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Characterization of the Macrophage Subset Affected and Its Response to a T Suppressor Factor (TsFmp) Found in Cryptococcosis

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Previous reports from our laboratory described the detection of a suppressor factor which inhibited the phagocytic activity of a macrophage subset in murine cryptococcosis and in classical models of immune tolerance. The suppressor factor was originally named PIL (phagocytosis-inhibiting lymphokine) but has recently been renamed TsFmp (T suppressor factor for macrophage phagocytosis) because it was found to resemble the antigen-specific I-J-restricted suppressor factors described by others. The current investigation revealed that TsFmp acted rapidly upon the macrophage (15 min or less) to exert its effect of inhibiting the phagocytic process. The time for the macrophage to recover from the effects of TsFmp was likewise very rapid. The ability of TsFmp to inhibit phagocytosis was limited to engulfment of particles by Fc and mannan receptors and did not extend to phagocytosis via complement receptors or by nonspecific mechanisms. The macrophage subset that responded to TsFmp was determined to be in the I-A$^+$ and I-J-IM$^+$ subset.

We previously described (2–4, 11) the detection of a T suppressor factor that inhibited the phagocytic activity of a macrophage subset in mice infected with *Cryptococcus neoformans* or in mice injected with soluble cryptococcal antigens. The suppressor cell responsible for the production of the suppressor factor was identified as an Lyt-2$^+$, I-J$^+$ T lymphocyte that secreted the factor into tissue culture medium only after stimulation with specific antigen. The suppressor factor had a molecular size of approximately 70,000 daltons and was genetically restricted in its activity. That restriction was mapped to I-J as defined by the difference between B10.A(3R) and B10.A(5R) mice. The factor was absorbed by cryptococcal antigen, and its activity could be blocked by anti-I-J alloantiserum.

The macrophage that responded to the factor was present among a very small subset (10 to 20%) of peritoneal exudate cells, but very little other information regarding the affected macrophage was known. Originally we named the factor PIL (phagocytosis-inhibiting lymphokine). However, because recent information regarding the size and molecular structure of the factor placed it in the class of antigen-specific suppressor factors that have been described in haptenic systems, we renamed the factor TsFmp.

The present investigation was aimed at further analysis of the response of the macrophage to the suppressor factor. Included was a kinetic analysis of the ability of TsFmp to inhibit the phagocytic process as well as determination of the time required for the macrophage to recover from the effects of TsFmp. The effect on phagocytosis via different membrane receptors was examined. Additionally, the susceptible macrophage was evaluated for its expression of the membrane markers I-A and I-J-IM.

MATERIALS AND METHODS

Abbreviations used. The following abbreviations and designations have been used: IgG, immunoglobulin G; EA, erythrocytes coated with IgG antibody; EAC, erythrocytes coated with IgM antibody and complement; I-J-IM, I-J-interacting molecule; PBS, phosphate-buffered saline; SCPA, soluble capsular polysaccharide antigen; sRBC, sheep erythrocytes; TsFeff, T effector suppressor factor; TsFmp, T suppressor factor for macrophage phagocytosis.

Animals. C3HeB/FeJ and C57BL/6J mice were purchased from the Jackson Laboratories, Bar Harbor, Maine. The mice were received when they were 8 weeks old and were used in experiments when they were 9 to 14 weeks old. The mice were maintained in the University of Oklahoma Health Sciences Center animal care facility, which is approved by the American Association for the Accreditation of Laboratory Animal Care. The animals were fed Purina Mouse Chow and water ad libitum.

Reagents. Sterile water for irrigation (no. R5000-01) was purchased from American McGaw, Irvine, Calif. Hybri-sure fetal calf serum (no. 12-10378-H), RPMI 1640 (no. 51-50178), HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (no. 59-20577), and sodium pyruvate (no. 59-20377) were purchased from Hazleton Research Products, Lexana, Kans. GIBCO Laboratories, Grand Island, N.Y., was the supplier of antibiotic-antimycotic (no. 600-5245), L-glutamine (no. 320-5039), and sodium bicarbonate solution (no. 670-5080). Dulbecco PBS (no. 17-5154) was purchased from Whattaker, MA Bioproducts, Walkersville, Md. Hybricare medium was purchased from the American Type Culture Collection, Rockville, Md. Human serum (type AB) was obtained from the Oklahoma Blood Institute, Oklahoma City. The blood was negative for human immunodeficiency virus antibody and all other tests required for human administration. Lots of human serum were preselected for their ability to support TsFmp production. Iron-supplemented newborn calf serum (no. A2151-4) was obtained from Hyclone, Logan, Utah. Lipopolysaccharide from *Escherichia coli* O55:B5 and Brewer thioglycollate medium (no. 0236-01) were supplied by Difco Laboratories, Detroit, Mich. Latex particles (0.0082 μm in diameter; no. LB-11) were purchased from Sigma Chemical Co., St. Louis, Mo.

Complete RPMI 1640 was used for macrophage culture

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and consisted of medium supplemented with 1% antibiotic-antimycotic, 200 mM L-glutamine, 10 mM HEPES buffer, 1% sodium pyruvate, and serum. In earlier experiments, 5% human serum supplemented with 10 μg of endotoxin per ml was used. Previous results showed that endotoxin supplementation was necessary to support adequate phagocytosis when human serum was used to culture macrophages (3). Later, the serum source was changed to 10% endotoxin-free fetal calf serum; no endotoxin supplementation was necessary, and in fact the phagocytic activity of macrophages was better than when human serum was used. For lymphocyte cultures, RPMI 1640 was supplemented with 5% human serum without endotoxin. Complete Hybricare medium consisted of medium supplemented with 1% antibiotic-antimycotic and 10% iron supplemented newborn calf serum.

Fungal strains. An encapsulated strain of *C. neoformans*, strain NU-2, and a nonencapsulated mutant, strain M7, were previously described (4). These strains were maintained in the laboratory by subculture on mycologic agar (Difco) and were also preserved under sterile mineral oil.

Antibodies. Anti-I-J alloantisera were obtained from Accurate Chemical Co. (anti-I-J^k) or the Transplantation Immunology Branch, National Institutes of Health (anti-I-J^b). These mouse alloantisera were used previously and were shown to eliminate suppressor cell activity from spleen cell populations of appropriate mouse strains when used at a concentration of 1:10 in the presence of rabbit complement (3). For that reason, a 1:10 dilution of each antiserum was used in cell separation procedures with the panning technique. Hybridoma cell lines HB 42 and HB 37, which produce anti-I-A^k and anti-I-A^b, respectively, were obtained from the American Type Culture Collection. These cell lines were subcloned, and clones producing high levels of mouse immunoglobulin were tested by indirect immunofluorescence on mouse peritoneal exudate cells to determine whether they exhibited appropriate strain specificity. Two subclones were selected, and supernatants from these cell lines were treated with 50% saturated ammonium sulfate to obtain the globulin fraction. After dialysis and suspension in PBS, dilutions of the fractions were tested by indirect immunofluorescence to determine the optimum dilution for treatment of macrophages in panning procedures. Fluorescent labeled goat anti-mouse immunoglobulin was purchased from Cooper Biomedical, Inc., West Chester, Pa. (no. 0711-0231). IgG anti-sRBC (no. 768-970) and IgM anti-sRBC (no. 768-990) were purchased from Cordis Laboratories.

Cryptococcal antigen. SCPA was obtained by repeated alcohol precipitation of supernatants obtained from a suspension of NU-2 cells after 5 min of treatment with a Braun cell disrupter. This procedure removed the capsule from the surface of the cell but did not disrupt the yeast cell itself. The methods for precipitation of the polysaccharide antigen have been previously described (3).

Preparation of EA and EAC complexes. In some experiments the ability of macrophages to phagocytize via Fc or complement receptors was evaluated. For Fc-mediated phagocytosis, sRBC that had been treated with IgG anti-sRBC (EA) were used as the phagocytic particle. These were prepared by treatment of washed sRBC (5% suspension) with a subagglutinating concentration of anti-sRBC (1:2,000) at 37°C for 30 min followed by three washes with PBS. EAC were prepared by treatment of washed sRBC with a subagglutinating concentration of IgM anti-sRBC (1:80). After washing the sRBC were treated with a 1:10 dilution of fresh mouse serum used as a source of complement for 30 min. The sRBC were then washed an additional three times and suspended (10^7 sRBC/ml) for use in phagocytic assays. For phagocytosis sRBC were suspended in RPMI 1640 without serum.

Preparation of TsFmp. Two procedures were used to prepare the TsFmp suppressor factor. The conventional procedure utilized spleen cells taken from animals injected intraperitoneally 1 week previously with 200 μg of SCPA. These cells were cultured at a concentration of 5×10^6 per ml in complete RPMI 1640 with or without 5 μg of SCPA per ml as described previously (3). Normal control supernatants were prepared in a similar fashion, except that spleen cells were harvested from normal mice. The normal controls showed that prior sensitization of mice with SCPA was required for suppressor factor production and that the polysaccharide antigen itself did not produce the effect. Later, a T-T hybridoma was produced (2) that secreted TsFmp. When supernatants were prepared from the hybrid cell line (F6.6.2) the cells were cultured at 2.5×10^5 cells per ml in complete Hybricare medium in the presence of 5×10^6 normal spleen cells as a source of accessory cells. Cultures were stimulated with 5 μg of SCPA per ml or were unstimulated. Additional control cultures consisted of the BW5147 fusion partner cultured in the presence or absence of cryptococcal antigen and normal spleen cells.

Assay of TsFmp. Peritoneal exudate cells were collected by peritoneal lavage of mice which were injected intraperitoneally 3 days previously with 0.25 ml of 10% aged thioglycollate medium. The cells were washed and suspended to 2×10^5 cells per ml of RPMI 1640 containing 10% fetal calf serum, and 0.5 ml of the cell suspension was dispensed into the wells of a eight-chambered Lab-Tek slide (Miles Laboratories, Inc., Elkhart, Ind.). The monolayers were allowed to adhere to the surface of the slides for various times as indicated. At the time of assay the medium was removed from the wells of the slides and replaced with 0.25 ml of test or control supernatant. During kinetic analysis the supernatants were incubated on the monolayers for various periods of time ranging from 15 min to 24 h. However, after the completion of these experiments the routine procedure involved treatment of the macrophage monolayers with supernatant for 1 h. After incubation of the monolayer at 37°C in 5% CO_2, the supernatants were removed and replaced with 0.5 ml of a suspension of nonencapsulated cryptococci (M7) at a concentration of 5×10^6 cells per ml, and the slides were incubated for an additional 2 h. Subsequently, the slides were washed by removing the plastic top and gently dipping the slides in beakers of warm PBS to remove extracellular cryptococci. The slides were then air dried and stained with Camco Quick Stain (Scientific Products Inc., Detroit, Mich.). In some instances phagocytosis via Fc-receptor-mediated, complement receptor, and nonspecific mechanisms was evaluated. In these experiments EA or EAC complexes and latex particles at 10^7 particles per ml were used instead of cryptococci. Assays utilizing EAC complexes were incubated for 2 h; however, ingestion of EA and latex was much quicker and required only 1 h of incubation. After phagocytosis of erythrocytes the slides were treated with 0.85% ammonium chloride solution before washing to lyse extracellular erythrocytes but otherwise were washed and stained as described for the M7 procedure. In all phagocytic assays four replicate determinations were performed for each experimental condition. At least 100 cells were evaluated, and the percentage of macrophages containing one or more particles was determined.

Separation of macrophage subpopulations by panning. Peritoneal exudate cells were separated based upon their expres-

sion of I-A and or I-J-IM by a panning procedure. The plates used for panning were treated with 10 μg of affinity-purified $F(ab')_2$ goat anti-mouse immunoglobulin per ml suspended in PBS for 18 h at 4°C. After the antibody solution was removed and plates were washed with PBS three times, the plates were used for cell separations. Cells (10^7 cells per ml) to be separated were treated with 50% fetal calf serum for 30 min on ice to block Fc receptors. They were then washed and treated at 4°C with anti-I-J or anti-I-A antiserum at dilutions previously shown to be appropriate (1:10). After 20 min of incubation the cells were washed and then diluted in PBS to a concentration of 1×10^7 per ml. The cell suspension (1.5 ml) was then placed in the tissue culture well (12-well plate) and incubated on ice for 1 h. After this incubation the nonadherent fraction was tested for its susceptibility to TsFmp as described above. The adherent fraction could not be evaluated because after this fraction was scraped from the bottom of the tissue culture well, it would not adhere to the Lab-Tek slides used in the phagocytic assay. The effectiveness of the I-A panning procedure was followed by indirect immunofluorescence analysis of the separated populations and was shown to remove the $I\text{-}A^+$ population. Before panning macrophage populations contained 16 to 33% $I\text{-}A^+$ cells. After panning with specific anti-I-A the nonadherent fractions were 5 to 6% $I\text{-}A^+$. $I\text{-}A^+$ cells in the adherent fraction were slightly enriched (17 to 40%) compared with unfractionated cells. This was attributed to the fact that even though the cells were panned at 4°C a substantial number of $I\text{-}A^-$ macrophages were able to adhere to the plastic surface. When macrophages were panned by using nonspecific anti-I-A antibodies, equal numbers of $I\text{-}A^+$ cells were found in the adherent and nonadherent fractions: these percentages were similar to those in the unfractionated population. These results indicated that the ability to adhere to plastic at 4°C was a random event in both the $I\text{-}A^+$ and $I\text{-}A^-$ populations. Because it is difficult to detect I-J-IM by conventional serologic procedures, we relied upon the fact that the dilution of anti-I-J used would remove suppressor cell activity from spleen cell populations (3) and that in the current experiments the affected macrophage was only removed by treatment with the appropriate anti-I-J reagent.

Statistical analysis. Data were analyzed by the Student *t* test with the Fisher test of variances. Phagocytosis in the presence of supernatants from normal cells cultured in the presence of SCPA were compared with those same cells cultured in the absence of antigen, as were the data from the paired supernatants of spleen cells from SCPA-injected mice. When hybridoma cells F6.6.2 were the source of TsFmp, unstimulated cells were compared with antigen-stimulated cells. Control BW5147 supernatants were compared in a similar fashion. This procedure of data analysis has been used routinely for several years (2–4) for two reasons. First, when conventional supernatants were used the overall level of phagocytosis occasionally varied when the supernatants from normal cells and SCPA-stimulated cells were compared. This variance was believed to be due to the presence of other factors in the cultures of SCPA-injected mouse spleen cells cultured without antigen that may serve as growth factors for the macrophage (3, 4). Second, it is our belief that this comparison is the most appropriate since the difference between the paired (positive and negative) supernatants is the only experimental condition where a single variable exists.

TABLE 1. Kinetics of TsFmp activity

Expt and time[a]	Supernatant[b]	% Phagocytosis (SEM)	P[c]
Expt 1			
0.5 h	N^-	55.8 (2.3)	
	N^+	56.5 (3.4)	
	S^-	59.3 (1.7)	
	S^+	42.0 (1.5)	<0.0005
1 h	N^-	59.3 (3.0)	
	N^+	57.8 (2.1)	
	S^-	63.0 (2.5)	
	S^+	51.0 (1.1)	<0.005
2 h	N^-	52.8 (3.4)	
	N^+	53.8 (1.4)	
	S^-	57.3 (3.0)	
	S^+	44.3 (1.3)	<0.005
24 h	N^-	44.3 (1.8)	
	N^+	44.0 (1.3)	
	S^-	47.8 (2.7)	
	S^+	36.8 (2.0)	<0.01
Expt 2			
15 min	N^-	42.5 (1.8)	
	N^+	38.3 (2.4)	
	S^-	40.0 (3.8)	
	S^+	32.8 (1.9)	<0.025
30 min	N^-	41.0 (3.0)	
	N^+	42.0 (3.0)	
	S^-	44.5 (2.4)	
	S^+	33.8 (2.0)	<0.0005
1 h	N^-	44.3 (2.5)	
	N^+	47.0 (1.9)	
	S^-	45.1 (2.8)	
	S^+	33.4 (3.3)	<0.005
2 h	N^-	47.8 (1.4)	
	N^+	43.0 (3.4)	
	S^-	47.8 (1.3)	
	S^+	33.9 (1.5)	<0.0005

[a] Time that supernatants were left on macrophage monolayers before phagocytic assay.

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

[c] S^+ supernatant compared with the S^- supernatant and N^+ supernatant compared with the N^- supernatant by the Student *t* test.

RESULTS

Kinetics of TsFmp activity. In our original description of the suppressor factor for macrophage phagocytosis, lymphocyte supernatants were incubated on macrophage monolayers for 3 days (11). Over several years we learned that the incubation period could be shortened to 24 h. It was of interest to know the minimal amount of time that TsFmp needed to be in contact with the macrophage for inhibition of phagocytosis to occur. Kinetic analysis of the effect is shown in Table 1. Initial experiments looked at the kinetics of the phenomenon over the first 24 h of culture; we found that inhibition occurred in as little as 30 min after TsFmp-containing supernatants were placed on macrophage monolayers. Further analysis showed that inhibition could occur after only 15 min of incubation of the suppressor factor on the macrophage monolayer. Because of the manipulations required in the performance of the experiments, it has not been possible to look at the 0- to 15-min period.

Kinetics of macrophage recovery from TsFmp. Of further interest was the time required for the macrophage to recover from the effects of TsFmp. For these experiments macrophage monolayers were cultured with TsFmp for 1 h. After the supernatant was removed from the monolayers, the cells

TABLE 2. Kinetics of macrophage recovery from the effects of TsFmp

Expt and time[a]	Supernatant[b]	% Phagocytosis (SEM)	P[c]
Expt 1			
0 h	N^-	47.5 (4.9)	
	N^+	42.0 (0.9)	
	S^-	52.0 (2.7)	
	S^+	39.8 (1.7)	<0.005
2 h	N^-	63.5 (1.2)	
	N^+	62.3 (3.6)	
	S^-	60.8 (2.6)	
	S^+	58.8 (1.3)	
4 h	N^-	57.8 (3.6)	
	N^+	53.5 (2.6)	
	S^-	62.8 (1.9)	
	S^+	57.4 (5.5)	
24 h	N^-	74.0 (3.4)	
	N^+	75.3 (2.6)	
	S^-	74.5 (1.1)	
	S^+	74.0 (2.6)	
Expt 2			
0 min	N^-	93.8 (1.5)	
	N^+	94.0 (0.9)	
	S^-	96.0 (1.1)	
	S^+	82.8 (0.9)	<0.0005
15 min	N^-	92.0 (2.7)	
	N^+	92.0 (2.2)	
	S^-	92.3 (0.7)	
	S^+	94.3 (1.4)	
30 min	N^-	92.5 (1.6)	
	N^+	93.8 (2.9)	
	S^-	92.3 (1.6)	
	S^+	92.5 (0.7)	
1 h	N^-	92.5 (1.7)	
	N^+	95.8 (0.5)	
	S^-	92.5 (0.9)	
	S^+	94.0 (1.1)	
2 h	N^-	96.7 (0.3)	
	N^+	96.3 (0.8)	
	S^-	95.5 (0.9)	
	S^+	95.0 (0.4)	

[a] Time that macrophage monolayers were cultured in complete medium after treatment with supernatant for 1 h and before phagocytic assay.
[b] See footnote *b* of Table 1.
[c] See footnote *c* of Table 1.

were washed three times with warm tissue culture medium and then fresh medium was added. The monolayers were incubated for various periods of time before the addition of the M7 suspension for analysis of phagocytosis. Initially the 0- to 24-h time period was examined; recovery was complete within 2 h. Examination of the 0- to 2-h time period revealed that the macrophage recovered from the effects of TsFmp after only 15 min of incubation in tissue culture medium (Table 2). The actual time for recovery may not be as fast as 15 min, since the phagocytic assay takes 2 h. Thus, we can only say that recovery occurs within 2 h and 15 min after removal of the suppressor factor.

Analysis of the phagocytic receptor inhibited by TsFmp. Previous experiments showed that TsFmp would inhibit phagocytosis of cryptococci or *Saccharomyces* cells (11); however, phagocytosis via other receptors had never been evaluated. Examination of the effects of TsFmp on Fc- or complement-receptor-mediated phagocytosis was evaluated by the ability of the suppressor factor to inhibit phagocytosis of EA or EAC complexes. Inhibition of non-receptor-mediated phagocytosis was examined by use of latex particles.

TABLE 3. Effect of TsFmp on the phagocytic activity of macrophages by various receptors

Particle[a]	Supernatant[b]	% Phagocytosis (SEM)	P[c]
M7	N^-	50.3 (0.7)	
	N^+	47.0 (1.1)	
	S^-	53.8 (2.8)	
	S^+	36.8 (1.7)	<0.005
EA	N^-	71.8 (1.6)	
	N^+	76.8 (1.6)	
	S^-	84.3 (1.7)	
	S^+	53.8 (5.0)	<0.005
EAC	N^-	67.3 (1.8)	
	N^+	64.7 (2.9)	
	S^-	65.3 (1.9)	
	S^+	67.8 (1.6)	
Latex	N^-	89.3 (0.9)	
	N^+	93.8 (0.6)	
	S^-	89.0 (1.7)	
	S^+	92.3 (2.3)	

[a] Monolayers were treated with supernatants; then phagocytosis via mannan receptors (M7), Fc receptor (EA), complement receptor (EAC), or nonspecific mechanisms (latex) was tested.
[b] See footnote *b* of Table 1.
[c] See footnote *c* of Table 1.

In preliminary experiments phagocytosis of M7 was determined to be mediated by mannan receptors by the ability of mannan to block macrophage phagocytosis of M7 (data not shown). The results of a typical experiment are shown in Table 3. Whereas TsFmp-containing supernatants could effectively inhibit phagocytosis of M7 yeast cells or of EA complexes, no inhibition occurred when EAC complexes or latex particles were used. Thus, the phagocytic activity per se of the macrophage subset was not affected; only phagocytosis via certain macrophage receptors was affected.

Expression of I-A and I-J-IM on the membrane of the affected macrophage. The macrophage subset that was affected by TsFmp was small, accounting for only 10 to 20% of the total macrophage population. The size of the subset and the I-J genetic restrictions previously reported for the effect of the factor upon the macrophage suggested that the macrophage may express I-A and/or I-J-IM on its membrane. Experiments to determine these possibilities were conducted by removing I-A- and I-J-IM-positive subsets by panning. The results of these experiments are shown in Tables 4 and 5. Treatment of C3HeB/FeJ macrophages with anti-I-A^k followed by panning on an anti-mouse immunoglobulin plate effectively removed the affected macrophage from the suspension of peritoneal exudate cells. However, treatment with anti-I-A^b did not remove the population. Likewise, when C57BL/6 macrophages were treated with anti-I-A^b the affected macrophage was removed, but it remained in the population when the macrophages were treated with anti-I-A^k.

Alloantisera prepared by cross-immunization of mouse strains differing only at the I-J locus contain antibodies reactive against I-J antigens as well as molecules on the macrophage membrane that interact with I-J and which have been called I-J-IM (7). For this reason I-J alloantisera were used to remove I-J-IM-positive macrophages from the peritoneal cell populations by panning (Table 5). Treatment of C3HeB/FeJ macrophages with anti-I-J^k followed by panning removed the susceptible subset from the population. Treat-

TABLE 4. Expression of I-A antigens on the macrophage responsive to TsFmp

Macrophage	Treatment[a]	Supernatant[b]	% Phagocytosis (SEM)	P[c]
C3H	None	BW^-	78.8 (2.3)	
		BW+	74.8 (3.4)	
		$F6^-$	77.5 (1.7)	
		$F6^+$	64.0 (1.9)	<0.005
	$I\text{-}A^k$	BW^-	49.5 (3.9)	
		BW^+	47.8 (0.7)	
		$F6^-$	47.3 (4.4)	
		$F6^+$	54.8 (2.4)	
	$I\text{-}A^b$	BW^-	55.0 (1.6)	
		BW^+	57.8 (1.7)	
		$F6^-$	59.8 (3.1)	
		$F6^+$	44.0 (2.1)	
C57BI	None	N^-	79.0 (2.8)	
		N^+	79.5 (4.5)	
		S^-	75.8 (2.9)	
		S^+	65.3 (0.8)	<0.01
	$I\text{-}A^k$	N^-	48.8 (2.1)	
		N^+	53.0 (2.6)	
		S^-	50.5 (3.1)	
		S^+	32.3 (2.8)	<0.005
	$I\text{-}A^b$	N^-	43.3 (2.1)	
		N^+	43.8 (2.6)	
		S^-	44.3 (1.1)	
		S^+	40.8 (0.5)	

[a] Peritoneal exudate cells were treated with anti-$I\text{-}A^k$ or anti-$I\text{-}A^b$ before panning on an anti-mouse immunoglobulin plate.

[b] Supernatant of BW5147 (BW), F6.6.2 (F6), or spleen cells from normal mice (N) or mice injected with SCPA (S) and cultured in the absence (−) or presence (+) of antigen (SCPA).

[c] $F6^+$ supernatant compared with $F6^-$ supernatant, BW^+ supernatant compared with BW^- supernatant, S^+ supernatant compared with S^- supernatant, and N^+ supernatant compared with N^- supernatant by the Student *t* test.

TABLE 5. Expression of I-J-IM on the macrophage responsive to TsFmp[a]

Macrophage	Treatment	Supernatant	% Phagocytosis (SEM)	P
C3H	None	BW^-	80.5 (4.3)	
		BW^+	79.8 (2.5)	
		$F6^-$	81.3 (2.5)	
		$F6^+$	65.0 (2.9)	<0.005
	$I\text{-}J^k$	BW^-	83.0 (1.1)	
		BW^+	84.0 (3.2)	
		$F6^-$	82.5 (1.8)	
		$F6^+$	81.3 (2.5)	
	$I\text{-}J^b$	BW^-	77.8 (1.1)	
		BW^+	76.8 (2.6)	
		$F6^-$	76.8 (1.1)	
		$F6^+$	64.0 (1.6)	<0.0005
C57BI	None	N^-	82.8 (4.7)	
		N^+	80.3 (2.6)	
		S^-	84.8 (2.7)	
		S^+	72.8 (0.9)	<0.005
	$I\text{-}J^k$	N^-	78.5 (1.2)	
		N^+	75.8 (1.1)	
		S^-	78.3 (2.2)	
		S^+	63.0 (1.5)	<0.005
	$I\text{-}J^b$	N^-	80.5 (1.3)	
		N^+	77.8 (2.0)	
		S^-	83.8 (2.8)	
		S^+	81.8 (2.0)	

[a] See footnotes *a*, *b*, and *c* of Table 4.

ment with anti-$I\text{-}J^b$ was ineffective. The reciprocal experiment with C57BL/6 macrophages treated with anti-$I\text{-}J^k$ or anti-$I\text{-}J^b$ showed that only the anti-$I\text{-}J^b$ treatment could remove the macrophage that was responsive to TsFmp.

DISCUSSION

We previously examined a suppressor cell mechanism that occurred in mice infected with *C. neoformans* and in mice injected with soluble cryptococcal antigens (2–4, 11). The suppressor cell was identified as belonging to the $Lyt\text{-}2^+$, $I\text{-}J^+$ subset. This suppressor cell required interaction with specific cryptococcal antigen in vitro before it secreted a soluble suppressor factor into tissue culture medium. The factor was originally named PIL; however, recent investigations (2) showed that the factor had a molecular size (70,000 daltons) and other characteristics (idiotypic and I-J restricted) that placed it in a category of factors that limit cutaneous hypersensitivity responses to haptens (1, 5) and the delayed-type hypersensitivity response in cryptococcosis (9, 12–14). For this reason we changed the name of the factor to be consistent with the nomenclature established for this group of antigen specific suppressor factors to TsFmp.

Although much was known about the identity of the lymphocyte that produced the suppressor factor and the factor itself, very little was known about the macrophage that responded to the factor. A kinetic study of the time required for TsFmp-macrophage interaction before inhibition of phagocytosis was inhibited showed that the interaction was very rapid (15 min or less). Thus, it was apparent that phagocytosis was inhibited as soon as the suppressor molecule interacted with the macrophage membrane and that no period of macrophage differentiation was required. This would rule out any hypothesis that TsFmp causes a change in the macrophage, such as activation, which would concomitantly reduce phagocytosis. Additionally, the macrophage was able to recover from the effects of TsFmp rapidly; only 15 min incubation in tissue culture medium after TsFmp treatment resulted in complete recovery from the effects of the factor. The actual time of recovery may have been longer than 15 min, since the phagocytic assay itself required 2 h of incubation. However, we can state that recovery occurred within 2 h and probably occurred more rapidly. If the interaction of TsFmp and the macrophage resulted in modulation of phagocytic receptors from the surface of the cell, one might expect that a period of time would be required for the cell to synthesize more receptors. This would be in keeping with what is known about the removal of immunoglobulin molecules from the surface of B cells during the modulation process (18). The rapidity of the recovery of the macrophage from the effects of TsFmp suggests that the phagocytic receptors are not modulated but that the receptors may be temporarily blocked by the suppressor factor. Proof of this hypothesis will require examination of the fate of the phagocytic receptor after interaction with TsFmp and the ability of the suppressor factor to inhibit binding of particles and/or receptor ligands. These experiments are planned for the near future. Rapid recovery from the effects of TsFmp may also indicate that the interaction between the suppressor factor and any macrophage receptor is one characterized with a low binding affinity.

Experiments designed to evaluate which phagocytic receptors were inhibited by TsFmp showed that phagocytosis via Fc or mannan receptors was inhibited, whereas phagocytosis by complement receptors or by nonspecific mecha-

nisms was not affected. The Fc and mannan receptors are very closely linked on the macrophage membrane and are known to comodulate (17). Since TsFmp only affected phagocytosis by this membrane complex, it is reasonable to suggest that the suppressor factor may interact at or very near these receptors. If the receptors are modulated, they must not be shed from the surface of the cell, since recovery is so rapid. On the other hand, a TsFmp interaction at or near this complex may provide stearic hindrance of these receptors and thereby inhibit the phagocytic process. Alternatively, the suppressor factor could cause inhibition of cellular enzymatic activities as does the glycosylation-inhibitory factor described for the regulation of IgE synthesis (6). If this were the case, then TsFmp could cause modification of certain receptors such that they could no longer bind their respective ligands. The possibility that the factor may inhibit cellular processes required for the phagocytosis of EA and M7 would require that different phagocytic processes were involved in the engulfment of the EAC and latex particles used in these experiments. It is of interest that Steele and co-workers (16) recently reported that T-suppressor factors from two independent suppressor cell networks shared common antigenic determinants with glycosylation-inhibitory protein and the phospholipase-inhibitory protein lipomodulin. This report is sure to lead to increased investigations into the possibility that the antigen-specific suppressor factors have the ability to inhibit enzymatic activities and that these properties contribute to their biologic activities in the generation of suppression.

Because TsFmp was known to be I-J restricted in its activity (2) and because we had previously shown that anti-I-J antisera would block the activity of TsFmp (2), we believed that the macrophage subset that responded to the factor could be similar in phenotype to the factor-presenting cells described by Dorf et al. (5, 8); those cells were I-A$^+$ and I-J-IM$^+$. These subsets were removed from the peritoneal exudate population by a cold (4°C) panning procedure; both anti-I-J and anti-I-A treatment could remove the affected macrophage. The factor-presenting cell in the anti-nitrophenyl suppressor pathway studied by Dorf et al. stimulated subsequent suppressor cells along the nitrophenyl pathway. Whether the macrophage under study in this investigation functions as a factor-presenting cell in a manner similar to the macrophages described by Dorf et al. is not known. Current investigations are directed at answering this question.

Another function that macrophages are known to exhibit after interaction with antigen-specific suppressor factors is that of synthesizing nonspecific suppressor molecules. Asherson et al. described a suppressor factor (TsFeff) in the anti-picryl suppressor pathway that had many characteristics in common with TsFmp and was able to arm macrophages or T-acceptor cells for the subsequent antigen-induced release of nonspecific suppressor factors (1, 10, 15, 19). The nonspecific macrophage factor inhibited lymphocyte proliferative responses to mitogens, interleukin 2 production, and cutaneous hypersensitivity responses to haptens. Other characteristics shared by TsFmp and TsFeff include the requirement for antigenic stimulation before the suppressor factor was produced by the T-suppressor cell, I-J restriction, and ability to be absorbed by specific antigen. TsFeff is known to be I-J positive, and we have indirect evidence to suggest that TsFmp is I-J positive (2).

This investigation has not defined the ultimate role of TsFmp in cryptococcosis. It seems unlikely that the suppressor molecule could significantly affect clearance of cryptococci, since only a small subset of macrophage is affected. On the other hand, it is known that this suppressor mechanism is common to other models of immune tolerance (4), and thus the role of the suppressor mechanism seems best attributed to an effect on the establishment or maintenance of the tolerant state. The suppressor factor and the macrophage that responds to the factor have similarities to cells described in both the picryl and nitrophenyl pathways, and the possibility that TsFmp is the same as the factors described in those systems or shares functional activities with those factors must be examined.

In previous investigations (11) we were able to suppress phagocytosis of a subset of cells obtained from nonstimulated peritoneal cavities (resident peritoneal cells). Thus, the population of cells obtained by thioglycolate stimulation was not unique to the peritoneal exudate. We attempted to examine the effects of TsFmp on fully activated macrophages (unpublished observations); however, these cells were not phagocytic, and thus no effect was seen. The possibility that the action of TsFmp on the cell may be related to its stage of activation (resident, primed, or activated) is of interest and one that our preliminary studies may not have adequately addressed, since the populations of cells used could have contained cells in several stages of activation. It should be noted that whether unstimulated cells or thioglycolate-induced cells were used, the number of responsive macrophages in the population did not increase, indicating that the thioglycolate peritoneal stimulant, which would have cells present that are at different stages of macrophage differentiation than resident cells, did not increase the number of cells responsive to the suppressor factor.

We previously reported that when the total phagocytic capacity was used to examine the effect of the suppressor factor on the macrophage population, a more substantial effect on the phagocytic process was detected (4). In these experiments some cells were found that engulfed fewer particles but that were not totally inhibited in their phagocytic activity. Because conventional supernatants were used, it is possible that the effect was due to other factors present in the supernatant. In addition, the time of incubation of supernatants on the macrophage monolayer was long enough to allow secondary effects to occur in the culture such as induction of the macrophage population to release other phagocytosis-inhibiting substances (for example, prostaglandin E_2). In the studies reported here, the phagocytic assay was modified as previously reported (2) to enhance the phagocytic activity of the macrophage populations. Under these conditions it was not possible to evaluate the total phagocytic capacity, since this determination required enumeration of the average number of yeast cells within each phagocytic macrophage. In our current assay most of the macrophages contain so many yeast cells that accurate counts of intracellular yeast cells are impossible.

The determination of the in vivo significance of the suppressor factor will come from experiments in which the fate of cryptococci is examined in animals after infusion of suppressor factor. Although preliminary experiments showed no effect on immediate clearance, we could not be sure that adequate amounts of suppressor factor were given. We must also examine the fate of yeast cells after infusion of suppressor factor over a longer term. If the suppressor factor is important in the establishment or maintenance of the tolerant state, its effect may require an in vivo induction period that could last several days. These issues will be the

focus of our further investigations of this T suppressor factor.

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