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A human soluble suppressor factor affecting lymphocyte responses *in vitro*

(differentiation of B cells/mitogenesis/immunoglobulin synthesis)

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ABSTRACT A soluble suppressor factor (SSF) has been demonstrated in the supernatant of normal human peripheral blood lymphocyte cultures that exhibits suppressive activity toward the proliferative response of normal lymphocytes to concanavalin A or alloantigens in mixed lymphocyte culture (MLC) or toward pokeweed mitogen-stimulated immunoglobulin synthesis and secretion *in vitro*. Suppression of the proliferative response in MLC reached maximal levels when added SSF-containing supernatant approximated 20% by volume of the culture medium. Suppression in the MLC was found to act at the proliferative stage. SSF acts independently of cytotoxicity and is stable at 56°C for 30 min but is inactivated at higher temperatures. Addition of SSF to the MLC as late as day 4 after initiation of the culture results in suppression of transformation. This factor(s) may regulate the magnitude of several immune responses in humans.

Ample evidence now indicates that human peripheral blood lymphocytes (PBL), after concanavalin A (Con A) stimulation, may develop suppressor T cell function that exerts a regulatory effect on lymphocyte transformation induced by mitogens and allogeneic cells (1–5), and on pokeweed mitogen (PWM)-stimulated synthesis and secretion of immunoglobulins (Ig) (6). Nadler *et al.* (7) suggested that programmed suppressor T cells exist among normal human PBL, and that the suppressive activity of these cells is amplified after appropriate stimulation.

The mechanism through which these nonspecific suppressor T cells exert their immunoregulatory activities has not been fully defined in humans. Some observations indicate that suppressor cells act on effector cells by cell–cell interactions (8). Other evidence suggests that suppressor cells regulate the immune response by secreting or generating soluble immune suppressor factors that prevent or interfere with functions of effector cells (9).

We (6) and several others (10–15) have reported that lymphocytes, after *in vitro* incubation, can develop suppressive activity that inhibits PWM-stimulated differentiation of B cells to Ig-synthesizing and secreting plasma cells. Such preincubated lymphocytes clearly act as suppressor cells that diminish the immune responses of freshly isolated normal lymphocytes. In the present study, we demonstrate that isolated lymphocytes incubated for 4 days *in vitro* without further stimulation elaborate and release into the supernatant substances that, when added to normal lymphocyte cultures, inhibit Con A- or alloantigen-induced proliferation and suppress PWM-stimulated IgG and IgM production.

MATERIALS AND METHODS

Isolation of Lymphocytes. Heparinized human venous blood (20 units of heparin per ml of blood) from normal volunteers was diluted with an equal volume of Hanks' balanced salt solution (HBSS, GIBCO). Lymphocytes were separated by Ficoll/Hypaque density gradient centrifugation as described by Böyum (16). After being washed three times with HBSS, the cells were resuspended in RPMI 1640 medium (GIBCO) supplemented with 20% heat-inactivated fetal calf serum, gentamicin (Schering) at 8.0 μg/ml, and fresh glutamine at 2 mM.

Preparation of Soluble Suppressor Factor (SSF). Lymphocytes, after being washed three times with HBSS, were resuspended in RPMI medium containing only gentamicin and glutamine. Suspensions of 2–5 $\times 10^6$ cells per ml were incubated at 37°C in a humidified 5% CO_2/95% air atmosphere. After 4 days of culture, the supernatant was separated from the cell suspension by centrifugation and stored at −70°C until used.

One-Way Mixed Lymphocyte Culture (MLC). Equal 0.1-ml samples of responder lymphocytes and allogeneic stimulator lymphocytes at 1.0×10^6 cells per ml of RPMI 1640 medium containing 20% fetal calf serum and gentamicin were mixed in the wells of a microtitration plate (Linbro). The cell mixture was cultured for 6 days in a humidified 5% CO_2/95% air incubator at 37°C. For the final 24 hr of incubation, 2 μCi (1 Ci = 3.7×10^{10} becquerels) of [^{3}H]thymidine (Radiochemical Centre, Amersham, England) was added to each well. The cultured cells were then collected with a cell harvester and incorporation of radioactive thymidine into lymphocyte DNA was determined in a Packard liquid scintillation counter.

Con A Stimulation of Lymphocytes. Samples (0.2 ml) of lymphocyte suspension at 0.5×10^6 cells per ml of RPMI 1640 medium supplemented as above together with Con A at a final concentration of 30 μg/ml were cultured in microtitration plates in a CO_2 incubator for 5 days. Two microcuries of [^{3}H]thymidine was added to each well during the last 24 hr of incubation. At the end of the fifth day, cells were harvested and radioactivity was measured as described above.

Stimulation and Detection of IgG and IgM Production *in Vitro*. Lymphocyte suspensions prepared as described above were adjusted to 1.0×10^6 cells per ml of supplemented RPMI 1640 medium. The cells were cultured with PWM at a final concentration of 10.0 μg/ml at 37°C in a 5% CO_2 incubator for 7 days. After 7 days of culture, the supernatant was separated by centrifugation. The IgG and IgM content in the supernatant was determined by a solid-state immunofluorescence assay (Immunofluor, Bio-Rad) as described (17).

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Abbreviations: Con A, concanavalin A; HBSS, Hanks' balanced salt solution; MLC, mixed lymphocyte culture; PBL, peripheral blood lymphocytes; PWM, pokeweed mitogen; SSF, soluble suppressor factor.

Calculation of Inhibition by SSF. Percent inhibition of lymphocyte transformation or Ig synthesis and secretion was calculated according to the following formula:

$$\% \text{ inhibition} = \left[1 - \frac{\text{cpm of [}^3\text{H]dThd or } \mu\text{g/ml of Ig in presence of SSF}}{\text{cpm of [}^3\text{H]dThd or } \mu\text{g/ml of Ig in absence of SSF}}\right] \times 100.$$

Note that a + sign preceding a value means enhancement above 100%.

RESULTS

Effect of SSF on Normal Lymphocyte Transformation. In these experiments, 50 μl of SSF was added to 0.2 ml of normal PBL suspension at 0.5×10^6 cells per ml at the initiation of culture. Con A at a final concentration of 30 μg/ml was added to induce lymphocyte transformation. The lymphocytes of 27 volunteers were analyzed. In 26 of these, Con A-induced lymphocyte transformation was suppressed by the SSF. The suppression ranged from 24.0% to 91.5% as compared to the response of the control cells incubated in the absence of the SSF with culture medium alone. Lack of suppression was observed in only one instance (Table 1). These studies clearly demonstrate that SSF can suppress Con A-induced lymphocyte transformation.

Effect of SSF on the MLC. One-way MLCs were set up according to the method described. Fifty microliters of SSF was added to 0.2 ml of culture containing 0.1×10^6 responding cells and 0.1×10^6 mitomycin-treated allogeneic stimulator cells. After 6 days of culture, the proliferative response in the MLC was found to be significantly suppressed by SSF. The MLC of every one of 20 volunteers tested showed significant suppression by SSF (Table 2). The percentage of suppression ranged from 17.1% to 84.2%; 11 subjects showed suppression greater than 40%.

To test the effect of various concentrations of SSF on the MLC, increasing dilutions of SSF were added to a final constant volume and constant number of cells in MLC. The results, recorded in Fig. 1, show that suppression increased in a linear fashion with concentrations of SSF up to 20%. With higher concentrations, the suppressive activity was relatively less; although the absolute value of suppressor activity continued to increase, the increase was not proportional to the amount added. This observation suggests that SSF at a concentration of 20% by volume in these preparations exerts a maximal suppressive effect. Consequently, further inhibition tests were performed with SSF fixed at 20% of the final volume.

The mechanism by which SSF acts on the MLC was next explored. To this end, SSF was added on successive days to a one-way MLC, beginning on day 0 and extending to day 6. The results recorded in Fig. 2 show that [^{3}H]thymidine incorporation in the MLC was significantly inhibited when SSF was added during any of the first 4 days of the culture, but no inhibitory effect was seen when SSF was added during the last 2 days of the culture (days 5 and 6). This result suggests either that SSF requires a period of contact before it can exert its suppressive effect, or that events initiated early in the MLC are susceptible to SSF. Thus, SSF does not seem to prevent stimulated cells from proliferating once the actual proliferative response has been initiated.

Effect of SSF on PWM-Stimulated Immunoglobulin Production. PWM is a polyclonal stimulator that induces transformation of both T and B lymphocytes. PWM further stimulates B cells nonspecifically to differentiate into plasma cells

Table 1. Effect of SSF-containing supernatant on the transformation of normal lymphocytes by Con A

Subject no.	SSF*	Proliferative response	
		cpm†	% inhibition
1	−	8,975 ± 807	
	+	3,306 ± 299	63.2
2	−	7,255 ± 798	
	+	3,994 ± 371	44.9
3	−	16,544 ± 2,482	
	+	8,642 ± 1,024	47.8
4	−	77,181 ± 6,946	
	+	57,993 ± 9,272	24.9
5	−	34,693 ± 347	
	+	26,360 ± 290	24.0
6	−	30,750 ± 3,997	
	+	22,167 ± 3,163	27.9
7	−	12,115 ± 1,696	
	+	4,452 ± 578	63.3
8	−	27,733 ± 1,890	
	+	21,238 ± 1,709	23.4
9	−	20,789 ± 2,910	
	+	1,980 ± 238	90.5
10	−	10,908 ± 654	
	+	1,615 ± 96	85.2
11	−	18,201 ± 1,254	
	+	1,543 ± 108	91.5
12	−	81,134 ± 7,789	
	+	27,108 ± 2,602	66.6
13	−	31,923 ± 1,596	
	+	13,851 ± 831	56.6
14	−	36,459 ± 2,916	
	+	16,520 ± 1,982	54.7
15	−	35,070 ± 4,909	
	+	25,211 ± 3,277	28.1
16	−	6,931 ± 554	
	+	2,863 ± 229	58.7
17	−	14,103 ± 1,128	
	+	15,077 ± 2,110	+6.9
18	−	27,010 ± 1,890	
	+	17,099 ± 1,709	36.7
19	−	25,244 ± 2,423	
	+	13,327 ± 1,186	47.2
20	−	24,996 ± 988	
	+	7,306 ± 296	70.8
21	−	127,336 ± 6,376	
	+	52,206 ± 4,698	59.0
22	−	76,687 ± 6,134	
	+	34,704 ± 1,735	54.7
23	−	8,698 ± 1,043	
	+	3,410 ± 204	60.8
24	−	179,691 ± 10,781	
	+	108,372 ± 6,502	39.7
25	−	9,550 ± 1,337	
	+	3,112 ± 186	67.4
26	−	90,537 ± 7,274	
	+	18,364 ± 2,201	79.7
27	−	20,146 ± 1,813	
	+	3,295 ± 263	83.6
		Mean ± SD = 53.5 ± 23.7	

* In the experiments summarized in Tables 1–4, SSF-containing supernatants were added where designated to a final concentration of 20%.

† Samples (0.2 ml) of PBL (0.5×10^6 cells per ml) were incubated for 4 days in the presence of Con A (30 μg/ml). Cultures were labeled with 2 μCi of [^{3}H]thymidine during the last 24 hr of incubation and the cells were harvested at the end of day 5. All counts are the mean of replicate samples ± SD.

Table 2. Effect of SSF-containing supernatant on MLC

Subject no.	SSF	[^{3}H]Thymidine incorporation in one-way MLC cpm*	% inhibition
1	−	10,842 ± 651	
	+	2,295 ± 160	78.8
2	−	3,074 ± 246	
	+	486 ± 29	84.2
3	−	4,908 ± 442	
	+	3,006 ± 336	38.8
4	−	3,798 ± 265	
	+	2,068 ± 249	45.6
5	−	5,753 ± 460	
	+	1,800 ± 108	68.7
6	−	3,710 ± 297	
	+	795 ± 67	78.6
7	−	1,424 ± 85	
	+	774 ± 46	45.6
8	−	1,344 ± 120	
	+	920 ± 56	31.5
9	−	1,290 ± 103	
	+	928 ± 74	28.1
10	−	1,916 ± 153	
	+	1,101 ± 132	42.5
11	−	1,648 ± 148	
	+	1,210 ± 98	26.6
12	−	1,467 ± 163	
	+	1,097 ± 69	25.2
13	−	4,974 ± 397	
	+	2,716 ± 217	45.4
14	−	2,455 ± 220	
	+	2,034 ± 244	17.1
15	−	2,616 ± 235	
	+	1,552 ± 132	40.7
16	−	22,868 ± 2,972	
	+	4,060 ± 487	82.2
17	−	10,656 ± 1,172	
	+	4,433 ± 623	58.4
18	−	8,137 ± 1,546	
	+	2,381 ± 262	70.7
19	−	6,154 ± 492	
	+	4,132 ± 372	32.9
20	−	4,848 ± 533	
	+	3,369 ± 404	30.5
		Mean ± SD = 48.6 ± 21.4	

* Equal 0.1-ml samples containing 0.1 × 10^6 responder or stimulator PBL were mixed and incubated for 5 days. During the last 24 hr of culture, 2 μCi of [^{3}H]thymidine was added and the cells were harvested at the end of day 6. All counts are the mean of replicate samples ± SD.

by a process that requires helper interaction of T and B lymphocytes. Lipsky *et al.* (10) found that human lymphocytes precultured *in vitro* for 7 days showed only weak responses to PWM stimulation; the number of plasma cells developed in the culture *in vitro,* detected by the reverse hemolytic plaque technique, was reduced as compared to that observed with cells that had not been precultured or were precultured for shorter periods. When freshly isolated lymphocytes were cocultured with lymphocytes that had been precultured for 7 days, the response of the fresh lymphocytes to PWM stimulation was inhibited, and the number of plasma cells developed was reduced. These results indicated that the precultured cells exert a suppressive effect on normal lymphocyte differentiation. In the present study, freshly isolated lymphocytes cultured in the presence of SSF for 7 days showed significant reduction in the amount of IgG and IgM produced (Table 3). This observation is consistent with our prior findings (6) and those of Lipsky *et al.* (10) wherein precultured PBL may act as suppressor cells in this assay. The present results indicate that SSF derived from lymphocytes cultured *in vitro* for 4 days can exert this same suppressor action on differentiation of B cells and reduce production of IgG and IgM.

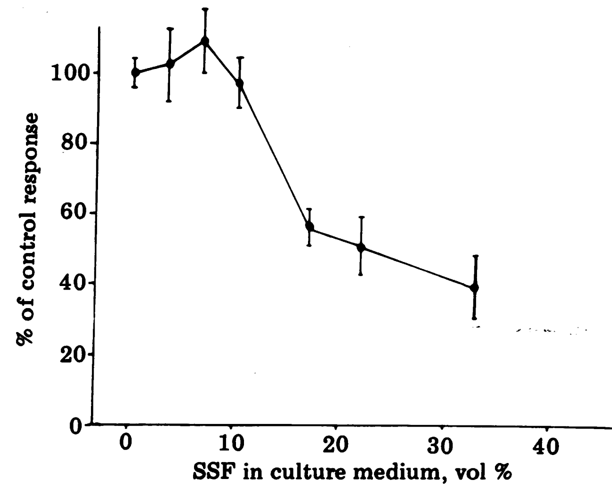

FIG. 1. Effect of SSF concentration on MLC. Various amounts of SSF were added to one-way MLCs consisting of equal 0.1-ml samples of 0.1 × 10^6 responder and stimulator PBL at the initiation of culture. At the end of the fifth day, 2 μCi of [^{3}H]thymidine was added to each well of a microtitration plate. After incubation for an additional 24 hr, the cells were harvested and the radioactivity incorporated into lymphocyte DNA was determined. Each point represents the mean ± SD for SSF from three individual donors.

Examination of Cytotoxic Effect of SSF. To address the possibility that the diminished proliferative response of PBL in the presence of SSF was a consequence of cytotoxicity, the viability of the lymphocytes at the end of culture was determined, using the trypan blue dye exclusion technique. The percentage of viable cells in the experimental group exposed to SSF was 88.4 ± 13.2%, while that of the control cells exposed only to added medium instead of SSF was 91.3 ± 7.3%. These findings clearly demonstrate that the inhibitory effect of SSF on lymphocyte transformation is not due to cytotoxicity. An-

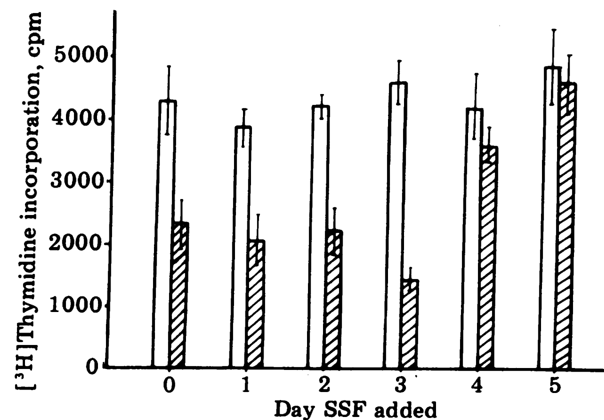

FIG. 2. Kinetics of SSF inhibition of proliferation in MLC. SSF at a final concentration of 20% was added to MLCs at initiation or on subsequent days as indicated. Culture conditions were as described in the legend for Fig. 1. Cells were harvested at the end of the sixth day and the incorporation of [^{3}H]thymidine by responder PBL in the presence of SSF (hatched bars) was compared to that in control cultures receiving medium alone (empty bars). The data respresent the mean ± SD of triplicate samples.

Table 3. Effect of SSF-containing supernatant on immunoglobulin production of PWM-stimulated normal lymphocytes

Subject no.	SSF	IgG production*		IgM production*	
		μg/ml	% inhibition	μg/ml	% inhibition
1	−	72.0		4.9	
	+	40.5	43.8	2.1	57.1
2	−	222.0		26.8	
	+	123.0	44.6	17.0	34.6
3	−	198.0		21.0	
	+	93.0	53.0	9.0	57.1

* PBL (1×10^6 cells per ml) were cultured for 7 days in the presence of PWM (10 μg/ml). At the end of the culture period, the concentrations of IgG and IgM secreted into the supernatant were measured by a solid-state immunofluorescence assay.

other experiment examining the influence of SSF on proliferation induced by PWM also illustrates that the decreased response is not attributable to a direct cytotoxic influence of SSF (Table 4). In these experiments, PWM was used as a stimulating mitogen at a final concentration of 10.0 μg/ml. Indeed, proliferative responses of the cells were significantly enhanced in 8 of 10 individuals tested, further demonstrating that SSF is not acting simply as a cytotoxic agent.

Analysis of Heat Lability. Summarized in Table 5 are experiments to analyze the influence of heat on the activity of SSF. It is evident from Table 5 that the suppressive effect of SSF was unaffected by heating to 56°C for 30 min. However, significant loss of suppressor activity occurred after incubation at 70°C for 30 min, and heating the supernatant at 80°C for 30 min resulted in a complete loss of the capacity to suppress Con A-induced proliferation.

DISCUSSION

Suppressor cells, which suppress immune responses of man and animals when induced *in vivo* or *in vitro*, have been demonstrated in many experimental systems. Important phenomena that appear to be mediated by suppressor cells include: induction of some forms of immunologic tolerance (18); participation in chronic allotype suppression (19); antigenic competition (20, 21); and the suppression of the immune response to T-independent antigens such as pneumococcal polysaccharide type III (22). Suppressor T cells have also been shown *in vitro* to abolish the blastogenic response of normal lymphocytes to mitogens and alloantigens, and to inhibit cell-mediated cytotoxicity (23). Our previous work has demonstrated the existence of suppressor cells in the peripheral blood of normal humans that can be induced or amplified by Con A to inhibit the proliferation of freshly isolated normal lymphocytes in response to mitogens, specific antigens, or allogeneic cells in MLC (1). In addition, we and others have found that Con A-stimulated suppressor cells inhibit stimulation of production of immunoglobulins by PWM (6).

In the present study, human PBL, isolated by Ficoll/Hypaque, when cultured for 4 days produce soluble factors that are released into the culture medium and are capable of inhibiting the responses of normal lymphocytes to Con A and to alloantigens in MLC. The inhibitory effect on the MLC was observed when the soluble suppressor factor was added relatively early in the culture period, and suppressive activity was not observed when SSF was added to the MLC after the fourth day. This finding indicates that the suppressor substance must

Table 5. Effect of heat-treated SSF on the proliferative response of normal lymphocytes to Con A

Treatment of SSF*	Proliferative response	
	cpm†	% inhibition
Medium control	30,195	
No treatment	12,621	58.3
50°C	11,802	60.9
70°C	18,122	40.0
80°C	29,196	3.3

* Samples were incubated for 30 min at the designated temperatures.

† Same as described in legend for Table 1.

Table 4. Comparison of the effects of SSF on the proliferative responses of normal lymphocytes to Con A and PWM

Exp.	SSF	Con A cpm	Con A % inhibition	PWM cpm	PWM % inhibition
1	−	14,103 ± 1,128		6,733 ± 554	
	+	15,007 ± 2,110	+6.9	7,100 ± 296	+5.5
2	−	27,010 ± 1,890		6,916 ± 371	
	+	17,099 ± 1,709	36.7	6,457 ± 578	6.6
3	−	31,923 ± 1,596		8,351 ± 805	
	+	13,851 ± 831	56.6	13,879 ± 1,201	+66.2
4	−	127,336 ± 6,376		25,298 ± 1,790	
	+	52,206 ± 3,678	59.0	47,877 ± 1,735	+89.3
5	−	76,687 ± 6,134		10,549 ± 1,696	
	+	34,704 ± 1,735	55.1	13,925 ± 1,254	+32.0
6	−	8,698 ± 1,043		2,140 ± 186	
	+	3,410 ± 204	60.8	3,550 ± 204	+65.9
7	−	179,691 ± 10,781		4,618 ± 374	
	+	108,372 ± 6,502	39.7	10,926 ± 1,257	+136.6
8	−	9,550 ± 1,337		2,335 ± 132	
	+	3,112 ± 186	67.4	5,341 ± 296	+128.7
9	−	90,537 ± 7,274		16,014 ± 1,186	
	+	18,364 ± 2,201	79.7	16,854 ± 1,709	+5.2
10	−	20,146 ± 1,813		3,149 ± 268	
	+	3,295 ± 263	83.6	6,163 ± 108	+95.7

Proliferative response*

* Same as in legend for Table 1, except PBL were also cultured in the presence of PWM (10 μg/ml).

be in contact with target cells for at least 2 days or that it exerts an influence on an initiation event in the MLC but not on a well-established proliferative process. It is of interest that the suppressive action of SSF may act as late as the fourth day of culture, when the responder lymphocytes have already begun to proliferate.

To rule out the possibility that the suppressive activity of SSF is merely a consequence of toxicity, we studied vital dye exclusion; the studies did not demonstrate any difference in viability between the SSF-treated and control cultures. In another experiment, when PWM was used as a mitogen to induce lymphocyte transformation, blastogenesis with SSF, instead of being suppressed, was significantly enhanced. Thus these inhibitory activities appear to be selective effects without the involvement of cellular toxicity.

Nonspecific suppressor factors have been demonstrated and partially purified from serum-free medium of spleen cell cultures of old overtly autoimmune NZB/W mice (24) and from the crowded growth of a human lymphocytoblastic cell line (12, 15). Both of these factors suppressed blastogenic responses to mitogens. Further, the factor from the NZB/W mouse spleen cells inhibited PWM-stimulated differentiation of B lymphocytes to plasma cells. Both of the factors were relatively stable to heat, remaining active after treatment at 56°C for 30 min but losing activity when heated to more than 80°C. The SSF demonstrated in the present study was also stable at 56°C for 30 min and lost activity when heated to more than 70°C for 30 min, indicating that the SSF derived from cultured human PBL possesses some of the properties observed with the factor derived from crowded cultures of a human lymphoblastoid cell line. Further investigations should permit isolation, purification, chemical analyses, measurement of molecular weight, definition of responses to enzymatic digestion, and analysis of the antigenic specificity of SSF.

For the present, we have shown that human PBL produce a soluble suppressor factor after incubation *in vitro* that can exert negative immunoregulatory actions without toxicity on a number of lymphocyte responses and immunological phenomena. The exact mechanism of the inhibitory activity of human SSF, its biological significance, and its chemical nature remain to be elucidated.

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