocytized less efficiently, with a maximal percent phagocytosis of 66%. The addition of splenic lymphocytes from normal or infected animals had no effect on the phagocytosis of either organism.

The addition of cryptococcal cell wall antigen to lymphocyte-macrophage cultures caused a significant decrease in PC phagocytosis of both *C. neoformans* and *S. cerevisiae*. Suppression ($P < 0.005$) was observed when lymphocytes of infected mice were present during the 72-h incubation or when clarified supernatants from antigen-induced lymphocytes were overlaid onto normal PC monolayers. The suppression only occurred when lymphocytes from infected animals were used and when cryptococcal antigen was present in lymphocytes cultured. The suppression of phagocytosis did not result when lymphocytes were obtained from normal mice. Because the supernatants of spleen cells harvested from infected mice without the addition of cryptococcal cell wall antigen did not inhibit phagocytosis, we were able to rule out the possibility that cryptococci or their products (present in these spleen cell preparations) were responsible for the phagocytic inhibition observed.

The suppressor factor was stable at 56°C at 2 h and sensitive to 30 min of trypsin digestion (Table 6), characteristics consistent with the physiochemical properties of antibody. However, the suppressor factor was produced by T cells (Tables 4 and 5), eliminating the possibility of it being humoral antibody.

Many T-cell factors that suppress other aspects of macrophage function have been described. Zembala and Asherson (18) reported a T-cell factor which inhibits the transfer of skin reactivity to irradiated recipients by sensitized lymph node cells. This factor was antigen specific and effective in the efferent phase of the immune response. Unlike the cryptococcal suppressor factor described in this report, their factor was resistant to trypsin digestion. Greene et al. (7) and Moorhead (11) described a factor which suppressed antigen-specific delayed-type hypersensitivity (DTH) which was similar to the factor described by Zembala and Asherson. Heat stability and trypsin sensitivity were not assessed in either study. Liew and Chan-Liew (10) isolated a factor which suppressed both the afferent and efferent phases of DTH to sheep erythrocytes. The source of their factor was a T cell, and the factor was stable to heating at 56°C for 30 min. Trypsin sensitivity was not reported.

Pierce and co-workers (15) investigated the

TABLE 7. Lymphokine modification of phagocytosis of *C. neoformans* by macrophages derived from various mouse strains

Supernatant	Antigen	% Phagocytosis of *C. neoformans* by:				
		C57BL/6J	B6AF1/J	CB6F1/J	A/J	BALB/cJ
C57BL/6J[a]	−	86 ± 2	83 ± 1	72 ± 2	83 ± 3	82 ± 2
C57BL/6J[b]	+	66 ± 2	65 ± 1	63 ± 3	83 ± 4	83 ± 2
B6AF1/J[a]	−	84 ± 1	82 ± 2	70 ± 1	82 ± 3	81 ± 3
B6AF1/J[b]	+	64 ± 1	68 ± 2	63 ± 2	83 ± 3	79 ± 1
CB6F1/J[a]	−	72 ± 2	70 ± 1	74 ± 3	84 ± 2	81 ± 3
CB6F1/J[b]	+	63 ± 3	63 ± 2	61 ± 1	82 ± 1	78 ± 2
BALB/cJ[a]	−	81 ± 1	81 ± 3	70 ± 2	83 ± 1	82 ± 1
BALB/cJ[b]	+	83 ± 1	79 ± 1	68 ± 2	82 ± 1	84 ± 1

[a] Supernatant obtained by incubation of lymphocytes from 21-day-infected mice.
[b] Supernatant obtained by incubation of lymphocytes taken from 21-day-infected mice plus cryptococcal antigen.

effects of concanavalin A-activated suppressor T cells and a secreted mediator. This factor, soluble immune response suppressor (SIRS), acted on the macrophage to decrease B cell proliferation or differentiation into antibody-secreting cells or both. This factor differed from the other factors discussed because the macrophage itself was not suppressed but was activated by SIRS to release other suppressive mediators. SIRS was produced by lymph node cells and was not antigen specific. Physiochemical characterization showed SIRS to be a glycoprotein, stable at 56°C for 60 min and trypsin sensitive. Although the physiochemical characteristics of SIRS were similar to those of the factor described here, the methods of induction and the activity of the factor were different.

The suppressor cell population and factor generated in this study share characteristics with other nonspecific suppressor T cells and factors. Like SIRS, the target cell for the suppressor factor was the macrophage. Both factors are required at macrophage culture initiation, but their effects are not observed until after day 1 of culture for our factor and day 3 of culture for SIRS. Both factors were shown to be produced by T cells. Unlike SIRS, the cryptococcal suppressor factor requires histocompatibility with its macrophage target to detect its activity. This characteristic is shared with the factors described by Rich and Rich (16) and Eisenthal et al. (4), which bore determinants encoded by the I region of the H-2 complex. More detailed genetic studies in H-2 congenic mice will be required before we can establish the locus responsible for the genetic restriction which we have detected. In our studies, BALB/c mice did not produce a detectable lymphokine nor did they respond to a (BALB/c × C57BL/6J)F1 lymphokine. BALB/cJ mice are more susceptible to infection by *C. neoformans*. This increased susceptibility may be a feature of other BALB/cJ background genes or H-2^d genes. The increased susceptibility of BALB/c mice may or may not be related to the inability to produce the suppressor factor studied here.

Because the suppressor factor here was not produced when a cell wall antigen of *S. cerevisiae* was used to stimulate lymphocytes obtained from infected animals, we believe that the factor is a product of the interaction of the cryptococcal cell wall antigen and lymphocytes obtained from animals infected with *C. neoformans*.

Kinetic experiments showed that the suppressor cell population appeared in week 2 of cryptococcal infection (Table 3). Both afferent (12) and efferent (Robinson et al., in press) suppressor cells or factors with different times of appearance or both have been discovered in the immune response to cryptococcosis. Murphy and Moorhead (12) injected cryptococcal antigen intravenously into mice to induce a population of lymph node cells that could, upon transfer, specifically suppress the DTH response to cryptococcal antigen and the splenic T lymphocytes responsible for the in vitro inhibition of the growth of *C. neoformans*. The suppressor cell that they found was detected in lymph nodes at 5 days after antigen injection, and it remained there for 7 days. It was not found in the spleens of tolerized mice. Mass cytolysis and nylon-wool subpopulation experiments showed that the suppressor cell was a T lymphocyte. The percent suppression of the DTH response was dependent upon the amount of antigen injected. This T-lymphocyte subpopulation may differ from the one found in our study since the lymph node suppressor cell population appeared 5 days after antigen injection, whereas the splenic suppressor cell population in this study appeared after 14 days of a cryptococcal infection. The lymph node suppressor cell specifically suppressed a DTH reaction and splenic T lymphocytes responsible for inhibiting the in vitro growth of *C. neoformans*, whereas the suppressor cell in this study nonspecifically suppressed the phagocytic capability of resident peritoneal macrophages. We have not studied its effect on the DTH reaction. Other suppressor cells described by our group (Robinson et al., in press) suppressed lymphocyte transformation responses to both specific and nonspecific antigens. The suppressive activity of these suppressor cells did not require antigen induction for their expression.

Although the suppression of phagocytosis that we observed was consistent from experiment to experiment, the magnitude of the suppression has not been great (i.e., 20 to 35%). We believe that this may result from the selective suppression of a particular subset of macrophages (perhaps those which are Ia positive). Further experiments are currently under way in our laboratory to answer this question. If the effect is upon a specific macrophage subset, the significance of this finding for the disease process may be related to the inactivation of a macrophage subset which is essential for the induction of protective T-cell immunity rather than a decrease in phagocytosis per se.

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