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Induction of a Macrophage-Suppressive Lymphokine by Soluble Cryptococcal Antigens and Its Association with Models of Immunologic Tolerance

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Soluble extracts of *Cryptococcus neoformans* were examined for their ability to induce a macrophage-regulatory T-suppressor cell known to appear in the spleens of mice infected with cryptococci. Suppressor cells were induced by injection of extracts of encapsulated or thinly encapsulated strains of cryptococci. Dose-response analysis showed that as little as 25 μg of soluble capsular polysaccharide antigen could induce significant suppressor cell activity, with maximum suppression occurring at a dose of 100 μg. The suppressor cells appeared within 1 week of injection of antigen and persisted for at least 2 months. Suppressor cells were induced in animals given tolerogenic doses of levan, human gamma globulin, and soluble capsular polysaccharide antigen. When these same antigens were administered in immunogenic form, no suppressor cell activity was detected. Therefore, the suppressive mechanism was common to models of immunologic tolerance and was not unique to cryptococcal disease or cryptococcal capsular polysaccharide antigen. The phagocytosis-inhibiting lymphokine produced by the suppressor cell population completely inhibited the phagocytic activity of only a portion of peritoneal exudate cells. Other macrophages in the population were not totally inhibited but exhibited a reduction in the number of yeast cells engulfed.

Our laboratory previously reported (4, 34) that animals infected with the yeast *Cryptococcus neoformans* developed both T-cell and adherent suppressor cells which limited immune responses to cryptococcal antigen and to an antigen unrelated to the yeast as evaluated by lymphoproliferative assays. Another suppressor cell described by our group (26) was antigen specific and exhibited the unique activity of inhibiting the phagocytic capabilities of macrophages. The suppressor cell was identified as a T lymphocyte which secreted a macrophage-suppressive lymphokine into the medium when cultured with specific cryptococcal antigen. The factor which was secreted was allospecific in its activity, since genetic identity between the lymphocyte which secreted the factor and the macrophage which responded to the factor was required. Only a portion of the macrophage population responded to the factor (about 20%). For this reason, the responsive cell was thought to be a particular subset of macrophage or at a discrete stage of differentiation.

Murphy and co-workers (27, 28, 30–32) described an antigen-specific suppressor pathway which occurred after the injection of solubilized cryptococcal antigen. A first-order, afferent suppressor cell (Ts1) appeared in the lymph nodes of mice 5 to 7 days after the administration of antigen. This first-order cell induced a second-order, efferent suppressor cell (Ts2), which appeared in the spleen 7 days after infusion of Ts1 cells. Ts1 cells were T cells bearing Lyt-1 and I-J markers on their surface, whereas the Ts2 population was composed of T cells having a Lyt-2$^+$ I-J$^+$ phenotype. Suppressor factors which were responsible for the regulatory effects of the suppressor cells were extracted from the cells without the need for prior antigenic stimulation. Ts1 cells were bifunctional in that they not only induced Ts2 cells but they inhibited the induction of effector cells for delayed-type hypersensitivity. Ts2 cells functioned by inhibiting the activity of delayed-type hypersensitivity effectors after they were established. The suppressor factors (TsF1 and TsF2) could be differentiated by the fact that TsF1 could bind cryptococcal antigen and TsF2 could not.

The current investigation was designed to determine whether the macrophage-inhibitory suppressor cell population previously found in *C. neoformans*-infected animals could be induced with soluble cryptococcal antigens. In addition, we wanted to evaluate the relationship of this immunosuppressive pathway to classic models of immunologic tolerance.

MATERIALS AND METHODS

Animals. C57BL/6J male mice were purchased from Jackson Laboratory, Bar Harbor, Maine. The animals arrived when they were 6 weeks old and were used in experiments when they were 8 to 12 weeks old. Mice were housed in the University of Oklahoma Health Sciences Center Animal Resource Facility which is approved by the American Association for the Accreditation of Laboratory Animal Care.

Organisms. The strains of *C. neoformans* used in this investigation were strain NU-2, a virulent human isolate originally obtained from the University of Nebraska Medical School, Department of Medical Microbiology; a pseudohyphal variant of NU-2 (NU-2P) isolated in the laboratories of G. S. Bulmer, University of Oklahoma Health Sciences Center, by coculture with the soil amoeba, *Acanthamoeba polyphaga* (33); and strain M7, a nonencapsulated mutant of *C. neoformans* originally described by Bulmer and Sans (9). All of the organisms were maintained by subculture on mycologic agar.

Reagents. Dulbecco minimal essential medium (DMEM; catalog no. 430-1600) and Dulbecco phosphate-buffered saline (PBS; catalog no. 450-1300) were purchased from GIBCO Laboratories, Grand Island, N.Y. Hazelton Research Products, (Denver, Pa.) was the supplier of fetal calf

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serum (catalog no. 12-10378). Brewer thioglycolate medium (catalog no. 0236-01) and mycologic agar (catalog no. 0405-17) were obtained from Difco Laboratories, Detroit, Mich. Incomplete Freund adjuvant (catalog no. RIA 871) was purchased from Calbiochem-Behring, La Jolla, Calif.

Antigens. Human gamma globulin (HGG; catalog no. G 4386) and levan (catalog no. L 4884) were purchased from Sigma Chemical Co., St. Louis, Mo. Urea extracts of fungi were prepared by the procedure of Bennett (3). Cryptococcal capsular polysaccharide was prepared as described by Farhi et al. (17) and is referred to as soluble capsular polysaccharide antigen (SCPA). Yeast cells (strain NU-2) were harvested from 72-h mycologic agar plate cultures with sterile PBS. After centrifugation at 400 × *g* for 10 min, the cell pellet was suspended in acetone and kept at 25°C with constant stirring. The acetone was changed every 8 h for a total of six changes, and the cells were dried in vacuo. Dried cells (10 g) were suspended in distilled water to a final concentration of 10% (vol/vol). Glass beads (20 g; 0.45 mm in diameter) were added to the cells, and the suspension was sonicated for 15 min with a Sonifier cell disrupter (model W140; Heat Systems-Ultrasonics, Inc., Plainview, N.Y.) at 50 power units with a standard tip. This procedure effectively removed capsular material from the cells without disruption of the yeast. After centrifugation at 12,000 × *g* for 30 min, the supernatant was collected and 10% sodium acetate, 1% acetic acid, and 2 volumes of absolute ethanol were added. The mixture was stored at 4°C for 48 h, and the precipitate was collected by centrifugation at 1,000 × *g* for 15 min. The precipitate was washed in 200 ml of absolute ethanol and collected by centrifugation at 1,000 × *g* for 15 min. The precipitate was then suspended in distilled water at a concentration of 1%. After centrifugation at 22,000 × *g* for 60 min, the supernatant material was deproteinized by eight extractions with chloroform–*n*-butanol (4:1 [vol/vol]). The deproteinized material was reprecipitated by the addition of 10% sodium acetate, 1% acetic acid, and 3 volumes of absolute ethanol. After incubation for 48 h at 4°C, the precipitate was collected by centrifugation at 1,000 × *g* for 15 min. The SCPA was suspended in distilled water and dialyzed against eight changes of distilled water. The SCPA suspension was lyophilized and stored in a tightly capped container at −20°C.

Chemical analysis. Protein content of extracts was determined by the procedure of Lowry et al. (24). The protein content of ultracentrifuged HGG was determined by the procedure described by Kalckar (21). Carbohydrate analysis was done by the phenol-sulfuric acid method of Dubois et al. (15).

Injection of mice. Mice were injected intraperitoneally with cryptococcal extracts so that equivalent amounts (100 μg) of carbohydrate were administered. In some experiments, SCPA was given in various doses up to 100 μg/ml. Immunization with SCPA was accomplished by subcutaneous injection of 0.125 μg of SCPA emulsified in incomplete Freund adjuvant. For induction of immunologic tolerance to HGG, mice were given an intraperitoneal injection of 2.5 mg of deaggregated HGG. The deaggregated protein consisted of the upper one-third of the supernatant after centrifugation of an HGG solution at 100,000 × *g* for 90 min (21). Animals immunized with HGG were given a subcutaneous injection of 100 μg of HGG emulsified in incomplete Freund adjuvant. Tolerance to levan was induced by a single intravenous injection of 1 mg of polysaccharide in 0.5 ml of PBS (25). Mice sensitized to levan were given an intravenous injection of 10 μg of levan in 0.5 ml of PBS. In most experiments, spleen cells were harvested 7 days after antigen injection; however, in some experiments, other times were used. The tolerogenic and immunogenic properties of the levan and HGG injection schedules were confirmed by assay of antibody production in passive hemagglutination assays as described by Johnson et al. (20) and Miranda (25). Tolerant animals were challenged with an immunogenic dose of antigen 1 week after tolerance induction, and serum samples were drawn 7 days later. Immune control animals were bled 7 days after immunization. Antibody production to SCPA was not evaluated because it is our experience that the C57BL/6 mouse strain used in this investigation does not produce antibodies when immunized with cryptococcal vaccines (19). Additionally, the SCPA of *C. neoformans* is known to be nonspecifically immunosuppressive (8).

Production of lymphokine. The production of the phagocytosis-inhibiting lymphokine was similar to that described previously (26) with some modifications. Spleen cells were dispersed by pressing through a 60-mesh wire screen and passage through a 22-gauge needle. The cells were washed three times in PBS and suspended (5×10^6 cells per ml) in DMEM containing 10% fetal calf serum (FCS) and 1% antibiotics. A urea extract of cryptococcal strain NU-2P was used at a final concentration of 1:100 in cultures of spleen cells taken from animals injected with cryptococcal antigen. The lot of urea extract used in this investigation contained 14 mg of protein and 13.6 mg of carbohydrate per ml. If animals were injected with HGG, 200 μg of HGG per ml was added to the culture medium. For levan-injected mice, 1 μg of levan per ml was used as the in vitro stimulus. Control cultures included spleen cells from antigen-injected mice without antigen in the in vitro culture phase, as well as cultures of normal spleen cells with or without added antigen. The cultures were incubated for 48 h at 37°C in an atmosphere of 5% CO_2. After centrifugation at 400 × *g* to remove cells, the supernatants were filtered through a 0.22-μm filter and layered over macrophage monolayers (see below).

Preparation of macrophage target cells. Macrophages were harvested from the peritoneal cavities of normal mice or from mice injected 5 days previously with 0.5 ml of 10% aged Brewer thioglycolate medium by a modified procedure of Cohn and Benson (11). DMEM (3 ml) containing 10 U of heparin per ml was injected into the peritoneal cavity, and the abdomen was gently massaged. The fluid was withdrawn with a 22-gauge needle and syringe. The cells were pelleted at 200 × *g* for 10 min and suspended in DMEM–10% FCS at a concentration of 1.5×10^6/ml.

Phagocytic assay. For assessment of phagocytic function, monolayers of macrophages were prepared in eight-chambered tissue culture slides (Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.). Macrophages (1.25×10^4 per well) were added to the wells, and the slides were incubated for 90 min at 37°C with 5% CO_2. The medium was removed from the wells, and 0.3 ml of lymphokine-containing or control supernatants was added to each of four replicate wells. After additional incubation for 48 h, the supernatants were removed. A 0.5-ml portion of a *C. neoformans* M7 suspension (10^6/ml) in DMEM–10% FCS was added to each well. The slides were again incubated for 2 h. The medium was removed, and the monolayers were washed five times to remove extracellular yeast cells. Slides were washed by removing the top section of the chamber and gently dipping the slide in a beaker of warm (37°C) PBS. This procedure prevented loss of cells from the monolayers. Examination of the average number of cells in 10 oil emer-

sion fields showed that equal numbers of cells were present after incubation with all supernatants. Monolayers were air dried and stained with Camco Quick stain (Cambridge Chemical Products, Fort Lauderdale, Fla.) The percent phagocytosis was determined by examination of the percentage of cells which contained one or more yeast cells. Values for four replicate determinations were averaged. For evaluation of the average number of yeast cells inside each phagocyte, the number of individual yeast cells inside 50 phagocytic cells was determined. The mean of four replicate tests was determined. The total phagocytic capacity (TPC) of the macrophage monolayer was calculated by the following formula: TPC = % phagocytosis × the average number of yeast cells per macrophage.

Statistical analysis. The difference between experimental groups was evaluated statistically by the Student's *t* test. Data having a *P* value of 0.05 or less were considered to be significant. Each experiment was performed at least three times with consistent results.

RESULTS

Because our previous results showed that a phagocytosis-inhibiting lymphokine was produced by antigen-stimulated lymphocytes of infected animals, it was of interest whether this activity would occur in animals injected with solubilized cryptococcal antigen. Animals were injected with urea extracts of encapsulated and nonencapsulated strains of *C. neoformans* as well as the SCPA of the encapsulated strain NU-2. Initially, the animals were injected with enough extract to give equivalent doses of 100 μg of carbohydrate. This dose was chosen because amounts in this range were known to induce suppressor cell activity in other systems (28). After 1 week, the animals were sacrificed and their spleen cells were placed in culture alone or with cryptococcal antigen (1:100 dilution of a urea extract of strain NU-2P). The urea extract of NU-2P was used at a dose known to produce lymphocyte proliferation in mice immunized to *C. neoformans* and is known to cross-react with all of the cryptococcal strains used in this study. After 2 days of culture, the supernatants were harvested and placed on macrophage monolayers to test their effect on the phagocytic activity of the macrophage. Phagocytosis assays performed 48 h later used the nonencapsulated strain M7 as the engulfed particle. When animals were injected with urea extracts of strains NU-2P or NU-2, suppression of phagocytosis by antigen-stimulated spleen cell culture supernatants was 37 and 46%, respectively. No suppression occurred with supernatants from mice injected with the nonencapsulated mutant M7. Injection of SCPA was also effective in inducing suppressor cell activity, with a resultant 37% reduction in phagocytosis (Table 1).

An analysis of the minimal dose of SCPA required to elicit suppressor cell activity is shown in Table 2. Mice were injected 7 days before sacrifice with 12.5, 25, 50, or 100 μg of SCPA. Spleen cell culture supernatants were then prepared and tested for the presence of the phagocytosis-inhibiting lymphokine. Significant suppression occurred when the mice were injected with 25 μg or more of SCPA, with maximal suppression occurring at the 100-μg dose. Normal control supernatants were not suppressive. In other experiments, we used SCPA doses above 100 μg/ml but did not achieve a greater degree of suppression (data not shown).

The kinetics of the expression of suppressor cell induction after intraperitoneal injection of 100 μg of SCPA is shown in Table 3. Significant suppressor cell activity was detected 4 days after injection of SCPA, with maximal suppression occurring at day 8. Other animals were tested for the

TABLE 1. Induction of suppressor cells for macrophage phagocytosis in mice injected with various cryptococcal extracts

Group[a]	Antigen[b]	% Phagocytosis ± SEM[c]	% Suppression
Normal	−	40.0 ± 1.8	
	+	39.8 ± 1.3	0
NU-2P-U	−	51.8 ± 3.0	
	+	32.8 ± 2.7[d]	37
NU-2-U	−	53.0 ± 3.5	
	+	28.5 ± 2.7[d]	46
M7-U	−	38.8 ± 0.5	
	+	42.5 ± 1.9	0
SCPA	−	54.0 ± 2.7	
	+	34.0 ± 0.5[d]	37

[a] Normal or antigen-injected mice.
[b] Supernatants were prepared with (+) or without (−) added antigen (NU-2P-U).
[c] Mean of four replicate determinations on thioglycolate-elicited macrophages.
[d] $P < 0.005$ compared with control (−) supernatant by Student's *t* test.

TABLE 2. Suppression of macrophage phagocytosis by spleen cell cultures of mice injected with SCPA

SCPA treatment (μg)	Antigen[a]	% Phagocytosis ± SEM[b]	% Suppression
0	−	42.3 ± 3.6	
	+	40.8 ± 4.6	3.6
100	−	57.3 ± 6.2	
	+	19.5 ± 6.4[c]	65.9
50	−	42.3 ± 3.2	
	+	24.7 ± 1.8[c]	41.6
25	−	47.8 ± 2.8	
	+	30.3 ± 1.3[c]	36.7
12.5	−	43.0 ± 0.6	
	+	44.3 ± 1.8	0

[a] Supernatants were prepared with (+) or without (−) added antigen (NU-2P-U).
[b] Mean of four replicate determinations on thioglycolate-elicited macrophages 7 days after injection of SCPA.
[c] $P < 0.005$ compared with control (−) supernatant by Student's *t* test.

TABLE 3. Kinetics of the appearance of suppressor cell activity after injection of SCPA

Days after injection[a]	Antigen[b]	% Phagocytosis ± SEM[c]	% Suppression
Normal	−	71.0 ± 0.6	
	+	71.5 ± 3.3	0
1	−	86.0 ± 2.0	
	+	78.3 ± 1.6	9
4	−	84.8 ± 2.3	
	+	68.0 ± 1.8[d]	20
6	−	87.5 ± 2.2	
	+	57.0 ± 4.3[e]	35
8	−	89.0 ± 3.6	
	+	51.3 ± 0.7[e]	42

[a] Injection of 100 μg of SCPA.
[b] Supernatants were prepared with (+) or without (−) added antigen (NU-2P-U).
[c] Mean of four replicate determinations on normal peritoneal cells.
[d] $P < 0.025$ compared with control (−) supernatant by Student's *t* test.
[e] $P < 0.005$ compared with control (−) supernatant by Student's *t* test.

TABLE 4. Duration of the expression of phagocytosis-inhibiting lymphokine

Group	Antigen[a]	% Phagocytosis ± SEM[b]	% Suppression
Normal	−	60.5 ± 2.3	
	+	57.8 ± 1.3	4
1 wk	−	67.0 ± 3.9	
	+	48.0 ± 3.5[c]	28
1 mo	−	68.3 ± 3.8	
	+	48.5 ± 2.7[c]	29
2 mo	−	68.8 ± 2.1	
	+	50.3 ± 4.1[c]	27

[a] Supernatants were prepared with (+) or without (−) added antigen (NU-2P-U).
[b] Mean of four replicate determinations on thioglycolate-elicited macrophages.
[c] $P < 0.005$ compared with control (−) supernatant by Student's t test.

duration of suppression over a 2-month period (Table 4). Over this time period, no drop in suppressor activity was detected.

Because the dose of SCPA used for induction of suppression in this model was one which is known to induce immune tolerance to the SCPA (29) and because we did not detect suppression after injection of the urea extract of the nonencapsulated strain (M7), we wanted to know whether this suppressor cell activity was unique SCPA or common to other models of immunologic tolerance. Animals were injected with tolerogenic forms of three different antigens. Two were polysaccharide in nature (SCPA and levan), and the third was a protein (HGG). After 1 week, the animals were sacrificed and their spleen cells were cultured with or without the antigen used for injection. At 48 h, supernatants were tested for their ability to inhibit phagocytosis of peritoneal macrophages. Significant suppression (Table 5) occurred when the tolerogenic forms of the antigen were administered, with 44, 40, and 35% suppression in the SCPA-, HGG-, and levan-tolerant animals, respectively. The same antigens given to mice in immunogenic form did not induce suppressor cell activity. Therefore, the suppressor cell which appeared in cryptococcus-infected animals was common to the state of immunologic tolerance and was not unique to cryptococcal antigen or to polysaccharide antigens. Animals injected with immunogenic forms of HGG and levan produced antibodies to HGG or levan, whereas tolerant animals failed to produce antibodies (data not shown).

Variations in the performance of the phagocytic assay have been noted depending on the source of macrophages used. Results from four experiments in which normal peritoneal cells or thioglycolate-elicited cells from spleen cell cultures of normal (N) and SCPA-treated (S) mice were tested against unstimulated (N^- and S^- supernatants) and antigen-stimulated supernatants (N^+ and S^+ supernatants) are shown in Tables 6 and 7. Several points may be made concerning these results. In general, normal peritoneal cells were more phagocytic than thioglycolate-induced cells, with a percent phagocytosis ranging from 60 to 80%. Phagocytosis was not suppressed as much in the normal peritoneal cell population by the S^+ supernatants. Thioglycolate-induced macrophages were consistently less phagocytic (40 to 70%), but showed greater suppression of phagocytosis by the S^+ supernatants. In some experiments, an enhancement of phagocytosis occurred when macrophages were cultured in control S^- supernatants compared with the phagocytic

TABLE 5. Ability of tolerogenic or immunogenic forms of antigen to elicit phagocytosis-inhibiting lymphokine

Group[a]	Homologous antigen	% Phagocytosis ± SEM[b]	% Suppression
Normal	None	54.3 ± 3.6	
	NU-2P	46.3 ± 2.3	15
	HGG	50.3 ± 1.0	7
	Levan	47.0 ± 2.3	12
SCPA-T	None	62.0 ± 4.1	
	NU-2P	34.5 ± 2.7[c]	44
HGG-T	None	57.0 ± 3.5	
	HGG	34.0 ± 2.5[c]	40
Levan-T	None	47.8 ± 2.8	
	Levan	31.3 ± 2.0[c]	35
SCPA-I	None	43.4 ± 1.8	
	NU-2P	43.8 ± 2.7	6
HGG-I	None	44.3 ± 3.5	
	HGG	44.3 ± 2.0	0
Levan-I	None	43.3 ± 2.5	
	Levan	42.0 ± 1.2	3

[a] T, Antigen injected in tolerogenic form; I, antigen injected in immunogenic form.
[b] Mean of four replicate determinations on thioglycolate-elicited macrophages.
[c] $P < 0.005$ compared with control (−) supernatant by Student's t test.

activity of the same macrophages cultured in the equivalent N^- supernatant. The phenomenon was most often observed when thioglycolate-induced macrophages were used in the assays. Among the experiments shown in Table 7, this occurred in three of four assays. Overall, our experience was that this occurred about 60% of the time. In contrast, enhancement by the S^- supernatant was detected in one of

TABLE 6. Effect of phagocytosis-inhibiting lymphokine on normal peritoneal macrophages

Supernatant[a]	% Phagocytosis ± SEM	% Suppression
Expt 1		
N^-	85.0 ± 2.9	
N^+	83.3 ± 3.3	
S^-	81.0 ± 2.7[b]	
S^+	54.7 ± 5.5[c]	34
Expt 2		
N^-	69.7 ± 1.9	
N^+	71.7 ± 2.4	
S^-	75.0 ± 1.2[b]	
S^+	53.3 ± 1.8[d]	29
Expt 3		
N^-	84.5 ± 1.5	
N^+	86.3 ± 4.5	
S^-	93.0 ± 2.1[e]	
S^+	70.5 ± 1.3[d]	24
Expt 4		
N^-	81.7 ± 0.9	
N^+	85.3 ± 1.5	
S^-	84.3 ± 2.3[b]	
S^+	65.5 ± 1.7[d]	22

[a] Supernatants of spleen cells from normal (N) or SCPA-treated (S) mice cultured without (−) or with (+) added antigen (NU-2P-U).
[b] Not statistically significantly different from the N^- supernatant by Student's t test.
[c] $P < 0.005$ compared with the S^- supernatant by Student's t test.
[d] $P < 0.0005$ compared with the S^- supernatant by Student's t test.
[e] $P < 0.05$ compared with the N^- supernatant by Student's t test.

four experiments in which normal peritoneal macrophages were used (Table 6) and, overall, about 40% of the time.

The lymphokine appeared to act on a small subpopulation of the macrophages (20 to 30% of the total population) when percent phagocytosis was evaluated. However, an examination of the total phagocytic capacity revealed that many more cells were affected than previously thought. This was seen when experiments were evaluated for suppression of TPC of the macrophage population. For example, in an experiment in which phagocytosis was suppressed by 37%, the TPC was inhibited 57% (Table 8). The TPC was calculated by multiplying the percent phagocytosis by the average number of cryptococci inside each phagocytic cell. It was apparent that some cells were unable to ingest as many yeast cells in the presence of the lymphokine as they were in its absence. Although these cells were still phagocytic, their capacity for engulfment of organisms was reduced.

DISCUSSION

In our initial attempts to elicit suppressor cell activity by injection of soluble cryptococcal antigens, we chose urea extracts of both encapsulated and nonencapsulated strains of *C. neoformans*. Suppressor cells were induced by injection of the urea extract of strain NU-2 and its pseudohyphal variant NU-2P, but not by the nonencapsulated mutant M7. The pseudohyphal variant usually has a dry-colony morphology but does possess small amounts of capsule when examined by electron microscopy. In addition, it is sometimes isolated from animals as a mucoid colony type. Therefore, it is considered to be thinly encapsulated. The fact that extracts of NU-2, a highly encapsulated strain, and NU-2P as well as SCPA (purified capsular polysaccharide) elicited suppressor cell activity, whereas the urea extract of M7 did not, led us to speculate that suppressor cell induction could be attributed to the capsular polysaccharide in each of these preparations.

TABLE 7. Effect of phagocytosis-inhibiting lymphokine on thioglycolate-elicited macrophages

Supernatant[a]	% Phagocytosis	% Suppression
Expt 1		
N^-	42.3 ± 6.5	
N^+	40.8 ± 9.2	
S^-	57.3 ± 10.7[b]	
S^+	19.5 ± 6.4[c]	66
Expt 2		
N^-	57.6 ± 1.3	
N^+	53.6 ± 8.5	
S^-	60.0 ± 12[d]	
S^+	21.7 ± 8.8[c]	64
Expt 3		
N^-	40.0 ± 1.8	
N^+	39.8 ± 1.3	
S^-	54.0 ± 2.7[e]	
S^+	34.0 ± 1.1[f]	37
Expt 4		
N^-	69.8 ± 1.7	
N^+	62.0 ± 1.6	
S^-	62.5 ± 2.9[b]	
S^+	40.0 ± 2.7[c]	36

[a] Supernatants of spleen cells from normal (N) or SCPA-treated (S) mice cultured without (−) or with (+) added antigen (NU-2P-U).
[b] $P < 0.025$ compared with the N^- supernatant by Student's t test.
[c] $P < 0.005$ compared with the S^- supernatant by Student's t test.
[d] Not statistically different from the N^- supernatant by Student's t test.
[e] $P < 0.005$ compared with the N^- supernatant by Student's t test.
[f] $P < 0.0005$ compared with the S^- supernatant by Student's t test.

TABLE 8. Effect of phagocytosis-inhibiting lymphokine on TPC

Supernatant[a]	% Phagocytosis ± SEM[b]	% Suppression[c]	Yeast/ macrophage ± SEM[c]	% Suppression of TPC
S^-	70.2 ± 1.2		3.1 ± 0.1	
S^+	44.3 ± 2.6[d]	37	2.1 ± 0.08	57
N^-	69.7 ± 1.9		3.0 ± 0.05	
N^+	71.7 ± 2.4	0	3.2 ± 0.04	0

[a] Supernatants of spleen cells from normal (N) or SCPA-treated (S) mice cultured without (−) or with (+) added antigen (NU-2P-U).
[b] Mean of four replicate determinations on normal peritoneal cells.
[c] Average number of yeast cells within each phagocytic macrophage.
[d] $P < 0.005$ compared with control (−) supernatant by Student's t test.

Dose-response analysis with the SCPA revealed that the dose requirement for induction of suppression was the same as that known to result in immunologic tolerance to the polysaccharide (29). For this reason, it was of interest to know if the suppressor cell under study would also appear in animals tolerant to other polysaccharide or protein antigens or both. When animals were given tolerogenic regimens of two additional antigens (levan and HGG), suppressor cells were found in spleens 1 week later. However, antigens administered in immunogenic form did not induce the macrophage-inhibitory suppressor cell. Animals were confirmed tolerant by their inability to produce antibody after challenge with immunogenic forms of antigen (data not shown). Cell-mediated responses were not specifically evaluated. The classic model of tolerance induction in the HGG system with the dose of antigen used here is well-known to produce tolerance of T cells before the onset of tolerance of B cells (5, 10). In addition, T-cell tolerance is longer-lasting than B-cell tolerance, which also requires higher doses of antigen for induction (10). Thus, tolerance at the T-cell level is present whenever B-cell tolerance is detected. Although polysaccharide antigens have routinely been considered to be thymus independent, more recent studies have shown that B-cell responses to these antigens are exquisitely regulated by T-cell systems (7, 23, 37). Even in situations in which immunity is apparent, T-cell suppressor activity may down-regulate selected responses of the immune system (7). Thus, suppression of antibody synthesis is generally associated with induction of T-cell regulators whether the antigens are protein or polysaccharide in nature. It was apparent that both protein and polysaccharide antigens were capable of inducing the suppressor cell as long as the antigen was given in such a way as to induce immunologic unresponsiveness. The failure to induce suppressive activity by the M7 urea extract was therefore best explained by the fact that an insufficient dose of a complex mixture of antigens was administered or that the in vitro eliciting antigen (a urea extract of NU-2P) was inappropriate either in dose or antigenic makeup. It was our expectation that inhibition of phagocytosis with antigen-stimulated supernatants of spleen cells taken from immunized mice might also occur, since gamma interferon or MAF or both should be produced. Because gamma interferon is known to activate macrophages, with a subsequent decrease in both Fc and mannan receptors (16), and to inhibit macrophage phagocytosis (1), it would not have been surprising to observe a reduction in phagocytosis by these supernatants. The lack of detection of such activity can be attributed to the production of an

insufficient amount of gamma interferon or to the production of various other factors with opposing effects. We believe that it is unlikely that we detected gamma interferon in the experiments described here. The factor which we studied was previously reported (26) to be allospecific, whereas gamma interferon does not require genetic identity between donor and responding cell.

The relationship of the lymphokine we described to the TsF1 and TsF2 factors of Murphy (28, 30–32) has not been directly tested. Several differences in the systems may be cited which would indicate that the lymphokine is different from TsF1 and TsF2. The Ts1 cell of Murphy is found in the lymph nodes and not in the spleen, making it unlikely that it is the same as our suppressor cell. Ts2 is found in the spleen and functions to inhibit the effector delayed-type hypersensitivity T cell, whereas the factor described here has a direct interaction with macrophages. It is possible, however, that the macrophage suppressor factor that we are studying represents an interaction of suppressor factor and the factor presenting cell as described by Dorf and Benacerraf (14) for the NP suppressor cell cascade. Inhibition of phagocytosis could be a secondary phenomenon coexistent with the activity of presenting the factor to another T cell. If this is the case, then TsF2 and our suppressor factor could be the same. A striking dissimilarity in the production of these factors should be noted, however. TsF2 is prepared by freeze-thaw extraction of isolated spleen cells. Our factor, on the other hand, required that spleen cells be cultured for 2 days in the presence of specific antigen. The factor was found as a product secreted into the culture medium. One might argue that it was released by dying cells in the cultures; however, if this were the case, it should have been found in the non-antigen-stimulated control cultures as well. Certainly, the question regarding the relationship of our factor with those described by Murphy cannot be answered with finality since different experimental protocols were used. Further characterizations of the suppressor cell and the factor released in our system are required.

In recent years, it has been realized that macrophages are heterogeneous in regard to their function and their physical attributes. These differences could either be due to the existence of distinct macrophage subpopulations or they may represent the presence of macrophages which are at distinct levels along a differentiation pathway. Two major subcategories of macrophages have been described based on their expression of Ia antigens, with the functional activity of antigen presentation attributed to the Ia-positive subset (12, 13, 18, 38). Ia-negative macrophages are responsible for beta-interferon-induced cytotoxicity and suppressor cell activity (6). Evidence from many different lines of investigation (2, 22, 35, 36) indicate that there is a conversion between Ia positivity and negativity depending on the stimuli and that the Ia-positive subset cannot be considered to be a stable subset. The fact that we detected an effect of the lymphokine on a subset of macrophages indicates that there may be a certain stage in the maturational pathway of the cells which can be affected by the suppressor molecule. In addition, the possibility that other macrophage functions are affected by the lymphokine is intriguing. Currently, investigations along these lines are in progress in our laboratory.

The variation in results which may occur when different macrophage sources are used can be seen in this investigation. When the macrophages were thioglycolate elicited, we often detected enhancement of phagocytosis when S^- supernatants of spleen cells were tested. There was an enhancement of phagocytosis by all non-antigen-containing supernatants compared with phagocytosis in medium alone (data not shown). This phenomenon is probably linked to the presence of factors in the conditioned medium which influence macrophage differentiation and may vary from supernatant to supernatant. The augmentation by the S^- supernatant was seen less frequently when normal peritoneal cells were used in the phagocytosis assay. These results suggest that a phagocytosis-stimulating factor was present in the S^- supernatant which exerted its effect on a population of cells found most often in thioglycolate-stimulated exudates. It was therefore essential to compare S^+ supernatant results with S^- supernatant results to account for the activity of opposing factors in the S^+ supernatants.

The relevance of the detection of this phagocytosis-inhibiting lymphokine to the clearance of cryptococci has not been determined. Initially, we thought that such a biologic significance seemed unlikely, since complete inhibition of phagocytosis occurred in a small number of macrophages. However, the results of this investigation show that the phagocytic activity of many more macrophages was slowed without being completely inhibited. If we take into account the cells affected in this manner, about 50 to 60% of the macrophage population was affected. These observations suggest that the factor may contribute to pathogenesis in cryptococcosis by inhibition of clearance of the organism.

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