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SUPPRESSION OF HUMAN NATURAL AND ANTIBODY-DEPENDENT CYTOTOXICITY BY SOLUBLE FACTORS FROM UNSTIMULATED NORMAL LYMPHOCYTES[1]

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Serum-free culture supernatants of unstimulated normal human peripheral blood mononuclear cells contain soluble suppressor factor(s) (SSF) that significantly inhibit natural (NK) and antibody-dependent cellular cytotoxic (ADCC) activities of allogenic lymphocytes against a variety of target cells. Lymphocytes precultured with increasing concentrations of SSF showed a dose-dependent suppressive effect on these cytotoxic functions that was optimal at a concentration of 20% volume/volume. Adherent cells were not required for the production of SSF. Suppression was evident even at higher effector: target cell ratios and the inhibition was not reversed by washing lymphocytes. SSF was not itself cytotoxic, was stable at 56°C, and its suppressive effect was maximal after 72 hr of incubation with effector lymphocytes. Initial estimate of the molecular weight of SSF by ultra-filtration was <20,000 daltons. Gel filtration of SSF on Sephacryl S-200 resulted in the elution of two peaks of activity; one in the region between markers of 13,700 and 25,000 daltons, and the other <13,700 daltons. Both fractions demonstrated significant suppressive activity on NK and ADCC functions of allogenic lymphocytes. SSF inhibition of NK activity could be partially reversed by incubating lymphocytes for 1 hr with human leukocyte interferon (IF) and almost completely reversed after 24 hr of IF treatment. A few selected monosaccharides (α-methyl-D-mannoside, L-fucose and L-rhamnose) showed a dose-dependent blocking effect on SSF activity, which suggests that SSF may act via receptor sites recognized by these sugars. As demonstrated for other lymphocyte functions, NK and ADCC activities may also be modulated by SSF elaborated by normal PBL.

In a wide range of human and animal studies, lectin or antigen-induced suppressor cells have been convincingly demonstrated to exert a major role in specific and nonspecific immunoregulation (1–10). Recently it has become evident that suppressor cells produce a number of soluble mediators that also exhibit regulatory activity on various immune functions (11–18).

Natural killer (NK)[3] cells and cells mediating antibody-dependent cellular cytotoxicity (ADCC) have been gaining attention because of a proposed role in defense against tumors and in the regulation of lymphoid cell reactions (19–22). If NK or ADCC activities are important in self-regulation of lymphoid cell functions, it seems likely that differentiation, maturation and function of NK and ADCC cells would also be subject to immunologic control. Cudkowitz and Hochman (23) showed that spleens of infant mice contained cells capable of inhibiting the lytic function of NK effector cells. We have recently demonstrated that a relatively faster sedimenting fraction of thymocytes separated by velocity sedimentation suppressed the NK activity of both autologous and allogeneic spleen cells in mice (24). In humans, Pollack and Emmons (25) have observed that the activity of peripheral blood mononuclear cells (PBMC) capable of mediating ADCC could be inhibited by a population of fresh autologous lymphocytes. A series of studies done in our laboratory have demonstrated that unstimulated cultured human PBMC can develop suppressor cell activities against 1) lymphocyte transformation, 2) polyclonal B cell activation, and 3) NK and ADCC reactions as a possible consequence of an autologous mixed lymphocyte culture reaction. However, little is known about the biochemical mechanisms that regulate the effector cells. Recently Shou *et al.* (26) demonstrated a soluble suppressor factor (SSF) from unstimulated culture supernatants of human PBMC that exhibits suppressor activity toward the proliferative response of normal lymphocytes to concanavalin A (Con-A), phytohemagglutinin (PHA), or alloantigens in mixed lymphocyte culture (MLC) and toward pokeweed mitogen (PWM)-stimulated immunoglobulin synthesis and secretion *in vitro*. Since NK and ADCC reactions are important in defense against tumors, allograft rejection, and the regulation of various lymphoid reactions (19–22), soluble regulatory mediators elaborated by the lymphocytes of healthy subjects may also play a role in normal immunologic homeostasis. The current investigations were undertaken to examine the role of SSF in the regulation of cytotoxic activities. These studies demonstrate that culture supernatants of unstimulated PBMC from healthy individuals exert significant suppressive effects on NK and ADCC reactions possibly through interactions with saccharide-specific receptors present on effector cells. Further, preliminary characterization of this SSF showed that it has a m.w. of < 20,000 daltons, and its inhibitory effect is reversed by interferon (IF). The results suggest that, as demonstrated for other lymphocyte functions, NK and ADCC activities may also be modulated by SSF elaborated by normal PBL.

MATERIALS AND METHODS

Lymphocyte donors. Peripheral blood from healthy donors of both sexes aged 25 to 40 years was drawn into a plastic syringe containing heparin (20 U/ml). Donors were apprised of the study and consents were obtained consistent with the policies of The University of Michigan and the National Institutes of Health.

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[2] Requests for reprints should be addressed to: Madhavan P. N. Nair, The University of Michigan, Department of Epidemiology, 2022 School of Public Health I, Ann Arbor, Michigan, 48109.
[3] Abbreviations used in this paper: ADCC, antibody-dependent cellular cytotoxicity; CEM, human T-lymphoblastoid cell line; E:T, effector to target; HBSS, Ca^{++}- and Mg^{++}-free Hanks' balanced salt solution; IF, human leukocyte interferon; K562, human erythroleukemia cell line; Molt-4, human T-lymphoblastoid cell line; NK, natural killer; PBL, peripheral blood mononuclear leukocytes; PBMC, peripheral blood mononuclear cells; SB, human B-leukemia cell line; SSF, soluble suppressor factor(s); v/v, volume/volume; Con A, concanavalin A; PHA, phytohemagglutinin; MLC, mixed lymphocyte culture; PWM, pokeweed mitogen; HEPES, N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid.

Isolation of lymphocytes. PBMC were isolated from heparinized venous blood by using a modified method of Böyum (27). Blood was diluted with an equal volume of Ca^{++}- and Mg^{++}-free Hanks' balanced salt solution (HBSS) (GIBCO, Grand Island, NY) and centrifuged at 400 × G for 30 min at 18°C. The mononuclear cell band was harvested, washed three times with HBSS, and resuspended in RPMI 1640 medium, containing 25 mM HEPES[3] buffer supplemented with 80 μg gentamicin (Schering Corp., Kenilworth, NJ) per ml and 300 μg fresh glutamine per ml. The viability of cells was routinely greater than 98% as assessed by trypan blue dye exclusion.

Removal of adherent cells. Mononuclear cells were depleted of adherent cells by passing through a G-10 column. PBMC were suspended in RPMI 1640 with 10% FCS and passed through a 7-ml column of Sephadex G-10 beads (Pharmacia Fine Chemicals, Piscataway, NJ), equilibrated in the same medium. After 45 min of incubation at 37°C, nonadherent cells were washed through with one bed volume of warm (37°C) medium. Five × 10^6 nonadherent cells were added to a suspension of carbonyl iron (Lymphocyte Separator Reagent, Technicon Instruments Co., Tarrytown, NY) at a volume ratio of 2:1 and the mixture was incubated at 37°C on a rotator for 30 min. Phagocytic cells were depleted by centrifugation on a Ficoll-Hypaque density gradient at 400 × G for 20 min. Mononuclear cells depleted of phagocytic cells, peripheral blood lymphocytes (PBL), were collected from the interface and washed three times with HBSS. The viability of cells was always greater than 95% as assessed by trypan blue dye exclusion.

Enrichment of NK cells. Enrichment of NK effector cells by using a discontinuous gradient of Percoll (Pharmacia) was carried out as described elsewhere (28). To prepare a discontinuous density gradient, Percoll solution was mixed in various concentrations with RPMI medium, and 2-ml aliquots (ranging from 50.0 to 37.5% in 2.5% increments) were gently layered into 15- × 130-mm round bottom, glass test tubes. Lymphocytes depleted of Sephadex G-10 (Pharmacia) adherent cells were layered on top of the gradient and centrifuged at 300 × G for 45 min at 20°C. Each fraction was collected separately and washed twice in RPMI 1640 medium. The least dense fraction banding at the 37.5% Percoll interface consistently showed the highest NK activity and was used routinely as a source of NK-enriched cells.

Preparation of SSF. SSF was prepared as previously described (26). Briefly, PBMC or PBL, after being washed three times with HBSS, were resuspended in RPMI 1640 medium containing only gentamicin and glutamine. Suspensions of 5 to 10 × 10^6 cells/ml were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air for 0 to 5 days. After incubation, supernatants were separated by centrifugation at 500 × G for 30 min and stored at −20°C. Supernatants collected at 0, 1, 2, 3, 4, and 5 days were tested for their effect on lymphocyte cytotoxicity. Since supernatants collected on the 4th day produced the maximal suppressive effect, this time of culture was henceforth used for all subsequent SSF preparations. Control supernatants prepared in the absence of PBL were treated similarly and termed as mock SSF.

Incubation of effector cells with SSF. Duplicate sets of cultures containing 2 × 10^6 PBL in 0.8 ml of RPMI 1640 plus glutamine, gentamicin, and 5% fetal calf serum were added to a tube containing 200 μl of SSF, mock SSF, or fresh medium (20% v/v unless otherwise indicated) and incubated in a humidified environment of 5% CO_2 in air at 37°C for 72 hr. Cells were then washed twice and resuspended in complete medium. Cell viability in SSF-treated cultures was comparable with that of mock SSF-treated cultures as determined by trypan blue dye exclusion and was found to be 80 to 90%. Treated and control cultures were tested for their ability to mediate NK and ADCC activities.

Tumor target cells. The human erythroleukemic cell line, K-562 (29), T-lymphoblastoid cell line (CEM) (30), and another T-lymphoblastoid cell line Molt-4 (31) were used as targets for NK cells. For the ADCC assay, we chose as targets an antibody-coated human B cell leukemia (SB) because of resistance to NK activity in a 4-hr ^{51}Cr release assay (32).

Preparation of target cells. Tumor target cells were serially passaged in complete medium and used in cytotoxicity assays no more than 48 hr after the last passage. To 0.8-ml aliquots of complete medium containing 5 × 10^6 washed tumor cells, 200 μCi of ^{51}Cr as sodium chromate (New England Nuclear, Boston, MA) was added. The cells were incubated at 37°C for 1 hr in a humidified atmosphere of 5% CO_2 in air with intermittent shaking. After incubation, the cells were washed three times with complete medium and resuspended to a concentration of 2 × 10^5 cells/ml.

Assay for NK activity. NK activity was determined in a direct ^{51}Cr release assay as previously described (3, 33–35). A fixed number of viable effector cells in complete medium were added to triplicate cultures of ^{51}Cr labeled target cells in final volumes of 0.2 ml in V-bottom microtitration plates (Costar, Cambridge, MA). After centrifugation at 40 × G for 2 min, they were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air for 4 hr. Percent cytotoxicity was calculated as follows:

$$\% \text{ cytotoxicity} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{total release} - \text{spontaneous release}} \times 100$$

where spontaneous release represents counts released from control wells containing only 2 × 10^4 target cells, and total release represents counts obtained from a lysate of 2 × 10^4 target cells.

Assay for ADCC. The ADCC activity of effector cells was determined by the original method of Perlmann and Perlmann (36) as modified by Handwerger and Koren (37). Briefly, 50 μl of varying concentrations of effector cells were added to 50 μl of complete medium containing ^{51}Cr-labeled SB target cells and 100 μl of a 2 × 10^{-4} dilution of rabbit anti-SB antisera previously found to yield maximal ADCC. Percent ADCC activity was calculated as described above for NK activity with the following exceptions. Spontaneous release represents counts released in control wells containing effector cells, labeled SB target cells and media instead of anti-SB antibodies, and total release represents counts obtained in an aliquot of 1 × 10^4 ^{51}Cr labeled SB target cells. Percent suppression of both NK and ADCC activities was calculated as follows:

$$\% \text{ suppression} = \frac{\%\text{ cytotoxicity of mock SSF-treated lymphocytes} - \%\text{ cytotoxicity of SSF-treated lymphocytes}}{\%\text{ cytotoxicity of mock SSF-treated lymphocytes}} \times 100$$

Interferon. Partially purified human leukocyte IF was obtained as a gift from Parke-Davis and Co., Detroit, MI. The specific activity was 6.3 × 10^4 U/mg protein. IF was diluted in RPMI 1640 medium and stored at −70°C before use.

Treatment of lymphocytes with interferon. PBL (2 × 10^6/ml) precultured either with SSF or mock SSF were washed twice and suspended in 1 ml of medium to which 500 U of IF (previously found to yield a maximal NK augmenting effect) was added. The cultures were then incubated for 1 hr or 24 hr at 37°C in a humidified atmosphere of 5% CO_2 in air, washed twice, and resuspended in medium. Control cultures were treated identically with the exception that IF was not added. Viability of IF-treated lymphocytes was unaffected as assessed by trypan blue dye exclusion. Both IF treated and control cultures were assayed for their NK activity.

RESULTS

Effect of SSF on NK and ADCC activities of lymphocytes. In view of the finding that supernatants of unstimulated human lymphocyte cultures contain SSF, which inhibit normal PBMC responses to mitogens, alloantigens, and polyclonal B cell activation, we investigated the effect of SSF on NK and ADCC activities of allogeneic PBL. When PBL were incubated with SSF for 72 hr, both NK and ADCC were suppressed at all effector to target (E:T) ratios tested (Table I). Suppression was dose-dependent for SSF concentrations ranging from 5% to 40%, v/v (Table II). However, suppression was often nonlinear above 20%, so this concentration was used in subsequent experiments. As shown in Table III, suppression of NK activity was demonstrable as early as 24 hr after addition of SSF to the cultures. Suppression of ADCC activity, however, was only evident 72 hr after SSF addition. Maximal suppression of both NK and ADCC activities required incubation with SSF for 72 hr and this inhibition of cytotoxicity by SSF appeared to diminish by 96 hr. To examine whether removal of SSF by washing would restore the cytotoxic potential of the lymphocytes, PBL were preincubated with SSF for 72 hr and then washed, precultured in complete medium for a further period up to 72 hr, and tested for their cytotoxic activity (Table IV). The results demonstrate that removal of SSF by washing did not restore the cytotoxic activity of SSF-treated lymphocytes. The viability of lymphocytes on each day of incubation after washing of the cells was equivalent in both SSF- and mock SSF-treated cultures as measured by trypan blue vital dye exclusion. Our data suggest that there is no spontaneous recovery of suppressed NK and ADCC activities after treatment of PBL with SSF.

Data presented in Figure 1 reveal that normal PBL precultured with SSF for 72 hr showed significant suppression of NK against each of three targets and ADCC against a single target. Normal PBL precultured separately with 38 different SSF samples showed a 42.5% mean suppression of their NK activity against K562, with a range of 13 to 68% suppression. Lymphocytes precultured separately with 15 SSF samples dem-

TABLE I

Effect of SSF on NK and ADCC activities[a]

Preculture Conditions	% Cytotoxicity[b]					
	NK[c]			ADCC[d]		
	E:T ratios[e]			E:T ratios		
	100:1	50:1	20:1	100:1	50:1	20:1
Fresh medium[f]	45.7 ± 2.4	34.9 ± 5.0	24.0 ± 4.2	36.2 ± 2.8	27.6 ± 0.7	14.8 ± 0.8
Mock SSF[g]	42.9 ± 4.7	31.5 ± 3.5	19.9 ± 2.7	37.0 ± 7.1	26.9 ± 0.9	15.2 ± 1.4
SSF[h]	29.0 ± 1.4	18.0 ± 1.4	9.7 ± 2.1	26.3 ± 4.1	18.8 ± 0.8	9.1 ± 0.7

[a] 2 × 10^6 lymphocytes precultured at 20% v/v with either fresh medium mock, SSF or SSF for 72 hr were washed and tested for NK and ADCC activities as described in *Materials and Methods*.
[b] % cytotoxicity was calculated as in *Materials and Materials*. Results are the mean ± SD of three separate experiments done in triplicate.
[c] NK activity measured against the human erythroleukemia cell line, K562.
[d] ADCC activity measured against the human B-lymphoblast cell line, SB, sensitized with rabbit anti-SB sera.
[e] Effector to target cell ratios.
[f] Fresh RPMI 1640 medium + glutamine + gentamicin.
[g] Medium incubated in absence of lymphocytes.
[h] Medium incubated with lymphocytes.

TABLE II

Effect of SSF concentration on suppression of NK and ADCC activities of PBL[a]

Targets[b]	% Suppression at SSF Concentration of:			
	5%	10%	20%	40%
K562[c]	21.8 ± 1.1[d]	25.6 ± 7.7	38.9 ± 6.1	45.4 ± 12.0
CEM[e]	10.7 ± 3.9	ND[f]	23.9 ± 11.3	61.6 ± 7.1
SB[g]	5.7 ± 3.4	25.2 ± 8.7	35.4 ± 3.1	50.5 ± 1.3

[a] 2 × 10^6 allogeneic PBL were precultured with different concentrations of either SSF or mock SSF for 72 hr., washed, and tested for their NK and ADCC activities.
[b] Target cells were labeled with ^{51}Cr as in *Materials and Methods*.
[c] Human erythroleukemic cell line as targets for NK activity.
[d] Values represent mean % suppression of cytotoxicity ± SEM of four separate experiments done in triplicate at an E:T ratio of 50:1. The spontaneous release for the NK and ADCC experiments was always <5% of the total labeling.
[e] Human T lymphoblastoid cell line as targets for NK activity.
[f] Not determined.
[g] Human B cell leukemia sensitized with rabbit anti-SB sera as targets for ADCC activity.

TABLE III

Kinetics of suppression of NK and ADCC activities of PBL precultured with SSF[a]

Time of Incubation	SSF Added	NK[b]		ADCC[c]	
		Cytotoxicity[d]	Suppression[e]	Cytotoxicity	Suppression
hr		%		%	
0	Mock	64.2	—	47.5	—
	SSF	62.8	2.2	47.1	0.8
24	Mock	44.5	—	52.4	—
	SSF	36.2	18.6	46.8	10.7
48	Mock	56.3	—	49.0	—
	SSF	34.6	38.5	44.1	10.0
72	Mock	42.3	—	27.8	—
	SSF	23.9	43.4	15.4	44.6
96	Mock	26.2	—	18.8	—
	SSF	17.0	35.0	13.4	28.7

[a] PBL precultured with SSF or mock SSF (20% v/v) for varying times were washed and tested for NK and ADCC activities at a 50:1 E:T ratio.
[b] NK activity measured against the human erythroleukemia cell line, K562.
[c] ADCC activity measured against the human B-lymphoblast cell line, SB, sensitized with rabbit anti-SB sera.
[d] % cytotoxicity was calculated as in *Materials and Methods*. Results are the mean of triplicate determinations from a representative experiment. Three additional experiments gave similar results.
[e] % suppression of cytotoxicity was calculated as in *Materials and Materials*.

TABLE IV

Effect of washing on the suppression of NK and ADCC activities of PBL precultured with SSF[a]

Post-wash	Incubation	NK[b]		ADCC[c]	
		Cytotoxicity[d]	Suppression[e]	Cytotoxicity	Suppression
		%		%	
0 hr	Mock	24.7	—	26.7	—
	SSF	15.1	38.8	17.4	34.8
24 hr	Mock	21.4	—	25.9	—
	SSF	14.4	32.7	17.9	30.8
48 hr	Mock	12.0	—	19.0	—
	SSF	8.0	33.3	12.1	36.3
72 hr	Mock	9.9	—	15.0	—
	SSF	6.5	34.3	10.2	32.0

[a] 2 × 10^6 lymphocytes were cultured with either SSF or mock SSF (20% v/v) for 72 hr, washed with Hanks' balanced salt solution, cultured in complete medium for up to 72 hr, and tested for NK and ADCC activities at a 50:1 E:T cell ratio.
[b] NK activity measured against the human erythroleukemia cell line, K562.
[c] ADCC activity measured against the human β-lymphoblastoid cell line, SB, sensitized with rabbit anti-SB sera.
[d] % cytotoxicity was calculated as in *Materials and Methods*.
[e] % suppression of cytotoxicity was calculated as in *Materials and Methods*. Results are from a representative experiment and three separate experiments gave identical results.

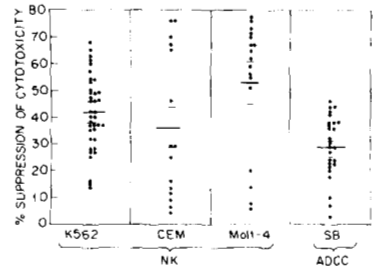

Figure 1. Suppression of NK and ADCC activities of PBL by culture supernatants. PBL (2 × 10^6/ml) were precultured with either SSF or mock SSF (20%, v/v) for 72 hr, washed twice, and their NK and ADCC activities measured. Suppression of cytotoxicity was calculated as in *Materials and Methods*. For the ADCC assay, SB targets were sensitized with rabbit anti-SB sera. An E:T ratio of 50:1 was maintained and each point represents different samples. Values are expressed as mean % suppression ± SEM.

onstrated a mean suppression of 36% of their NK activity against CEM targets, the range of suppression being 5 to 76%. A greater mean suppression, 53.4% (range: 6 to 77%), was obtained when 17 SSF samples were assayed for their ability to suppress the NK activity of PBL against Molt-4 target cells. In the ADCC reaction, normal lymphocytes precultured separately with 26 SSF samples showed a marked decrease in their cytotoxicity against antibody coated SB targets, the mean suppression being 29.3% with a range of 3 to 46%. In summary, 57 of 70 SSF samples exhibited > 20% suppression of NK activity of allogeneic lymphocytes and 21 of 26 SSF samples demonstrated > 20% suppression of ADCC activity. These studies demonstrate that unstimulated PBMC from apparently healthy individuals produce and elaborate SSF into the culture media that exhibits a significant suppressive effect on the NK and ADCC activity of allogeneic effector cells.

To examine the effect of SSF on NK enriched cells, Percoll

separated lymphocytes were precultured with either mock SSF or SSF for 72 hr, washed, and tested for their NK activity. Data presented in Table V demonstrate that NK-enriched cells, when precultured with SSF, showed a marked decrease in their cytotoxicity against K562 target cells. These data suggest that SSF may act directly on NK cells, and other cell populations are not required for its activity.

As demonstrated by Table VI, SSF produced by either PBMC or PBL showed comparable suppressive effects on NK activity of allogeneic lymphocytes. Thus production of SSF is independent of monocytes.

Molecular weight analysis of SSF. An initial estimate of the m.w. of SSF was made by ultrafiltration through a UM20 Diaflo membrane (Amicon Corp., Lexington, MA), which allows the passage of globular molecules with a m.w. < 20,000 daltons. The data of Table VII demonstrate that the UM20 filtrate showed a comparable suppressive effect to that of unfiltered SSF, whereas the UM20 retentate (> 20,000 daltons) showed negligible suppressive effects on both NK and ADCC activities of allogeneic lymphocytes. This suggests that the m.w. of SSF is < 20,000 daltons.

Further determinations of the m.w. of SSF was made by gel filtration. The elution profile of a typical Sephacryl S-200 SF (Pharmacia) column (2.5 × 45 cm) is shown in Figure 2.The elutate was monitored for protein by absorbance at 280 nm. The first peak (F1) eluted between markers for 13,700 and 25,000 daltons, whereas the second (F2) and third (F3) peaks eluted after the 13,700 dalton marker. The control fractions (PBS elutate) and the peak fractions (F1, F2 and F3) were individually pooled, concentrated, sterilized with a 0.22-μ filter, and assayed for their suppressive activity at a 20% final concentration (Fig. 2). Lymphocytes precultured separately with either control fractions or third peak pooled fractions (F3) showed negligible suppression of their NK or ADCC activities. Lymphocytes precultured separately with the pooled fractions, F1 or F2, showed 48% ($p < 0.02$) and 42% ($p < 0.02$) suppression of their NK activity and 45% ($p < 0.02$) and 30% ($p < 0.03$) suppression of their ADCC activities respectively, compared to lymphocytes precultured with mock SSF. The results suggest that SSF may consist of two distinct active moieties.

TABLE V

Inhibitory effect of SSF on percoll gradient-enriched NK effector lymphocytes[a]

	Cytotoxicity[b]	Suppression[c]
	%	
Mock SSF	33.6	—
SSF 1	14.5	56.8
SSF 2	18.1	46.2
SSF 3	18.7	44.4
SSF 4	26.4	21.4
SSF 5	9.5	71.7

[a] Percoll gradient-enriched lymphocytes (2 × 10^6/ml) were cultured with either SSF or mock SSF (20% v/v) for 72 hr, washed, and tested for their NK activity against K562 target cells at a 10:1 E:T ratio.
[b] % Cytotoxicity was calculated as in *Materials and Methods*.
[c] % Suppression of cytotoxicity was calculated as in *Materials and Methods*.

TABLE VI

Suppression of NK activity of lymphocytes precultured with SSF derived from PBMC and lymphocytes depleted of adherent cells[a]

Experiment	SSF Source	Cytotoxicity[b]	Suppression[c]
		%	
1	Mock	26.7	—
	PBMC[d]	12.5	53.1
	PBL[e]	13.6	49.0
	PBMC	15.2	43.0
	PBL	16.3	38.9
2	Mock	24.0	—
	PBMC	18.0	26.8
	PBL	13.0	44.7
	PBMC	11.9	51.3
	PBL	13.2	46.3
3	Mock	43.8	—
	PBMC	33.6	23.3
	PBL	32.3	26.2
	PBMC	30.8	29.7
	PBL	31.4	28.3

[a] 2 × 10^6 lymphocytes cultured with either SSF or mock SSF (20% v/v) for 72 hr were washed and tested for NK activity at 50:1 E:T cell ratio.
[b] % Cytotoxicity was calculated as in *Materials and Methods*.
[c] % Suppression of cytotoxicity was calculated as in *Materials and Methods*.
[d] PMBC separated as described in *Materials and Methods*. Estimation of latex-ingesting phagocytic cells and nonspecific esterase-positive cells varied from 12 to 15% and 11 to 14%, respectively.
[e] Total PBL were depleted of monocytes/macrophages (M) by passage through a G-10 column followed by removal of carbonyl iron-ingesting cells. Residual lymphocytes always contained <1% nonspecific esterase-positive cells.

TABLE VII

Inhibitory effect of ultrafiltered SSF on cytotoxicity of lymphocytes[a]

	NK[b]		ADCC[c]	
	Cytotoxicity[d]	Suppression[e]	Cytotoxicity	Suppression
	%		%	
Mock SSF				
Unfiltered	30.8	—	51.9	—
Filtrate[f]	34.4	—	48.8	—
Retentate[g]	40.0	—	47.0	—
SSF I				
Unfiltered	17.9	42.0	29.4	43.3
Filtrate	21.4	37.8	30.8	36.9
Retentate	39.2	2.0	44.9	4.4
SSF II				
Unfiltered	20.9	32.3	36.8	29.0
Filtrate	23.8	30.8	37.4	23.4
Retentate	36.7	8.2	ND[h]	—

[a] SSF was passed through a UM20 Diaflo membrane (Amicon Corp.) with constant stirring, and assayed for suppressive activity.
[b] NK activity measured against the human erythroleukemia cell line, K562.
[c] ADCC activity measured against the human B lymphoblast cell line, SB, sensitized with rabbit anti-SB sera.
[d] % Cytotoxicity was calculated as in *Materials and Methods* at a 50:1 E:T ratio.
[e] % Suppression of cytotoxicity was calculated as in *Materials and Methods*.
[f] SSF passed through the filter (<20,000 daltons).
[g] Materials retained by the filter (>20,000 daltons), dissolved to original volume and sterilized by a 0.45-μ filter.
[h] Not determined.

Effect of IF on suppression of NK activity by SSF. We investigated the role of IF, an agent known to enhance natural cell-mediated cytotoxicity (38–43) on the suppressive effect of SSF on NK cells (Table VIII). Lymphocytes precultured with mock SSF and then pretreated with IF (500 U) for 1 hr demonstrated enhancement of their NK activity, compared to the NK activity of lymphocytes precultured with mock SSF alone. Lymphocytes precultured with, for example, SSF 12 showed 40.5% suppression of their NK activity which was partially reversed to 32.7% suppression (i.e., a 20% recovery of cytotoxic activity) by treatment with IF for 1 hr. Similar results were observed in additional experiments with the use of Molt-4 target cells (data not presented). However, lymphocytes precultured with SSF and incubated with IF for 24 hr showed almost a full recovery of their cytotoxic potential, e.g., lymphocytes precultured with SSF 12 showed a 35.5% suppression of their NK activity, which was significantly reversed to 6.4% suppression (i.e., a >82% reversal of their suppressive effect). By contrast, the simultaneous preculture of PBL with SSF and IF for 72 hr failed to abrogate the suppressive effect of SSF on NK and ADCC activities of allogeneic PBL (data not presented).

Effect of monosaccharides on SSF activity. As a preliminary analysis of SSF-specific receptors or recognition structures on cytotoxic effector cells, we examined a few selected monosac-

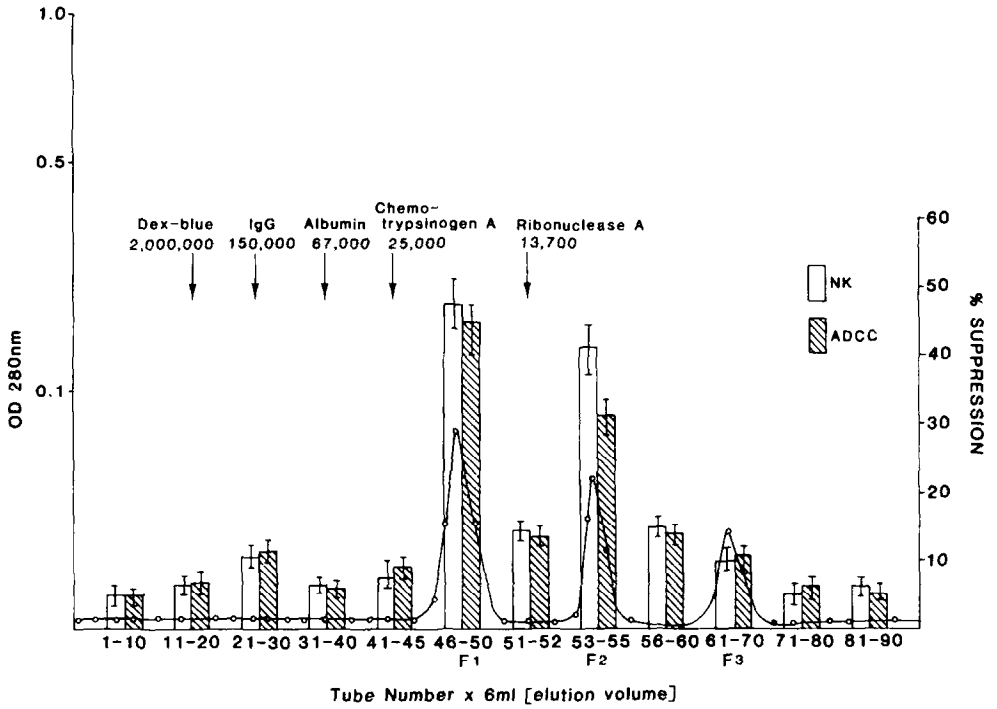

Figure 2. Molecular sieve chromatography of SSF. A 3- to 4-ml SSF sample was passed over a 2.5- x 0.5-cm column of superfine Sephacryl S-200 (Pharmacia) equilibrated with 0.01 M phosphate-buffered (pH 7.4) saline. The eluates were collected in 6-ml aliquots and monitored for protein by absorbance at 280 nm. The protein-containing fractions and control eluate fractions were individually concentrated to their original volume and tested for suppressor activity. The protein concentration of pooled peak fractions F1 and F2 was 12.5 and 62.5 μg/ml, respectively, as estimated by Lowry's method. The column was previously calibrated with four markers of known molecular weight: rubonuclease A (13,700), chemotrypsinagen A (25,000), albumin (67,000), and immunoglobulin G (150,000). The void volume was determined with blue dextran 2000 (Pharmacia).

TABLE VIII

Effects of interferon on inhibition of NK activity by SSF[a]

Treatment of Effectors	Incubation with IF			
	1 hr		24 hr	
	Cytotoxicity[b]	Suppression[c]	Cytotoxicity	Suppression
	%		%	
Mock	30.3	—	29.5	—
Mock, then IF	42.7	−40.9[d]	41.7	−41.4
SSF 12	18.0	40.5	19.5	35.5
SSF 12, then IF	28.7	32.7	39.7	6.4
SSF 13	15.4	49.1	16.2	45.0
SSF 13, then IF	28.3	33.7	36.8	11.7

[a] 2 × 10[6] lymphocytes were cultured with either SSF or mock SSF (20% v/v) for 72 hr and washed and treated with 500 U of IF for 1 hr or 24 hr. IF-treated cells were washed twice and tested for NK activity against K562 targets at a 50:1 E:T ratio.

[b] % Cytotoxicity as calculated in *Materials and Methods*. Results are the mean of triplicate determinations from a representative experiment. Three additional experiments gave similar results.

[c] % Suppression of cytotoxicity as calculated in *Materials and Methods*. IF diluted in RPMI 1640 medium and used at an optimum concentration (500 U) as previously determined.

[d] Minus sign indicates enhancement.

TABLE IX

Monosaccharide reversal of SSF activity[a]

Monosaccharide Added	Concentration	NK Activity[b]		Suppression[c]
	mM	%		%
Mock SSF[d]	—		27.1	—
None	—		16.7	38.5
L-Fucose	10	(26.8)[e]	17.8	33.5
	25	(27.7)	27.3	1.4
	50	(25.0)	28.6	−14.4[f]
L-Rhamnose	10	—	17.9	—
	25	(24.0)	19.5	18.7
	50	(25.1)	21.7	13.5
α-Methyl-D-mannoside	10	(25.2)	16.3	35.3
	25	(26.7)	19.8	25.8
	50	(28.2)	26.4	6.3

[a] Lymphocytes (2 × 10[6] ml) were precultured with 20% (v/v) SSF and varying concentrations of the indicated monosaccharides for 72 hr, washed, and assayed for their activity against K562 targets at a 50:1 E:T ratio.

[b] % Cytotoxicity calculated as in *Materials and Methods*.

[c] % Suppression of cytotoxicity was calculated based on the cytotoxicity of PBL precultured with monosaccharide alone.

[d] A 20% (v/v) concentration of mock SSF prepared as in *Materials and Methods* was substituted for SSF.

[e] Numbers in parentheses are % cytotoxicity of PBL precultured with varying concentrations of monosaccharide without added SSF.

[f] Minus sign indicates enhancement.

charides for their ability to block the suppression of cytotoxicity mediated by SSF. Lymphocytes were precultured with either mock SSF, SSF alone, various concentrations of either L-fucose, L-rhamnose, or α-methyl-D-mannoside (αM), or SSF plus various concentrations of monosaccharides for 72 hr and washed and examined for NK activity against K562. Data from a representative experiment are presented in Table IX. Lymphocytes precultured with SSF demonstrated a significant suppression (38.5%) of their NK activity compared to the cytotoxicity of lymphocytes precultured with mock SSF. However, this suppressive effect was reversed completely by the presence of 50 mM L-fucose (14.4% enhancement) or αMM (6.3% suppression). Only partial reversal of SSF-mediated suppression was obtained with an equal concentration of L-rhamnose (13.5% suppression). When added alone, concentrations of monosaccharides ranging from 10 to 50 mM had no effect on the NK activity of normal lymphocytes and was comparable to mock SSF. All monosaccharides tested, including L-rhamnose, exhibit dose-dependent blocking effects on the suppression mediated by SSF. Concentrations ≤ 10 mM did not affect SSF activity. These data suggest that SSF may exert its suppressive effect on cytotoxic effector cells through interactions with receptor sites recognized by L-fucose and αMM.

DISCUSSION

We previously demonstrated that normal human PBMC preincubated in media develop suppressor cell activities against 1) lymphocyte transformation, 2) polyclonal B cell differentiation, and 3) NK and ADCC functions of allogeneic and autologous lymphocytes. We report herein that serum-free culture supernatants of unstimulated normal PBMC contain soluble suppressor factor(s) that exhibits significant suppressive effects on NK or ADCC activities of allogeneic lymphocytes against a variety of target cells. Suppression is operationally defined herein as the direct inhibition of cytotoxic effector lymphocytes after preincubation with SSF for 72 hr. Suppression is not caused by selective toxicity by SSF because the percentage of viable lymphocytes recovered in cultures incubated with SSF was equivalent to the viability of mock SSF-treated cultures (data not shown) and SSF-induced suppression could be completely reversed by overnight incubation of SSF-treated lymphocytes with IF (Table VIII). Suppression caused by pH shifts in the medium is also unlikely, since the baseline killing observed with mock SSF was comparable to fresh medium (Table I). SSF does not appear to suppress the cytotoxicity of lymphocytes via a lymphotoxin, although Jeffes and Granger (44) have postulated that lymphotoxin in sublethal dose ranges may suppress various immune functions. This suggests that the lack of cell death in cultures preincubated with SSF does not rule out the possibility that low concentrations of lymphotoxin may be responsible for its inhibitory activity. Our preliminary estimate of the m.w. of SSF, < 20,000 daltons, indicates that it is distinct from α- and β-lymphotoxin that have m.w. of 80,000 to 100,000 and 40,000 daltons, respectively (45). Further, the suppressive activity of SSF is not likely caused by prostaglandins because preparations derived from lymphocytes depleted of monocytes/macrophages are comparable in their inhibitory activity as SSF prepared from total mononuclear leukocytes (Table VI). Thus inhibition by SSF appears to be caused by selective suppression without nonspecific toxicity.

Other investigators have reported that cultured lymphocytes produce soluble suppressor factor(s) that can inhibit various immune responses *in vitro* both in murine and human models (46–49). Broder *et al.* (50) recently demonstrated that a soluble factor obtained from cultured normal T cells activated prosuppressor cells present in the peripheral blood of a leukemic patient to functionally active suppressor cells in a coculture experiment. In an extensive study, Green *et al.* (11) and Fleisher *et al.* (12) showed that Con A-stimulated human PBMC, elaborated two distinct suppressor factors that could differentially suppress mitogen induced T cell proliferation and PWM stimulated B cell differentiation. In their studies, however, supernatants from unstimulated cultures termed mock supernatants also showed inhibition of these activities. Suppressor factors in supernatants from unstimulated PBMC cultures may thus be similar to suppressor factors produced by mitogen-stimulated cultures. Rigorous comparison of these systems is pending.

SSF as originally reported by Shou *et al.* (26) manifested suppressor activity toward lymphocyte responses to mitogens, polyclonal B-cell stimulation and alloantigens in MLR. Lederman *et al.* (51) also showed recently that unstimulated nonadherent lymphocytes from healthy individuals elaborate a substance into culture medium containing human serum that suppresses lymphocyte proliferative responses to mitogens (PHA, PWM, and Con A) and antigens (streptokinase-streptodornase, SKSD). The SSF of the present study, identical with the originally described material (26), bears many similarities with the suppressor factor of Lederman *et al.* (51). Both are heat stable at 56°C, < 20,000 daltons m.w. and do not require adherent cells for production. The stimulus leading to the elaboration of SSF in our culture supernatant is unknown. It is possible that this activity may be induced by an autologous mixed lymphocyte reaction as we have previously reported (3). Experiments are currently investigating this possibility.

IF seems to exert a major role in immunoregulation (52–54). IF or IF inducers augment NK activity both *in vitro* and *in vivo* (38–43) and IF appears to enhance NK by more than one mechanism. IF may activate noncytotoxic pre-NK cells to mature cytotoxic effector cells (55), or activate mature NK cells that are transiently inactive as suggested by Perussia and Trinchieri (38), or potentiate endogenous lytic mechanisms by developing new receptors or modulating pre-existing cell surface molecules or their avidity (38–41). Interestingly, Altman *et al.* (56) have recently shown in the murine system that IF does not play any significant role in the biologic activities mediated by soluble suppressor factors toward various *in vitro* reactions like cytotoxic T lymphocyte activation, mitogen stimulation, plaque formation, and antibody responses to T-dependent antigens. We have shown, however, that SSF-induced suppression can be partially reversed by 1 hr and almost completely reversed by 24 hr incubation with IF (Table VIII). This suggests that IF and SSF may have complimentary immunoregulatory activities.

Several monosaccharides, when added directly to the 4-hr ^{51}Cr release assay, have been previously found to block murine natural cell-mediated reactions (57). In the present study, we failed to observe any decrease in the cytotoxicity of human lymphocytes precultured with monosaccharides. This may be caused by the absence of saccharides in the reaction mixture, since they were removed by prior washing and is consistent with the finding of Ades *et al.* (58) who found no inhibition of the NK activity of effector cells precultured with the monosaccharides, fucose, xylose, and mannose. Recently R. B. Herberman (NIH) observed that several monosaccharides did not affect the target-binding ability of lymphocytes (personal communication); this, too, is consistent with our finding that monosaccharides did not affect the cytotoxicity of the effector cells. Further, we observed that SSF-mediated suppression is largely reversed by αMM, a monosaccharide known to bind Con A, suggesting that SSF binds to sites recognized by the lectin. SSF-mediated suppression is reversed completely by L-fucose and partly by L-rhamnose. A number of factors may be involved in the mechanism of reversal of SSF activity, which includes the ability of monosaccharides to bind, at least in part, to the same putative receptor sites recognized by SSF or, alternatively, to bind directly to SSF itself.

It is becoming increasingly clear that immune regulation is a complex series of checks and balances, a system of specific and nonspecific regulatory cells and their soluble factors. SSF-mediated suppression of natural cytotoxicity may thus be a normal, nonpathologic, homeostatic mechanism functioning to prevent immunologic overkill in response to a foreign antigen or neoplastic cell. If NK or ADCC cells are involved in immune surveillance of malignancies, then enhancing their effects in premalignant and malignant states by diminishing NK or ADCC specific suppressor cells could have a role in the prevention or treatment of cancer and immunodeficiencies. Evidence indicates that the identity of the effector cells involved in the resistance to marrow transplantation is similar to natural cytotoxic cells, thus a reciprocal clinical situation where effector

cells could be therapeutically suppressed with SSF might be effective in prolonging marrow allografts in the treatment of malignant disease and immunodeficiencies. Studies are currently underway to isolate and characterize culture-induced suppressor cells and to biochemically analyze their SSF product(s).

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