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Characterization of a Suppressor Factor That Regulates Phagocytosis by Macrophages in Murine Cryptococcosis

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A T-suppressor factor which inhibits the phagocytic activity of a macrophage subset has been further characterized. This suppressor factor was first described for a murine model of cryptococcosis but was later found to be common to models of immunologic unresponsiveness. The suppressor factor was produced when suppressor cells were cultured in the presence of specific cryptococcal antigen. It could not be extracted from spleen cells and was not induced by antigen in cultures of lymph node cells. The suppressor factor was filtered through Amicon filters of 100-kilodalton (kDa) exclusion limit but was retained by filters excluding molecules of less than 50 kDa. By Sephadex G-100 chromatography, the factor eluted just ahead of bovine serum albumin (68 kDa). The activity of the suppressor factor could not be inhibited by anticryptococcal antibody, but it was inhibited by anti-I-J alloantiserum of the same genotype as the lymphocyte which produced the factor. Absorption with an encapsulated strain of *Cryptococcus neoformans* removed the suppressor factor from culture supernatants, while absorption with a nonencapsulated mutant or an unrelated yeast cell had no effect. On the basis of these observations, it was apparent that the suppressor factor was idiotypic in nature and that I-J and/or the I-J-interactive molecule played a role in the function of the suppressor factor. The requirement for antigenic stimulation for the production of suppressor factor in vitro distinguished it from the T-suppressor factor 3 described by others which regulates delayed-type hypersensitivity in cryptococcosis.

A common feature of cryptococcal disease is the development of a state of immunologic anergy. This has been attributed to the presence of suppressor cells for the specific response to cryptococcal antigens (3, 4, 12–14) as well as nonspecific suppressors which limit responses to unrelated antigens (16). Among those suppressor cells which have been found to be antigen specific is the cell which produces a suppressor factor which inhibits macrophage phagocytosis and has been previously described by our group (3, 4, 11). The factor is secreted into tissue culture medium after stimulation with specific antigen and interacts with a macrophage subpopulation, resulting in inhibition of macrophage phagocytic activity for yeast cells. The lymphocyte which produces the suppressor factor is a Lyt-2^+, I-J^+ T lymphocyte, and the interaction between the macrophage and the factor is genetically restricted by the I-J region of the major histocompatibility complex (3).

Other antigen-specific suppressor cells have been described by Murphy et al. (12–14) for animals injected with cryptococcal antigens. These suppressor cells operate to limit delayed-type hypersensitivity responses and function in a cellular cascade in a manner which is analogous to that of the haptenic systems described previously (7). The suppressor factors which function in these pathways are extracted from spleen cells. They are I-J^+ and may be idiotypic or anti-idiotypic in nature.

The current investigation was undertaken to further characterize the suppressor factor previously described by our group. In addition, we wanted to determine the possible relationship this suppressor factor had to other antigen-specific factors reported for cryptococcosis.

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MATERIALS AND METHODS

Abbreviations. The following abbreviations were used: TsF3, T-suppressor factor 3; SCPA, soluble capsular polysaccharide antigen; PBS, phosphate-buffered saline; PEC, peritoneal exudate cells; Ts2, T-suppressor cell 2; Ts3, T-suppressor cell 3; TsF2, T-suppressor factor 2; Ts1, T-suppressor cell 1; NP, 4-hydroxy-3-nitrophenylacetyl; and TsFeff, T-effector suppressor factor.

Animals. C3HeB/FeJ and C57BL/6J mice were purchased from Jackson Laboratory, Bar Harbor, Maine. The mice were 8 weeks old upon arrival and were used in experiments when 10 to 14 weeks old. The mice were housed in the University of Oklahoma Health Sciences Center animal care facility. This facility is approved by the American Association for the Accreditation of Laboratory Animal Care. The animals were fed Purina mouse chow and water ad libitum.

Fungal strains. An encapsulated strain of *Cryptococcus neoformans*, NU-2, was originally obtained from the Department of Medical Microbiology, University of Nebraska Medical School, Omaha. Strain M7 is a nonencapsulated mutant and was originally described by Bulmer and Sans (5). *Saccharomyces cerevisiae* was originally obtained from Glenn Bulmer (University of Oklahoma). The fungal strains were maintained in our laboratory by subculture on mycological agar (Difco Laboratories). When used in experiments, yeast cells were harvested after 72 h of growth at room temperature on mycological agar plates.

Antisera. Rabbit anti-*C. neoformans* antiserum was a gift from Nan Scott, Veterans Administration Medical Center, Oklahoma City. It had a 1:12,800 titer by enzyme-linked immunosorbent assay testing (8) against soluble capsular polysaccharide antigen (serotype A,D) of cryptococci. Normal rabbit immunoglobulin G (IgG) was purchased from Cooper Biomedical (6012-0080). Anti-I-J^k was purchased from Accurate Chemical Company. Anti-I-J^b was obtained from the Transplantation Immunology Branch, National

Institutes of Health, Bethesda, Md. These mouse alloantisera have been used previously (3) and were shown to exhibit appropriate strain specificity in eliminating suppressor cell activity when used in conjunction with rabbit complement.

Reagents. Sterile water for irrigation (cat. no. R5000-01) was obtained from American McGaw, Irvine, Calif. RPMI 1640 (cat. no. 12-10378H), Hybri-sure fetal calf serum (cat. no. 12-10378H), HEPES (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid) (cat. no. 59-20577), sodium pyruvate (cat. no. 59-20377), and hypoxanthine-aminopterin-thymidine (HAT) supplement (cat. no. 59-77076) were purchased from Hazleton Research Products, Lenexa, Kans. Antibiotic-antimycotic (cat. no. 600-5245), L-glutamine (cat. no. 320-5039), and sodium bicarbonate solution (cat. no. 670-5080) were obtained from GIBCO Laboratories, Grand Island, N.Y. Dulbecco PBS (cat. no. 17-5154) and hypoxanthine-thymidine (HT) supplement (cat. no. 17-782A) were purchased from Whittaker, M.A. Bioproducts, Walkersville, Md. Hybricare medium was obtained from the American Type Culture Collection, Rockville, Md. Type AB human serum was obtained from the Oklahoma Blood Institute, Oklahoma City, Okla. The blood was negative for human immunodeficiency virus antibody and all other tests required for human administration. Lots of human serum were preselected by their ability to support proliferative responses of mouse lymphocytes and to support the production of suppressor factor.

Antigens. SCPA serotype A,D was prepared from strain NU-2 of *C. neoformans* as previously described (4).

Induction of suppressor cell activity. Mice were injected intraperitoneally with 200 μg of SCPA suspended in 0.2 ml of Dulbecco PBS. The animals were sacrificed 7 days later, and their spleens were removed for the preparation of suppressor factor-containing or control supernatants.

T-T hybridomas. In some experiments suppressor factor was derived from T-T hybridomas. The hybridomas were obtained by fusion of 6-thioguanine-resistant BW 51-47.G.1.4 (ATCC TIB 48) lymphoma cells (4×10^7) with plastic nonadherent spleen cells (4×10^7) of SCPA-injected C3HeB/FeJ mice with 40% polyethylene glycol at 37°C for 45 s. After the cells were slowly diluted over 5 min with 10 ml of Hybricare medium, the cells were centrifuged at $200 \times g$ for 10 min and suspended in medium containing HAT supplement and 10% fetal bovine serum at a concentration of 3×10^5 lymphoma cells per ml. The cells were distributed in 0.2-ml amounts into the wells of a 96-well plate and were incubated at 37°C in an atmosphere of 5% CO_2. The cells were fed three times per week with hypoxanthine-aminopterin-thymidine medium for 3 weeks and then with hypoxanthine-thymidine medium for 1 week. Finally, Hybricare medium containing 10% fetal calf serum was used for feeding of fusion products. The fusion products were tested for their ability to produce suppressor factor in vitro in the presence or absence of 5 μg of SCPA per ml and in the presence or absence of normal spleen cells, which were used as a source of accessory cells. Three of the clones were found to produce suppressor factor if they were cultured for 24 h in the presence of normal splenic accessory cells and cryptococcal antigen. These clones were subcloned by a limiting dilution procedure, and subclone F6.6.2 was chosen for use in this investigation. The suppressor factor produced by clone F6.6.2 was found in tissue culture medium after culture for 24 h with SCPA. It was not found when levan, a nonspecific antigen, was used to stimulate its production. In addition, it exhibited the same genetic restrictions as previously reported (3) for the factor prepared by the conventional procedure (manuscript in preparation).

Suppressor factor production. Conventional suppressor factor-containing or control supernatants were prepared as previously described (3). Spleen cells (5×10^6 per ml) were cultured in RPMI 1640 containing 5% human serum, 200 mM l-glutamine, 10 mM HEPES buffer, 1% sodium pyruvate, and 1% antibiotics with or without 5 μg of SCPA per ml. Twenty-four hours later, the spleen cells were removed by centrifugation at $400 \times g$. The supernatant fluid was used immediately or after storage at −20°C for no longer than 2 weeks. In some experiments supernatants were obtained from mesenteric and peripheral lymph node cells as described above. Extraction of suppressor factors from spleen cells was done by a procedure similar to that described by Murphy and Moorhead (13). Spleen cells were suspended (5×10^7 per ml) in serum-free RPMI 1640. The cells were lysed by repeated freezing and thawing in liquid N_2 and warm water, and the preparation was centrifuged at $100,000 \times g$ to remove cellular debris. The extract was then diluted 1:10 in complete tissue culture medium and tested for suppressor factor activity. When hybrid cells (clone F6.6.2) were used for suppressor factor production, 2.5×10^5 hybridoma cells were mixed with 5×10^6 normal spleen cells per ml of Hybricare medium containing 5% human serum and 5 μg of SCPA per ml. The control supernatant consisted of the same cell mixture cultured without cryptococcal antigen. In addition, supernatants prepared with the BW 5147 fusion partner instead of the hybridoma cell line were included as negative controls.

Preparation of macrophage target cells. Macrophages were obtained from mice injected 3 days previously with 0.25 ml of aged 10% thioglycolate medium. PEC were harvested by the procedure of Cohn and Benson (6). The fluid withdrawn from the peritoneal cavity was centrifuged at $200 \times g$ for 10 min, and the cells in the pellet were suspended in RPMI 1640 containing 10% fetal calf serum, 200 μM l-glutamine, 10 mM HEPES buffer, 1% sodium pyruvate, and 1% antibiotics.

Phagocytic assay. Macrophages at a concentration of 2×10^5 per ml were distributed (0.5 ml) into the wells of eight-chambered tissue culture slides (Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.) and were incubated overnight at 37°C in an atmosphere of 5% CO_2 before testing of supernatants for suppressive activity. On the next day, the tissue culture medium was removed and 0.3 ml of suppressor factor-containing or control supernatant or fractions thereof were added to the monolayer. The monolayers were incubated for an additional 1 h at 37°C in 5% CO_2. The supernatants were then removed, and 0.5 ml of *C. neoformans* M7 (5×10^6 per ml) suspended in RPMI 1640 containing 10% fetal calf serum was added to each well. The slides were incubated for 2 h, and the monolayers were gently washed in warm (37°C) PBS. The monolayers were allowed to air dry and were stained with Camco Quick Stain (Cambridge Chemical Products). The percent phagocytosis was determined by counting the percentage of cells which contained one or more yeast cells intracellularly. At least 100 cells were evaluated, and the mean of four replicate determinations was determined. It was possible to detect activity when supernatants were diluted (1:2 to 1:3), but activity was lost when higher dilutions (1:5) were used. The phagocytic assay was modified slightly from that described previously (3). Kinetic analysis revealed that the factor suppressed phagocytosis when incubated on macrophage monolayers for 1 h or less (submitted for publication). Thus, we were able to culture spleen cells for factor production in 5%

human serum and then test the supernatants on macrophage monolayers which were precultured overnight in medium containing fetal calf serum. It was determined that macrophage preculture in 10% fetal calf serum (containing less than 0.1 ng of endotoxin per ml) resulted in better and more consistent phagocytosis than when the cells were cultured in 5% human serum and small amounts of endotoxin (unpublished results). Although the calf serum contained virtually no endotoxin, it apparently provided another factor(s) that supported macrophage phagocytic activity and that was not present in human serum. Direct comparisons of the two assay procedures showed that equivalent results were obtained (data not shown).

We believe that only intracellular yeast cells were counted in the phagocytic assay. This is based upon the observation that no yeast cells adhered to macrophage monolayers if the slides were incubated at 4°C to inhibit phagocytosis (results not shown). The interaction between the yeast cells and the macrophage receptors is apparently not strong enough to be maintained during the washing steps used in the assay procedure. Although in a previous publication we reported our data as the average number of yeast cells per macrophage, this procedure has not been possible under routine conditions. In order to consistently detect suppression, we have chosen to optimize phagocytosis. When this is done, many of the macrophages ingest so many yeast cells that accurate quantitation of intracellular yeast cells is impossible. For this reason, we have routinely evaluated the number of phagocytic cells.

Fractionation by Amicon filtration. Suppressor factor-containing or control supernatants were fractionated by filtration through filters of various pore sizes (XM100, CF50A, and CF25; Amicon Corp., Lexington, Mass.) to obtain information regarding the approximate molecular size of the suppressor factor. The material passing through the filter was then tested for suppressor activity.

Sephadex column chromatography. Before separation by gel filtration, supernatants were filtered through an Amicon XM100 filter to obtain the fraction containing molecules less than 100,000 daltons. This fraction was then concentrated 10× by use of a Minicon-A25 concentrator (Amicon). The concentrated supernatant (2 ml) was applied to a Sephadex G-100 column (2.5 by 75 cm) equilibrated with PBS. Fractions were collected in PBS and tested directly for suppression of phagocytosis.

Absorption of suppressor factor by yeast cells. The ability of suppressor factor to bind antigen was tested by absorption of supernatants with *S. cerevisiae* or encapsulated (NU-2) or nonencapsulated (M7) strains of *C. neoformans*. The fungi were grown on mycological agar for 72 h and harvested from the surface of the agar with sterile PBS. After being washed three times, the yeast cells were suspended in PBS and counted. The volume of yeast cells required to obtain 4×10^8 cells per ml of supernatant to be absorbed was transferred to a centrifuge tube, and the cells were pelleted. The supernatant fluid was removed, and test supernatants were added to the yeast cell pellet. After suspension of the yeast cells, the tubes were incubated on ice for 1 h with occasional agitation. The yeast cells were then removed by centrifugation, and the supernatant was filtered through a membrane filter (0.45 μm; Millipore Corp., Bedford, Mass.) prior to being tested for suppressive activity.

Blocking of suppression with antiserum. Anti-I-J and anti-*C. neoformans* antisera were used to block the interaction between the suppressor factor and the macrophage in vitro. Supernatants were first treated with antiserum for 30 min at room temperature. In the case of anti-I-J antiserum, the supernatants were treated at a final dilution of 1:80. Rabbit anti-*Cryptococcus* globulin and normal rabbit globulin were used at final concentrations of 0.15 μg per ml. For the immune globulin, this concentration corresponded to a 1:1,000 dilution of the stock solution which had a 1:12,800 titer against SCPA by enzyme-linked immunosorbent assay testing performed by the method of Eckert and Kozel (8).

TABLE 1. Suppressive activity in stimulated lymph node cultures and extracted spleen cells

Culture or extract[a]	Supernatant[b]	% Phagocytosis[c]
Spleen	N−	67.3 ± 1.9
	N+	64.8 ± 2.8
	S−	72.8 ± 1.8
	S+	58.0 ± 2.5[d]
Lymph node	N−	68.3 ± 1.3
	N+	69.5 ± 2.0
	S−	73.8 ± 2.2
	S+	74.8 ± 3.9
Spleen extract	N	69.5 ± 5.2
	S	75.3 ± 6.2

[a] Cultures were derived from C57BL/6J spleen or lymph node cells from normal (N) or SCPA-injected (S) mice. Spleen extract preparations were from spleen cells of normal (N) or SCPA-injected (S) mice.

[b] Supernatants of spleen cells or lymph node cells from normal (N) or SCPA-treated (S) mice cultured without (−) or with (+) antigen (SCPA).

[c] Tested on C57BL/6J PEC (mean ± standard error of the mean of four replicate determinations); data are representative of three experiments.

[d] $P < 0.001$ compared with control supernatant without antigen by Student's t test.

RESULTS

Comparison of methods for preparation of suppressor factor. The suppressor cell that we described has phenotypic characteristics (Lyt 2^+, I-J^+) similar to those of the Ts2 and Ts3 suppressor cells described for haptenic systems (1, 7) and for cryptococcosis (12–14). We wanted to know if the suppressor cell and its factor merely represented another activity of one of the already described factors, TsF2 or TsF3, which limit delayed-type hypersensitivity responses. We previously cited the requirement for in vitro antigenic stimulation of the suppressor cell in our system as the one feature which distinguished it from the suppressor cells described in these other models (3). In the case of suppressor factors TsF2 and TsF3, spleen cells were extracted by repeated freezing and thawing. Direct comparison of factor preparation procedures in our system had not been made. In addition, Murphy and Moorhead (13) detected Ts1 cells in lymph nodes, but Ts2 and Ts3 cells were found in spleens of experimental mice. We had no information regarding the anatomical distribution of the suppressor of macrophage phagocytosis. Experiments to compare these characteristics were carried out, and the results of a typical experiment are shown in Table 1. The only condition which resulted in elaboration of the suppressor factor for macrophage phagocytosis was when spleen cells of SCPA-injected mice were cultured in the presence of SCPA. The factor could not be extracted from the spleens of SCPA-injected mice and was not produced by lymph node cells in culture with cryptococcal antigen. Thus, this characteristic remained a feature which distinguished the suppressor of macrophage phagocytosis from those that function in the regulation of delayed-type hypersensitivity responses.

TABLE 2. Fractionation of suppressor factor activity by Amicon filtration

Filter size limit (Da)[a]	Supernatant[b]	% Phagocytosis[c]
None (unfractionated)	N−	59.8 ± 4.5
	N+	58.0 ± 4.7
	S−	61.0 ± 4.0
	S+	49.0 ± 1.1[d]
100,000	N−	75.6 ± 2.7
	N+	76.0 ± 3.0
	S−	81.0 ± 3.0
	S+	60.5 ± 2.8[e]
50,000	N−	68.8 ± 1.9
	N+	70.8 ± 2.2
	S−	70.5 ± 4.3
	S+	71.3 ± 1.9
25,000	N−	56.5 ± 3.6
	N+	56.0 ± 1.6
	S−	51.8 ± 3.6
	S+	49.5 ± 3.8

[a] Fraction passing through Amicon filters XM100 (100,000 Da), CF50A (50,000 Da), and CF25 (<25,000 Da).

[b] Supernatants of C3HeB/FeJ spleen cells from normal (N) or SCPA-treated (S) mice cultured without (−) or with (+) antigen (SCPA).

[c] Tested on C3HeB/FeJ PEC (mean ± standard error of the mean of four replicate determinations); data are representative of four experiments.

[d] $P < 0.025$ compared with control supernatant without antigen by Student's t test.

[e] $P < 0.005$ compared with control supernatant without antigen by Student's t test.

Determination of the molecular size of the suppressor factor. Initial experiments were performed to obtain an estimate of the size of the suppressor factor by filtration of the supernatants through Amicon filters of various pore sizes. XM100 (exclusion limit, 100 kilodaltons [kDa]), CF50A (exclusion limit, 50 kDa), and CF25 (exclusion limit, 25 kDa) filters were employed. The fractions which passed through the filters were then tested for their ability to inhibit macrophage phagocytosis (Table 2). While the suppressor factor passed through the XM100 filter, it did not pass through filters having exclusion limits of 50 or 25 kDa. This indicated that the factor was in the 50- to 100-kDa molecular mass range. Further fractionation was accomplished on a Sephadex G-100 column (Fig. 1). The active molecule was found to elute just ahead of the bovine serum albumin marker (M_w, 68,000), indicating that the suppressor factor had a molecular size of approximately 70 kDa.

Blocking of suppressor factor activity by anti-*C. neoformans* antibodies. The TsF2 suppressor factors described by Dorf and Benacerraf (7) have been shown to be anti-idiotypic in nature and were absorbed by antiserum prepared against the specific antigen used for induction of the suppressor cascade. To determine whether the suppressor factor for macrophage phagocytosis was anti-idiotypic, we attempted to block its activity with anti-*C. neoformans* antibody (Table 3). The IgG fraction of rabbit anticryptococcus serum was used at a final concentration of 0.15 μg of protein per ml. The IgG fraction of normal rabbit serum served as a negative control. Treatment with anticryptococcal antibody did not interfere with suppressor factor activity. In other experiments, immunoglobulins were added at a concentration of 1.5 μg per ml, and no inhibition of suppressor factor activity was detected (data not shown). When conventional supernatants were tested in similar experiments, equivalent results were obtained. These data suggested that anti-idiotypic determinants were not associated with the suppressor factor.

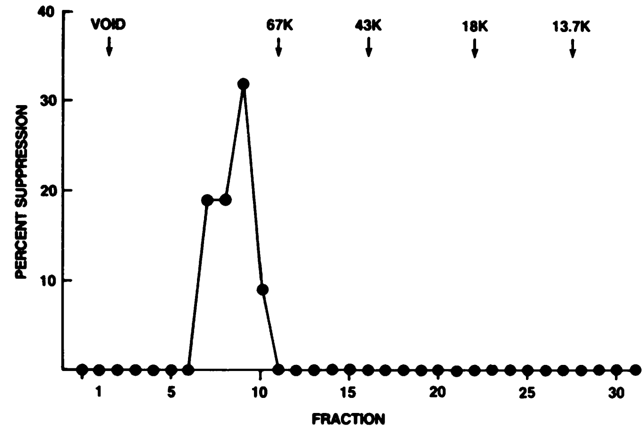

FIG. 1. Fractionation of the suppressor factor by Sephadex G-100 chromatography.

Absorption of suppressor factor by cryptococcal antigen. Many of the I-J-restricted suppressor factors which have been described in the literature could bind to the specific antigen used in their induction. To examine the possibility that the suppressor factor for macrophage phagocytosis was idiotypic, we used encapsulated cryptococcal cells to absorb suppressor factor from culture supernatants. Controls included use of the nonencapsulated mutant of *C. neoformans*, M7, and an unrelated yeast, *S. cerevisiae*. The results of a typical experiment are shown in Table 4. Absorption with the encapsulated strain of *C. neoformans*, NU-2, effectively removed the suppressor factor from supernatant fluids of the F6.6.2 cell line, whereas absorption with the nonencapsu-

TABLE 3. Blocking of suppressor factor activity by anti-*C. neoformans* antibodies

Treatment and supernatant[a]	% Phagocytosis[b]
No treatment	
BW 5147−	76.5 ± 3.0
BW 5147+	77.8 ± 1.3
F6.6.2−	83.5 ± 1.2
F6.6.2+	70.3 ± 2.0[c]
Normal IgG	
BW 5147−	76.3 ± 1.3
BW 5147+	75.0 ± 0.9
F6.6.2−	78.0 ± 0.4
F6.6.2+	68.3 ± 2.8[d]
Anti-*C. neoformans* IgG	
BW 5147−	80.0 ± 2.1
BW 5147+	79.0 ± 1.1
F6.6.2−	80.0 ± 1.7
F6.6.2+	70.0 ± 1.6[c]

[a] Supernatants of BW 5147 or F6.6.2 cells mixed with normal C3HeB/FeJ spleen cells and cultured without (−) or with (+) antigen (SCPA); supernatants were treated with 0.15 μg of the IgG fraction from normal or *C. neoformans*-immunized rabbits per ml.

[b] Tested on C3HeB/FeJ PEC (mean ± standard error of the mean of four replicate determinations); data are representative of three experiments.

[c] $P < 0.005$ compared with control supernatant without antigen by Student's t test.

[d] $P < 0.025$ compared with control supernatant without antigen by Student's t test.

TABLE 4. Absorption of suppressor factor by yeast strains

Yeast strain and supernatant[a]	% Phagocytosis[b]
None	
BW 5147−	69.0 ± 1.9
BW 5147+	64.3 ± 4.1
F6.6.2−	69.8 ± 2.5
F6.6.2+	54.5 ± 0.6[c]
C. neoformans NU-2	
BW 5147−	67.8 ± 2.3
BW 5147+	64.8 ± 2.6
F6.6.2−	66.1 ± 1.8
F6.6.2+	68.5 ± 4.8
C. neoformans M7	
BW 5147−	60.8 ± 1.1
BW 5147+	65.0 ± 4.6
F6.6.2−	64.3 ± 2.2
F6.6.2+	53.0 ± 2.8[d]
S. cerevisiae	
BW 5147−	66.8 ± 3.7
BW 5147+	63.0 ± 2.2
F6.6.2−	68.5 ± 2.4
F6.6.2+	53.3 ± 1.3[c]

[a] Supernatants of BW 5147 or F6.6.2 cells mixed with normal C3HeB/FeJ spleen cells and cultured without (−) or with (+) antigen (SCPA); supernatants were absorbed with 4×10^8 yeast cells per ml for 1 h at 4°C.
[b] Tested on C3HeB/FeJ PEC (mean ± standard error of the mean of four replicate determinations); data are representative of six experiments.
[c] $P < 0.005$ compared with control supernatant without antigen by Student's *t* test.
[d] $P < 0.01$ compared with control supernatant without antigen by Student's *t* test.

TABLE 5. Blocking of suppressor factor activity by anti-I-J antisera

Treatment and supernatant[a]	% Phagocytosis[b]
None	
BW 5147−	83.3 ± 1.5
BW 5147+	87.0 ± 2.9
F6.6.2−	84.5 ± 1.9
F6.6.2+	74.3 ± 1.2[c]
Anti-I-J^b	
BW 5147−	81.5 ± 1.4
BW 5147+	82.8 ± 3.3
F6.6.2−	85.5 ± 1.6
F6.6.2+	74.5 ± 2.2[c]
Anti-I-J^k	
BW 5147−	90.0 ± 1.1
BW 5147+	89.3 ± 1.7
F6.6.2−	85.3 ± 2.4
F6.6.2+	84.3 ± 3.6
None	
B6 N−	88.5 ± 1.6
B6 N+	85.3 ± 2.2
B6 S−	87.8 ± 1.9
B6 S+	76.0 ± 1.3[c]
Anti-I-J^b	
B6 N−	85.0 ± 3.2
B6 N+	81.8 ± 1.8
B6 S−	85.5 ± 1.3
B6 S+	82.0 ± 3.2
Anti-I-J^k	
B6 N−	84.0 ± 1.1
B6 N+	82.0 ± 2.0
B6 S−	84.3 ± 1.7
B6 S+	75.8 ± 2.9[d]

[a] Supernatants of BW 5147 or F6.6.2 cells mixed with normal C3HeB/FeJ spleen cells and cultured without (−) or with (+) antigen (SCPA). C57BL/6 (B6) supernatants were derived from spleen cells of normal (N) or SCPA-treated (S) mice cultured without (−) or with (+) antigen (SCPA). Supernatants were treated with a 1:80 dilution of anti-I-J^b or anti-I-J^k for 30 min before testing.
[b] Tested on syngeneic PEC (mean ± standard error of the mean of four replicate determinations); data are representative of three experiments.
[c] $P < 0.005$ compared with control supernatant without antigen by Student's *t* test.
[d] $P < 0.025$ compared with control supernatant without antigen by Student's *t* test.

lated strain (M7) or *S. cerevisiae* did not. Similar results were obtained when supernatants were prepared by the conventional procedure. These data showed that the suppressor factor could bind to the capsular polysaccharide used for its induction and was therefore idiotypic in nature.

Blocking of suppressor factor activity by anti-I-J antiserum. The role of I-J molecules and/or the I-J-interactive molecule in the interaction between the suppressor factor and the target macrophage was examined in experiments in which anti-I-J alloantisera were used to block suppressor factor activity. The results of one such experiment are shown in Table 5. Supernatants from BW 5147 and F6.6.2 (I-J^k) cultures were treated with anti-I-J^k or anti-I-J^b, as were conventionally prepared culture supernatants in the C57BL/6J (I-J^b) mouse strain. The supernatants were then tested on syngeneic macrophage monolayers to determine their ability to inhibit ingestion of yeast cells. The activity of the F6.6.2 cell line cultures in the presence of SCPA was blocked by the anti-I-J^k antiserum but not by the anti-I-J^b antiserum. Suppressor factor activity in supernatants prepared from the C57BL/6J mice was blocked by anti-I-J^b antiserum treatment but was not affected by anti-I-J^k antiserum. In other experiments, conventionally prepared suppressor factor from C3H mice could only be blocked by anti-I-J^k.

DISCUSSION

Our laboratory previously described a suppressor cell mechanism for a murine model of cryptococcosis which functioned to limit the phagocytic activity of a macrophage subset (3, 4, 11). The suppressor cell was found in the spleens of animals injected with *C. neoformans* or in the spleens of animals injected with soluble cryptococcal antigens. The suppressive mechanism was also detected in animals made immunologically tolerant of both a protein antigen (human gamma globulin) and another polysaccharide antigen (levan). The suppressor cell could not be detected in the spleens of mice given immunogenic doses of these antigens.

The suppressor cell was characterized as a Lyt 1^-, Lyt 2^+, I-J^+, and I-A^- T lymphocyte. After stimulation with specific cryptococcal antigen in vitro, the cell released a soluble suppressor factor into the tissue culture medium. The interaction of the suppressor factor with the affected macrophage was genetically restricted, and this restriction was associated with the I-J locus of the major histocompatibility complex. Many of the features listed above are characteristics shared with the TsF2 and TsF3 suppressor factors described by Dorf and Benacerraf (7) for the NP haptenic system and by Murphy and co-workers (12, 14) for crypto-

coccosis. However, one feature, the method for preparation of the suppressor factor, differed. That is, the suppressor factor that we study required stimulation with cryptococcal antigen in vitro before it could be detected in tissue culture medium. The TsF2 and TsF3 factors were prepared by extraction of spleen cells without need for additional incubation with antigen. Nonetheless, we had never attempted to obtain our suppressor factor by extraction procedures. Attempts to detect the suppressor factor by freeze-thaw extraction methods were unsuccessful. We cannot totally eliminate this method as a possible way to obtain the factor, since our inability to detect the factor could always be due to a concentration effect. In addition, we did not prove that our method was completely analogous to that of Murphy and co-workers by direct comparison with the delayed-type hypersensitivity system. However, recently developed T-T hybridomas which produce the suppressor factor for macrophage phagocytosis also require antigen stimulation in the presence of syngeneic accessory cells before the suppressor factor can be detected in the supernatant. This is in contrast to the TsF3 hybridomas described by Dorf and Benacerraf (7) and Fidel and Murphy (9), which constitutively produce suppressor factor found in culture supernatants without need for antigenic stimulation.

In our previous publications (3, 4), we described the rationale behind the data analysis in which N+ supernatants were compared with N− supernatants and S+ supernatants were compared with S− supernatants (for definitions, see Table 1, footnote *b*). This scheme was used because the degree of phagocytosis of macrophages cultured in supernatants from normal mice sometimes differed from that of those cultured in supernatants from SCPA-treated mice. Since one would expect to see a mixture of factors which were different in these two populations, the analysis described above was determined to be most appropriate. This phenomenon was extensively discussed in previous publications (3, 4). The use of the hybrid clone F6.6.2 and the BW 5147 cell line in this investigation has eliminated much of the variation seen with conventionally prepared supernatants. We have chosen to statistically compare BW 5147+ with BW 5147− and F6.6.2+ with F6.6.2− as a routine procedure since this is the condition in which only one variable (presence or absence of antigen) occurs. Slight variations in the overall level of phagocytosis between the BW 5147 and F6.6.2 supernatants have not been statistically significant.

The molecular size of the suppressor molecule was first estimated by filtration through Amicon filters and then was more precisely determined by using Sephadex chromatography procedures. The molecular mass of the suppressor factor was determined to be approximately 70 kDa. This is consistent with the two-chain suppressor factors described for the picryl and NP pathways and for cryptococcosis. Such factors have been shown to have one chain which is either idiotypic or anti-idiotypic and a second chain which is I-J$^+$ (1, 7). While we have no direct proof that the suppressor factor under study is I-J$^+$, we were able to block the activity of the factor by treatment of culture supernatants with appropriate I-J alloantisera. This blocking effect is most likely due to an interaction of anti-I-J with the suppressor factor as well as antibodies to the I-J-interactive molecule which is on the macrophage membrane. Antibodies to both of these structures are found in the anti-I-J alloantisera (7, 10).

The inability to block suppressor factor activity with anticryptococcal antibodies at concentrations far in excess of those which result in positive enzyme-linked immunosorbent assays and the ability to absorb suppressor factor from supernatants with encapsulated *C. neoformans* were taken as evidence that the suppressor factor under study was idiotypic in nature. Therefore, this factor most closely resembles the TsF3 factor of Dorf and Benacerraf (7) and the TsFeff described by Asherson (1) for the antipicryl system. Although SCPA was added to cultures to induce suppressor factor from suppressor cells, we do not know the final concentration of polysaccharide present in the supernatant after removal of the cells. It is possible that some, if not all, of the antigen would adhere to accessory cells (now known to be necessary for induction of factor [unpublished results]) and thus be removed along with the cells. If SCPA does remain in the supernatant, the levels of suppressor factor are apparently above the binding capacity of the polysaccharide. If this were not the case, then the factor, bound to high-molecular-weight polysaccharide, would not pass through the Amicon XM100 filter (exclusion limit, 100 kDa). It is known that SCPA can bind to nonencapsulated *C. neoformans* cells, and therefore any SCPA-factor complexes would be absorbed by the nonencapsulated M7 control. However, there should be sufficient free factor present to allow detection of suppressor factor activity, as seen in Table 4 where suppression occurred after absorption with the nonencapsulated strain M7. Because the factor was absorbed by encapsulated cryptococci and was not absorbed by the nonencapsulated strain, its ability to interfere in the phagocytic assay by direct interaction with the yeast particle seems unlikely as the nonencapsulated strain was used as the phagocytic particle. Additionally, the suppressor factor was removed from the monolayer prior to the initiation of the phagocytic assay.

Asherson and colleagues found that the TsFeff of the antipicryl system required in vivo activation with specific antigen 24 h before removal of spleen cells from mice (2). The requirement for antigen stimulation is similar to that of the factor that we have described. The TsFeff was capable of arming another T cell, called the T-acceptor cell, which subsequently released a nonspecific suppressor factor (nsTsF-1). This latter factor was the first of two nonspecific factors which were found to operate in this pathway and which ultimately suppressed cutaneous sensitivity responses (1, 17). A direct comparison of the TsF3 factor of the NP suppressor cascade with the TsFeff in the antipicryl pathway (2) revealed that each was capable of arming T-acceptor cells for release of nsTsF-1. The differences between the antipicryl and the anti-NP pathways include the lack of a Ts2 cell (or anti-idiotypic loop) in the picryl pathway and a necessity for antigen stimulation in the picryl pathway. Whether the antigen-induced suppressor factor that we have described functions in a manner similar to TsFeff remains to be determined.

The exact mode of action of the suppressor factor is not currently known. We do know that the factor acts rapidly (within 15 min of incubation with the macrophage) to inhibit phagocytosis and that the macrophage can rapidly recover from the effects of the factor once it is removed. Phagocytosis via interaction with mannan or Fc receptors is affected, while phagocytosis via complement receptors or by nonspecific mechanisms is not (submitted for publication). Taken together, these observations show that the factor does not exert a toxic effect on the macrophage. Further investigations regarding the mechanism of inhibition, including the fate of mannan receptors and the possibility that phagocytic

particles are inhibited from binding to the macrophage membrane, are currently under way.

The macrophage subset affected by the suppressor factor is small and accounts for only 10 to 20% of the total peritoneal exudate population. Dorf and Benacerraf (7) detected macrophages called factor-presenting cells (FPC) in the NP pathway. These cells bound suppressor factor and functioned to induce the next cell in the suppressor pathway. The FPC were shown to be I-A$^+$ and reactive with anti-I-J alloantiserum, presumably due to their expression of the I-J-interactive molecule. If the macrophage subset that we have studied is similar phenotypically to the FPC, then the size of the affected cellular subset is reasonable since there are only 10 to 15% I-A$^+$ cells in the peritoneal exudate. To date, we have defined this subset according to its susceptibility to suppressor factor. However, recent evidence showed that the macrophage was both I-A$^+$ and I-J$^+$ (submitted for publication). While this investigation has not defined the overall function that our suppressor factor may play in the induction or maintenance of the tolerant state, it has confirmed the class of suppressive molecule under study. Because the factor has structural characteristics in common with TsF3 and TsFeff (factors which have known interactions with macrophages), other activities, such as FPC or suppressor macrophage function, need to be considered. The ability of the suppressor factor to inhibit the phagocytic activity of a small macrophage subset may only be secondary to those other activities which the subset may exhibit. The TsFeff in the picryl system can arm not only T-acceptor cells but also macrophages to release nonspecific suppressor factors (15). We have not tested the suppressor factor in our system to determine if the macrophage releases immunosuppressive molecules. It is possible that the affected macrophage functions as an FPC in a manner similar to those described for the NP pathway. Certainly, an overall effect on the clearance of cryptococci seems unlikely, because the cellular population affected is so small and because the factor inhibits phagocytosis mediated by mannan and Fc receptors but not that mediated by complement receptors (unpublished data). Current investigations in our laboratory are focused on identifying the subset of macrophage affected and determining whether other macrophage activities might be attributed to this macrophage subset.

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