

T CELL-SPECIFIC SUPPRESSOR FACTOR(S) WITH REGULATORY INFLUENCE ON INTERLEUKIN 2 PRODUCTION AND FUNCTION¹

MICHAEL KRAMER AND ULRICH KOSZINOWSKI²

From the German Cancer Research Center, Institute of Immunology and Genetics, P.O. Box 101949, D 6000 Heidelberg, and the Federal Research Institute for Animal Virus Diseases, Department of Immunology, P.O. Box 1149, D 7400 Tübingen

In this study we report that alloantigen-activated spleen cells produce both amplifying and suppressive factors under the same conditions. Both types of soluble mediators—as detected in different assay systems—were present in the supernatants of *in vivo* sensitized and *in vitro* restimulated spleen cell populations and were separable by gel filtration. As shown by others, the amplifying factor (IL 2) was eluted in the size range of 30,000 m.w. The suppressive factor(s) (SF) was eluted in the size range of 10,000 m.w. SF was shown to inhibit the proliferative response of T cells to alloantigen, as well as the generation of regulatory T cells and cytotoxic T cells from their precursors when added at the beginning of the *in vitro* culture. Furthermore, SF inhibited the release of IL 2 from producer T cells but had no detectable effect on the interaction of IL 2 with receptive T cells. In addition it was shown that SF does not affect the generation of PFC from their precursors after activation by T cell-independent antigens. The results indicate that SF selectively acts on T cells and that it is involved in the regulation of the immune response by modulating early events in T cell activation.

The biologic and biochemical characterization of soluble mediators that modulate the activation and differentiation of T cells is of central importance for the understanding of cellular interactions leading to immune responses. In the past the detailed biochemical analysis of regulatory factors was precluded by the small quantity of biologically active materials obtained. Many assay systems used for the demonstration of activating or inhibitory properties have been carried out using nonselected cell populations. This has complicated the detailed analysis of the target cells and the mode of action of factors during the regulatory process. Recent progress in defining the activity of lymphokines is based on testing biochemically characterized substances (1–11) on cell populations of defined state of activation or on defined cell lines (12–17). Furthermore, the possibility of obtaining biologically active factors from cell lines has helped to define the producer cells and the conditions for optimal production (18–25). Thus, it seems now that cytotoxic T cell activation and proliferation is dependent upon at least 2 mediators, interleukin 1 and interleukin 2.

In light of these observations it is of interest to study the

regulatory events that negatively influence the activation, proliferation, and maturation of T cells initiated by antigen, mitogen, or amplifying factors (26–32).

In this paper we show that supernatants of alloantigen-stimulated spleen cells contain distinct factors that regulate T cell activation and maturation. The demonstration of mediators with amplifying or with suppressive activity in those supernatants depends mainly on the *in vitro* assay systems used. We demonstrate that amplifying and suppressive activities are separable. In addition, the biologic effects of the inhibitory activity on various lymphocyte functions are shown. The partially purified suppressive factor(s) inhibits the proliferation of T cells as well as the maturation to effector cells in the presence of antigens but does not act on the induction of T cell-independent B cell responses.

MATERIALS AND METHODS

Animals. Six- to 8-wk old C57BL/6 (H-2^b) and DBA/2 (H-2^d) mice were purchased from the Zentralinstitut für Versuchstierzucht, Hannover, West Germany. BALB/c nu/nu were obtained from Bomholtgard Ltd., Rye, Denmark. The animals were vaccinated against ectromelia virus and were rested at least 3 wk before being used in experiments.

Media. Mixed lymphocyte cultures were set up in RPMI 1640 supplemented with L-glutamine (2 mM final concentration), streptomycin and penicillin (50 U/ml), 2-mercaptoethanol (2×10^{-6} M), and 10% fetal calf serum. Mishell-Dutton cultures for the generation of PFC were performed with Schwartz's modified Click's medium (33).

Preparation of biologically active factors. Murine spleen cell suspensions of DBA/2 mice were aseptically prepared, washed twice in saline, and adjusted to 2×10^6 cells/ml; 0.1 ml of this suspension was injected i.v. into C57BL/6 mice.

Biologically active supernatants were prepared as previously described by Nadler and Hodes (32). In short: 4 to 8 days after priming, the spleen cells of DBA/2 primed mice were prepared and mixed with inactivated (2000 R) DBA/2 splenic stimulator cells. The culture was carried out in 10-ml tubes (17 x 100 mm, no. 2059, Falcon Plastics, Oxnard, CA) containing 1 ml of cell suspension in culture medium supplemented with 2% FCS. The cell concentration was 2×10^7 ml, and a stimulator:responder cell ratio of 1:1 was used. After 24 hr of culture in a humidified atmosphere of 5% CO₂ the cells were pelleted at 1500 rpm for 10 min. Supernatants were harvested and centrifuged once again at 6000 rpm for 30 min to remove remaining cell fragments.

Gel chromatography. For gel filtration the biologic activity was concentrated by ammonium sulphate precipitation at 80% saturation. The precipitated material was suspended in phosphate-buffered saline (PBS) and dialyzed overnight against 200 vol of the same buffer. Chromatography was performed at 4°C using degassed PBS as running buffer. Bio Gel P 30 (no. 150; 1340, Bio-Rad Laboratories, Richmond, CA) was expanded and equilibrated in PBS. Four milliliters of concentrated supernatant were layered on top of the Bio Gel P 30 column (1.5 x 90 cm, Pharmacia Fine Chemicals, Uppsala, Sweden) and were eluted with PBS at a flow rate of 20 ml/hr. The protein content of 20 to 25 6-ml fractions was determined using a spectrophotometer (Gilford 250) calibrated for absorbance at 280 nm. The column was calibrated with the molecular weight standards (Sigma) chymotrypsinogen (m.w. 23,000) and cytochrome c (m.w. 13,000). The void volume was determined with dextran blue, and the column volume was indicated by DNP-alanine.

Assays for lymphokine activity. IL 2 activity of supernatants or partially purified samples was determined by the ability to promote the growth of Con A-activated T cell blasts. This assay has recently been described by Andersson *et al.* (34). In short: T cell blasts were prepared by culturing

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² Correspondence to: Dr. U. Koszinowski, Federal Research Institute for Animal Virus Diseases, Department of Immunology, P.O. Box 1149, D 7400 Tübingen, Germany.

1×10^6 spleen cells in 50 ml culture medium containing 5 $\mu\text{g/ml}$ Con A (Pharmacia Fine Chemicals; Lot. no. 11130). After 72 to 96 hr of culture the cells were harvested and washed in BSS containing 5% FCS. Blast cells were then purified by spinning the cells onto a cushion of Lymphoprep Ficoll Solution (Nyegaard & Co, AS, Oslo) for 20 min at 1500 rpm at room temperature. The cells were washed twice in HBSS containing 5% FCS and 20 mg/ml alaphamethyl-p-mannoside. Blast cells were then seeded into round-bottom 0.2-ml microtiter plates (Greiner Labortechnik, no. 650-160, Nürtingen, Germany) at a concentration of 1×10^4 cells per well. Proliferation was assayed by the uptake of ^3H -thymidine ($6\text{-}^3\text{H}$ thymidine, Amersham Buchler, England). The cultured cells were pulsed at day 3 for 4 hr with 1 $\mu\text{Ci/well}$. Cultures were then harvested onto glass fiber filters in a microharvesting apparatus (Skatron Cell Harvester, A.S., Lierbyen, Norway), and radioactivity on dried filters was determined by scintillation counting. Each dilution of a sample with biologic activity was tested in 6 replicates. The assay of co-stimulator activity required for the mitogenic response of mouse thymocytes *in vitro* was carried out according to Shaw *et al.* (4).

Mixed lymphocyte culture (MLC). The mixed lymphocyte culture was set up by culturing 1×10^7 responder spleen cells together with 1×10^7 2000-R irradiated stimulator cells in 2 ml in flat-bottom tissue culture plates (Costar No. 3524). Cells were cultured at 37°C in a humidified 5% CO_2 atmosphere. After 4 days of culture the proliferative response was determined by the uptake of ^3H thymidine, and the generation of CTL was assayed in the $^{51}\text{chromium}$ release assay.

In experiments designed to determine only the proliferative response, cell culture was carried out in round-bottom microtiter plates (Greiner Labortechnik, no. 650-160, vol. 0.2 ml/well) containing 2×10^5 cells of either responder or stimulator cell type. At day 4 cells were pulsed for 4 hr by adding 1.0 μCi of ^3H -thymidine per well.

$^{51}\text{Chromium}$ release assay. *In vitro* grown P-815 (H-2^b), EL-4 (H-2^b), and L 929 (H-2^k) cell lines were used as target cells. Virus-infected target cells were prepared as described previously (35). Labeling with $^{51}\text{chromium}$ (Sodium-Chromate, Amersham Buchler) was performed for 2 hr at 37°C . 5×10^6 target cells were mixed together with 200 μCi of $^{51}\text{chromium}$ in a total volume of 0.4 ml. After repeated washings the target cells were incubated at 1×10^4 cells/well together with graded numbers of attacker cells in round-bottom microtiter plates (Greiner Labortechnik, no. 650-160). Controls and calculations of percent specific $^{51}\text{chromium}$ release were carried out as described (36). Cytolytic activity is either depicted at different effector-to-target cell ratios or calculated as lytic units per 10^6 effector cells (35).

Generation of PFC. Spleen cells of BALB/c nu/nu mice were cultured in flat-bottom tissue culture plates (Costar, no. 3596, vol. 0.2 ml/well) at a concentration of 1×10^6 cells/well using a modification of the original Mishell Dutton technique (37). As antigen TNP-Ficoll (generously supplied by Dr. M. Simon, Heidelberg) was added at different concentrations. Schwartz's modified Click's medium was used as culture medium. After 4 days of culture in a humidified atmosphere of 2% CO_2 , antibody-secreting cells were detected by a local hemolysis assay in gel, using TNP-modified SRBC as target cells (33).

Suppressor cell assay. The assay is a modification of the system described by Al-Adra and Pilarski (38). The activity of antigen-nonspecific suppressor cells was assayed during the induction phase of secondary virus-specific CTL *in vitro*. Secondary Sendai virus-specific CTL were generated *in vitro* as described previously (39). In short: C 57BL/6 cells preactivated by 2 days of mixed lymphocyte culture with DBA/2 stimulator cells were added in graded numbers to second cultures in order to suppress the generation of virus-specific effector cells from their precursors. The cytolytic activity of the CTL generated under these conditions was tested as described (35).

Helper cell assay. The assay of helper cell activity is based upon a system described by Pilarski (40). In short: 1×10^6 responder cells were co-cultured with 1×10^6 glutaraldehyde-fixed CBA stimulator cells in round-bottom microtiter plates (0.2 ml/well). Helper cells were added in a ratio of 1 helper cell per 8 responder cells. Before the addition the helper cells were washed 3 times in HBSS and irradiated (1500 R). For each value, 4 replicate cultures were set up. After 4 days of culture the individual cultures were diluted by successive 1:2 dilutions. The cytolytic activity was then determined by adding 1×10^5 $^{51}\text{chromium}$ -labeled target cells per well.

Control cultures contained either responder cells and irradiated stimulator cells, or responder cells only, or helper cells and glutaraldehyde-treated stimulator cells. Helper cells were generated in a first culture by a 2-day MLR of C57BL/6 spleen responder cells with DBA/2 stimulator cells. This protocol is identical to that of suppressor cell generation. The main difference between the assay showing suppression and that demonstrating help are the different regulator:responder cell ratios used and the irradiation of helper cells.

RESULTS

Production and separation of IL 2 and suppressive factor(s). Previous experiments have shown that supernatants of Con A-

activated spleen cells in addition to IL 2 contain factors that inhibit the generation of cytotoxic T lymphocytes (CTL) from their precursors (41). Similar activities were observed in supernatants from *in vitro* MLR cultures of previously *in vivo* sensitized responder cells. Supernatants produced under these conditions support T cell blast growth but inhibit responder T cells from proliferating and generating CTL *in vitro*. (A detailed report on the induction conditions will be published elsewhere).

Gel chromatography of ammonium sulphate-treated concentrated supernatants was carried out to separate IL 2 from the suppressive material. Two assay systems were used to detect IL 2 and/or suppressive factor (SF)³ activity in the eluted fractions: a) induction of proliferation of T cells in mixed lymphocyte cultures, and b) continuous proliferation of Con A-activated T cell blasts. In Figure 1 a typical activity profile of the fractions eluted from a Bio Gel P 30 column is shown.

Data show that amplifying and suppressive activities can be separated by this method. IL 2 is eluted as a narrow peak in the size range of 30,000 m.w. (Fig. 1B) when tested on T cell blasts. When tested in the mixed lymphocyte reaction (MLR), a broader peak of amplifying activity is observed, which is probably due to additional substances present in those fractions that augment the proliferative response in the MLR. Material inhibitory for the induction of proliferation in the MLR is constantly eluted as a single peak in the molecular size range of about 10,000 daltons.

The Bio Gel P 30 column fractions that contained the highest suppressive activity (herein referred to as SF) were pooled, stored in aliquots at -20°C , and used in all further experiments described. The release of SF was found not to be dependent on FCS components since material with identical functional and biochemical characteristics was also obtained from cultures carried out in serum-free medium (42).

A titration of the suppressive activity inhibiting ^3H -thymidine uptake in C57BL/6 anti-DBA/2 MLR is depicted in Figure 2. About 50% inhibition of proliferation was detected at 25% SF concentration. Therefore in all further experiments presented in this paper SF was used at either 25% or 50% final concentration in the culture medium.

Effects of SF on the proliferation and generation of T effector cells. Cultures of C57BL/6 splenic responder cells were stimulated with irradiated DBA/2 cells in the presence or absence of SF, and the proliferative response as well as the generation of effector cells (CTL and regulatory cells for CTL induction: T helper and T suppressor cells) was monitored during the following 4 days. In the presence of SF, proliferative responses of C57BL/6 lymphocytes were reduced to background levels (C57BL/6 lymphocytes incubated with syngeneic irradiated stimulator cells; Fig. 3A). It is therefore reasonable to assume that the unresponsiveness of the cultures containing SF is due to inhibition of cell replication rather than to a mere alteration of the response kinetics. Suppression of proliferative responses was maximal if SF was added at the beginning of the culture period and decreased with delayed addition (data not shown). Similarly, the effect of SF on the generation of CTL in a C57BL/6 anti-DBA/2 MLR was examined (Fig. 3B). The control cultures developed cytolytic activities of approximately 4×10^4 lytic units per 10^6 cells at day 4, but no measurable cytotoxic potential was detected in SF-containing cultures.

The suppression of proliferative and cytotoxic responses by SF either reflects a general inhibition of lymphocyte activation

³ Abbreviations used in this paper: IL 2, interleukin 2; MHC, major histocompatibility complex; SF, suppressive factor.

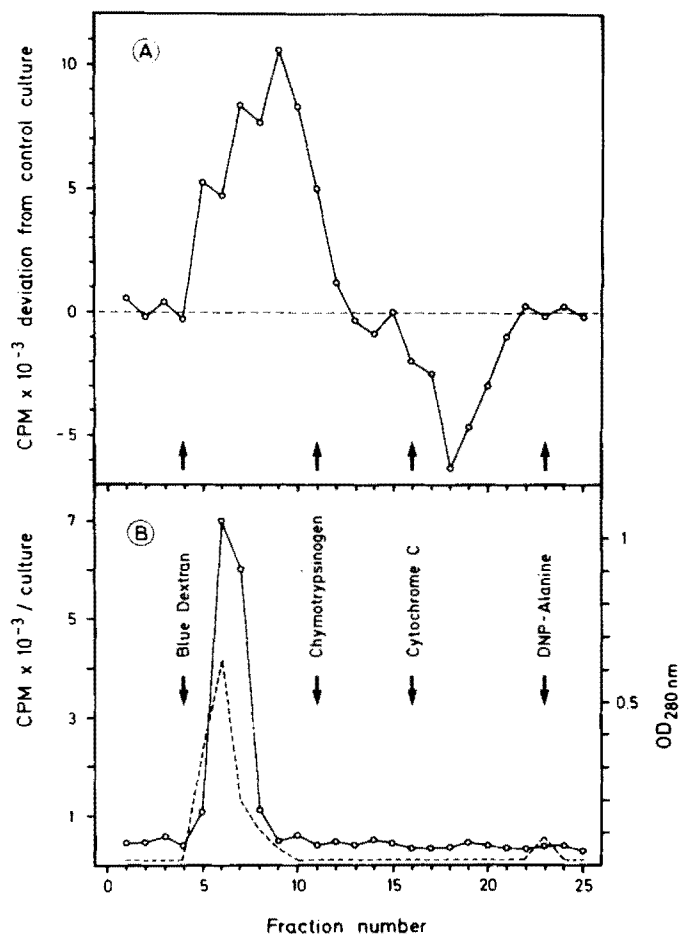


Figure 1. Lymphokine activities assayed after Bio-Gel P-30 gel exclusion chromatography of concentrated medium conditioned by alloantigen-stimulated murine spleen cells. The 80% $(\text{NH}_4)_2\text{SO}_4$ precipitate from 275 ml conditioned medium was dissolved in 20 ml PBS and dialyzed twice against 200 vol of the same buffer. Four-milliliter portions were layered on the column and eluted fractions were tested for biologic activities at 50% final concentration during the assay. A, \circ — \circ , Effects on ^3H -thymidine uptake in a C57BL/6 anti-DBA/2 MLR. Data are plotted as deviation of cpm from cpm in control cultures that were set up in medium supplemented with 50% of the running buffer (PBS). Cpm in this control were 12185 ± 300 . B, \circ — \circ , Effects on ^3H -thymidine uptake of C57BL/6 T cell blasts; —, absorbance at 280 nm.

and differentiation or is due to factor-induced triggering of regulatory cells that negatively influence the activation of effector cell precursors. Accordingly, the effect of SF on the generation of T helper cells for CTL precursors was tested, using an assay system similar to that described by Pilarski (40). C57BL/6 lymphocytes were sensitized *in vitro* against irradiated DBA/2 splenocytes in the presence or absence of SF. After 2 days the cells were irradiated and transferred to the detection system containing C57BL/6 responder cells and glutaraldehyde-treated CBA stimulator cells. In previous experiments using this protocol we could demonstrate T helper cell effects. The generation of T helper cell function in the detection system requires a second exposure to antigenic determinants that were present in the first culture (40). Therefore, we conclude that non-major histocompatibility complex (MHC) determinants (minor H-antigens, M-locus) are responsible for the activation of helper cell function in the combination used. The advantage of this modification is the exclusion of MHC-specific CTL that are transferred from the first culture and that may react with determinants on the stimulator cells. As can be seen in Figure 4A, cultures containing responder cells and glutaraldehyde-treated stimulator cells developed only marginal cytolytic activity. Addition of helper cells resulted

in the restoration of the cytotoxic response to levels comparable to that of the controls (responder cell plus irradiation stimulator cells). However, when SF was present during the first culture, no helper activity could be demonstrated. This indicates that the generation of T helper cells is prevented by SF.

It is known from the literature that during primary MLC both T helper cells and specific and nonspecific suppressor cells are generated (38). Consequently we tested the effects of SF on the generation of nonspecific suppressor cells able to inhibit the generation of CTL.

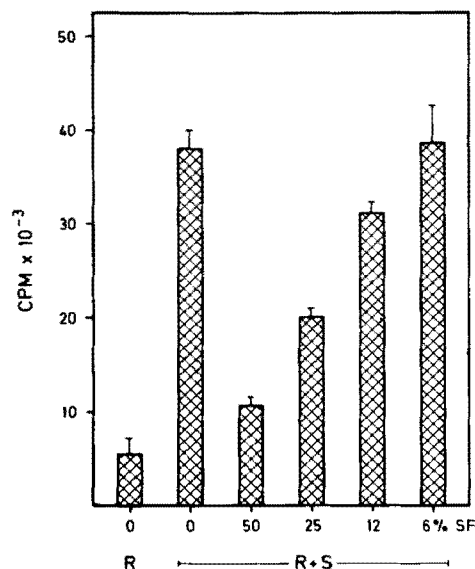


Figure 2. SF activity of inhibitory fractions is dose dependent. Pooled fractions with inhibitory activity were added at different concentrations (6 to 50%) to a mixed lymphocyte reaction of 2×10^5 C57BL/6 responder cells (R) and 2×10^5 DBA/2 stimulator cells (S). Control cultures (R+S) were carried out in medium containing 50% PBS. The ^3H -thymidine uptake during 4 hr of six replicate cultures \pm SEM was determined at day 4.

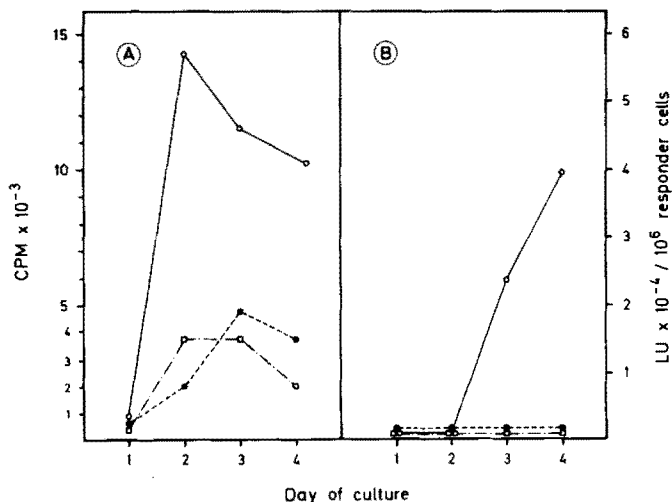


Figure 3. Effects of SF on MLR kinetics and CTL generation. A, Effect on MLR: \circ — \circ , C57BL/6 responder cells and irradiated DBA/2 stimulator cells in medium supplemented with 50% PBS; \square — \square , C57BL/6 responder and syngeneic irradiated stimulator cells; \bullet — \bullet , C57BL/6 responder and irradiated DBA/2 stimulator cells in presence of medium supplemented with 50% SF. B, effect on CTL generation: \circ — \circ , cytotoxic activity of C57BL/6 responder cells stimulated with irradiated DBA/2 stimulator cells in medium supplemented with 50% PBS; \square — \square , C57BL/6 responder cells incubated with syngeneic stimulator cells; \bullet — \bullet , C57BL/6 responder cells and DBA/2 stimulator cells incubated in presence of medium supplemented with 50% SF. P-815 cells served as target cells.

C57BL/6 cells were sensitized *in vitro* against irradiated DBA/2 splenocytes. After 2 days the cells were harvested and added without irradiation to a second culture containing C57BL/6 responder cells and viral antigen. Since the detection system contains virus-infected syngeneic stimulator cells, suppression is unlikely to be mediated by cytolytic elimination of stimulator cells through alloreactive CTL transferred from the first culture. As can be seen in Figure 4B, the generation of suppressor cells is inhibited in presence of SF. Only lymphocytes sensitized in the first culture in the absence of SF were able to suppress the generation of virus-specific CTL in the second culture. These results argue strongly against selective effects of SF on a particular functional T cell subset and exclude activation of suppressor cells by SF.

Since the generation of all T cell functions tested so far was inhibited by SF, it could be argued that all lymphoid cells—T and B cells—are paralyzed nonspecifically by toxic substances present in the SF fractions or that the suppression is due to specific inhibition of cells from the T cell lineage. The toxicity for T cells was tested in the co-stimulator assay (4). SF, at the concentration used, inhibited the induction of thymocyte proliferation by mitogen- and IL-2-containing medium. Despite that,

Inhibition of helper and suppressor cell generation

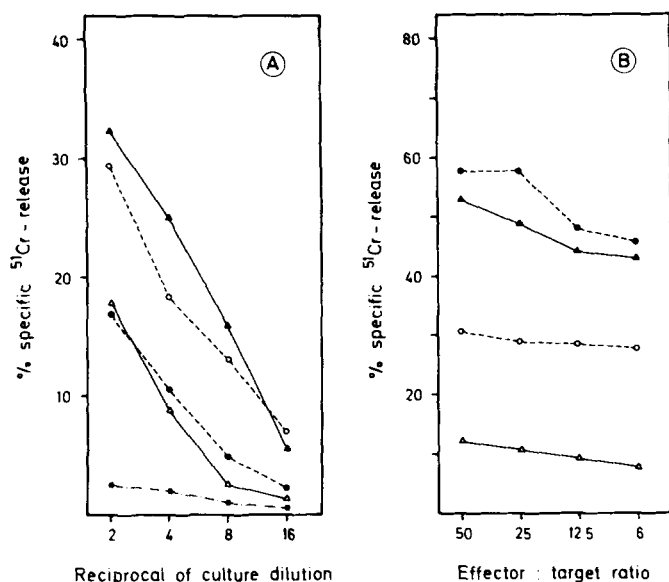


Figure 4. Effects of SF on T helper and T suppressor cell generation. A, effects on helper cell generation. Detection system for help: CTL that lysed L929 target cells were generated in the MLC of C57BL/6 responder co-cultured with CBA stimulator cells. The MLC was carried out in quadruplicate microcultures and the total number of effector cells harvested on day 4 was tested at four attacker to target cell ratios. ○—○, Activity of responder cells incubated without stimulator cells; ▲—▲, activity of responder cells incubated with irradiated stimulator cells; △—△, activity of responder cells incubated with irradiated and glutaraldehyde stimulator cells; □—□, 1500 R irradiated T helper cells (C57BL/6 responder cells preincubated for 48 hr with irradiated DBA/2 stimulator cells in medium supplemented with 50% PBS) were added to cultures that contained glutaraldehyde-treated stimulator cells. Helper to responder cell ratio was 1:8. ●—●, T helper cells added to the detection system are derived from 48-hr cultures carried out in medium supplemented with 50% SF. B, effects on suppressor cell generation. Detection system for suppression: C57BL/6 responder cells primed with Sendai virus *in vivo* were restimulated *in vitro* with 1 μ g/ml inactivated SV and CTL activity generated was tested at various attacker to target cell ratios on SV-infected EL-4 target cells. ▲—▲, Activity of SV-specific effector cells after restimulation with viral antigen; △—△, activity of SV-primed cells without restimulation by viral antigen *in vitro*; □—□, T suppressor cells added (C57BL/6 responder cells preincubated for 48 hr with DBA/2 stimulator cells in medium supplemented with 50% PBS) to cultures containing responder cells and antigen. Suppressor to responder cell ratio was 1:2. ●—●, T suppressor cells added to the detection system were derived from 48-hr cultures carried out in medium supplemented with 50% SF.

by comparing the effects of PBS and Con A with those of SF, it was found that SF did not affect cell recovery and viability (Table I).

Effect of SF on B cell responses to T independent antigens. In order to study the effects of SF on the activation of B cells, splenocytes from BALB/c nu/nu mice were incubated with the T cell-independent antigen TNP-Ficoll in the presence or absence of SF. At day 4 of *in vitro* culture the number of IgM-producing cells was determined in the Jerne plaque assay. It was found (Table II) that SF did not significantly affect the generation of plaque-forming cells at a concentration known to inhibit T cell responses. This confirms again that SF is not toxic for all differentiating lymphocytes and suggests that SF acts predominantly on cells of the T cell lineage.

Effect of SF on the production and function of IL 2. Data from the literature suggest that amplifying factors such as IL 1 and IL 2 are essential for the generation of T effector cells from their precursors. We therefore tested whether SF has any effects on the release of IL 2 from *in vitro* sensitized lymphocytes. C57BL/6 responder cells were incubated with DBA/2 stimulator cells in the presence or absence of SF. Supernatants from these cultures were collected daily and tested for IL 2 activity in the T cell blast proliferation assay (34). Figure 5 depicts the results of one representative experiment. In the supernatants of control MLC, IL 2 activity is present at optimal concentrations after 48 hr of culture. In supernatants obtained from cultures containing SF no IL 2 is detectable after 2 days. There are at least 2 alternative explanations for these findings. First, SF remaining in the supernatant from the first culture interferes with the effects of IL 2 on receptive T cell blasts in the detection system. Second, SF inhibits the release of IL 2 in primary MLC. To investigate these alternatives the effect of SF on the interaction of IL 2 with T cell blasts was studied.

SF does not interfere with the IL 2 effects on T cell blasts. In Figure 6A it can be seen that the amount of ³H-thymidine uptake of a given number of T cell blasts is correlated with the IL 2 concentration in the culture medium. An IL 2 concentration that corresponds to 1.5% of the activity of the reference supernatant results in the uptake of approximately 15,000 cpm

TABLE I
Effects of SF on the proliferation of thymocytes^a

Additions to Thymocyte Culture	cpm ^b	Total Cell Recovery ^c	Viability ^d
PBS ^d	1250 \pm 170	0.54 \times 10 ⁵	63% \pm 5.0
PBS + Con A	1770 \pm 380	0.67 \times 10 ⁵	60% \pm 4.9
PBS + Con A + IL-2	10300 \pm 890	2.77 \times 10 ⁵	66% \pm 7.1
SF + Con A + IL-2	2200 \pm 300	0.70 \times 10 ⁵	64% \pm 3.2

^a Thymocytes from C57BL/6 mice at a concentration of 5×10^4 cells/well in 200 μ l medium were incubated for 96 hr in the presence of various additions. During the final 4 hr ³H-thymidine was added.

^b Data represent the mean value \pm SD of six replicate determinations.

^c SD always less than 10%.

^d IL-2 was provided by addition of 12.5% vol/vol of a Con A supernatant from rat spleen cells. PBS and SF were added at 50% vol/vol and Con A to give a final concentration of 3 μ g/ml.

TABLE II
No effect of SF on the generation of PFC^a

Responder Cells	Antigen	Inhibitor	PFC/Culture
BALB/c nu/nu spleen cells			4 \pm 2
BALB/c nu/nu spleen cells	TNP-Ficoll (1:10 ⁷)	25% PBS	120 \pm 10
BALB/c nu/nu spleen cells	TNP-Ficoll (1:10 ⁷)	25% SF	128 \pm 17
BALB/c nu/nu spleen cells	TNP-Ficoll (1:10 ⁶)	25% PBS	75 \pm 17
BALB/c nu/nu spleen cells	TNP-Ficoll (1:10 ⁶)	25% SF	115 \pm 28

^a Spleen cells of BALB/c nu/nu mice were incubated for 96 hr together with different dilutions of a stock of TNP-Ficoll in medium supplemented with either PBS or SF. Direct plaque-forming cells were detected by a local hemolysis assay in gel by using TNP-modified SRBC. For each value six replicate cultures were carried out.

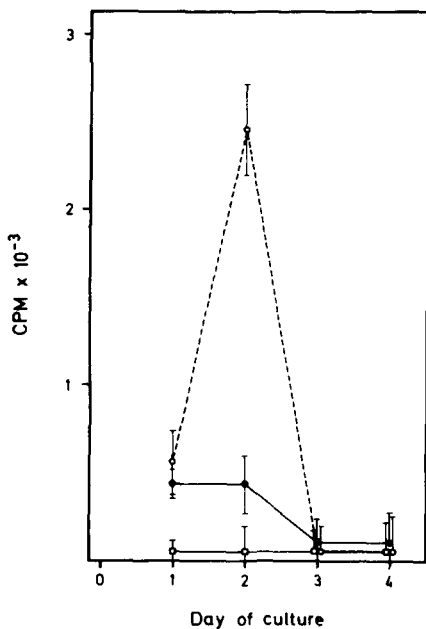


Figure 5. Effect of SF on the IL-2 release in the MLR. ○—○, Culture supernatants from C57BL/6 anti-DBA/2 lymphocyte cultures were harvested daily and tested for IL-2 activity in the T cell blast proliferation assay. The medium was supplemented with 50% PBS. ●—●, Culture supernatants from cells incubated in medium supplemented with 50% SF. ■—■, Culture supernatants from cultures carried out in absence of DBA/2 stimulator cells in medium supplemented with 50% PBS. Counts per minute in control cultures containing 25% PBS and 25% culture medium not conditioned by cells (621 ± 43) were subtracted.

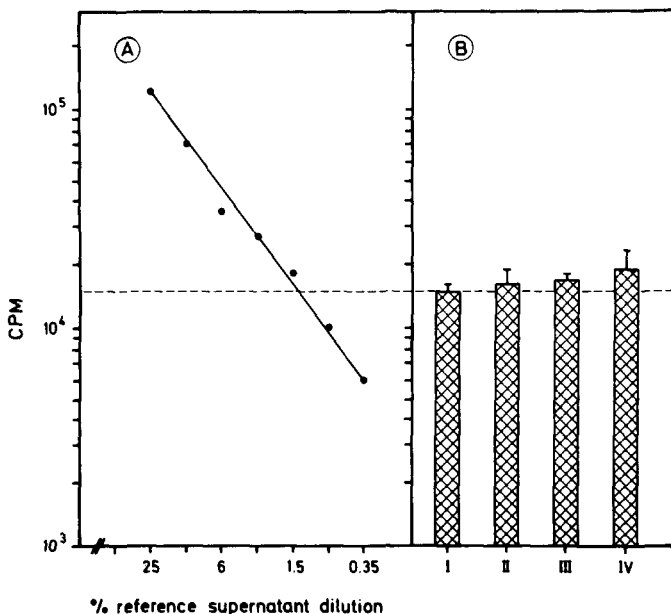


Figure 6. No effect on SF on the responsiveness of T cell blasts to IL-2. A, relationship between the concentration of IL-2 and ³H-thymidine uptake of T cell blasts. Con A-activated C57BL/6 T cell blasts (10^4 /well) were incubated for 72 hr in graded concentrations of a reference supernatant and ³H-thymidine uptake during the final 4 hr of culture was determined. B, SF does not inhibit the activity of IL-2 on T cell blasts. ³H-thymidine uptake of T cell blasts in medium containing 25% Bio-Gel P-30 purified murine IL-2 supplemented with dilutions of PBS and/or SF. I) 25% IL-2, 25% PBS; II) 25% IL-2, 25% SF; III) 25% IL-2, 12.5% SF, 12.5% PBS; IV) 25% IL-2, 6% SF, 19% PBS.

per individual culture containing 1×10^4 cells during the last 4 hr of a 72-hr culture period. In Figure 6B it is shown that proliferative responses of only 1×10^4 Con A-activated T cell blasts are not affected by concentrations of SF that clearly inhibit the proliferation of cells in the MLR and in the co-

stimulator assay (see also Table I and Fig. 2). These results suggest that the reduced IL 2 activities detected in the supernatants of SF-treated MLR are most likely due to inhibition of IL 2 release rather than to the mere interference of remaining SF with IL 2 effects on Con A-activated T cell blasts.

DISCUSSION

During activation of lymphocytes *in vitro* by either mitogens or alloantigens, soluble mediators that support T cell maturation and growth are generated and released into the culture medium. The main results of our previous and present experiments are that under similar conditions soluble mediators are released that inhibit lymphokine production and T cell activation. Both amplifying and inhibitory activities can be detected in the supernatants from the same cultures using different assay systems.

In this study we describe the biochemical separation of both activities and the functional characterization of the suppressive material. Separation was achieved by gel chromatography. The suppressive activity elutes as a single peak in the size range of approximately 10,000 m.w. The protein nature is indicated by the destruction of functional activity after protease treatment (unpublished results). These characteristics exclude low molecular substances such as prostaglandins, cyclic AMP, and glucocorticoids known to be inhibitory for T cell activation (43). The size range of 10,000 m.w. separates SF from a number of other regulatory molecules that have been chemically characterized, such as soluble immune response suppressor (SIRS/m.w. 48,000 to 67,000) (44), inhibitor of DNA synthesis (IDS/m.w. 75,000 to 80,000) (45), lymphoblastogenesis inhibition factor (LIF/m.w. 68,000) (46), low m.w. suppressors (LMW/m.w. <1000) (47), and the chalcones (m.w. 30,000 to 50,000) described by Houck *et al.* (48). The size of the factor we describe is similar to that of the suppressive material found by Lafferty *et al.* (49) in the supernatant of Con A-stimulated spleen cells.

Another classification defines regulatory factors in terms of antigen specificity (43) or with respect to MHC-encoded structures as integral components for factor activity (50) or in terms of MHC control of responder, producer, or acceptor status (51). Previous experiments indicate that the factor described in this report is not antigen specific nor H-2 or even species restricted (41), and it is therefore distinct from the MLR suppressor factor described by Rich and Rich (29). The nonrestricted activity on CTL generation is reminiscent of the factor described by Truitt *et al.* (52), although the activity of that factor is lost in medium containing 2-mercaptoethanol.

The functional activity of the chromatographically purified material is characterized by the inhibition of the generation of effector T cells, i.e., CTL, and regulatory T cells for CTL induction. Furthermore the production of IL 2 is inhibited. In this connection we observed that the growth-promoting activity of IL 2 on T cell blasts is not affected by the presence of SF. Since the viability of SF-treated thymocytes is not reduced and the generation of PFC from splenocytes of nude mice against TNP-Ficoll is also not inhibited, nonspecific toxic effects on differentiating lymphocytes in general are excluded. The predominant effect on T cells is also strengthened by the findings of previous experiments showing that supernatants that completely abolished the generation of CTL gave maximal help to spleen cells from nude mice responding to SRBC (41). The apparent specificity for T cells separates SF from factor(s) of similar size that also act on B cells (31).

The inhibition of T cell proliferation could be explained by

inhibition of IL 2 production, by prevention of T cells from becoming responsive to IL 2, or by a combination of both effects. We favor the third explanation, since in further experiments we found that the proliferative response of resting T cells to mitogen or antigen is blocked by SF even in the presence of abundant concentrations of mitogen and activating lymphokines (53). The data indicate a qualitative distinction between resting and activated T cells with respect to their responsiveness to IL 2 and SF. It is suggested that resting T cells are not responsive to IL 2 (54, 55). Responsiveness to IL 2 by incubation with either antigen or mitogen is acquired through the surface expression of a functional receptor for IL 2, which can be demonstrated in absorption experiments (56). SF seems not to influence the functional interaction of IL 2 with receptor but the expression of the receptor during activation.

The data may be of some importance for the technique of frequency analysis of T cells that is presently carried out in many laboratories. This is routinely done by disseminating graded numbers of cells in the presence of antigen, filler cells, and conditioned medium containing IL 2. The quality of the conditioned medium is usually tested by its growth-promoting activity on activated T cells or T cell lines (23, 34). However, the suppressive activity we describe is not detectable in these assays. Supernatants at their optimal growth-promoting activity in these tests are considered to be useful for precursor frequency analysis. Since it is sufficiently documented that IL 2 acts preferentially on activated T cells, factors that inhibit activation will also inhibit expansion even in the presence of abundant concentrations of growth factors. Taken together, our findings advise the use of at least partially purified growth-supporting media that are tested for more than just one biologic activity.

We describe a chalone of the immune system as a substance that is produced by a given tissue and that inhibits proliferation of cells of the same type as those from which it is obtained (56). To our knowledge this is the first lymphocyte-derived factor described that inhibits IL 2 production but not IL 2 function, as do other factors described recently (57). The mode of action is different from that of glucocorticoid hormones because the inhibition cannot be overcome by the addition of IL 2 (26, 27). It is, however, very similar to the action of the fungus metabolite cyclosporin A, which inhibits the acquisition of responsiveness to IL 2 in resting T cells at low concentrations (1 to 5 $\mu\text{g}/\text{ml}$) and, with increasing concentrations (10 $\mu\text{g}/\text{ml}$), also inhibits the production of IL 2 (28). SF does not interfere with the clonal expansion of antigen-specific effector T cells triggered by contact with antigen preceding SF release. Thus SF may act as a homeostatic mechanism for T cell responses by limiting the generation of an uncontrolled range of effector cells to a given antigen.

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