Proc. Natl. Acad. Sci. USA
Vol. 77, No. 4, pp. 2205–2208, April 1980
Immunology

In vitro production of immunosuppressive factors by murine sarcoma virus-transformed mouse fibroblasts

(proliferation inhibitory peptides/lymphocyte suppression)

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Communicated by H. Sherwood Lawrence, February 1, 1980

ABSTRACT **Murine sarcoma virus-transformed mouse fibroblasts produce potent immunosuppressive factors (ISF) *in vitro*. The partially purified ISF inhibited thymocyte proliferation induced by concanavalin A or phytohemagglutinin plus lymphocyte activating factor (Interleukin 1), lipopolysaccharide-induced spleen cell proliferation, the *in vitro* splenic anti-sheep erythrocyte plaque-forming cell response, and the generation of alloantigen-specific cytotoxic T cells. The effect of ISF on thymocyte proliferation was not readily reversible and required only a 4-hr exposure of the thymocytes to ISF to inhibit cell proliferation. Although ISF shares several biochemical properties with a murine sarcoma virus-transformed cell-derived sarcoma growth factor (e.g., acetic acid solubility and sensitivity to dithiothreitol), the two factors could be resolved by gel filtration on Bio-Gel P-60. Two peaks of ISF activity were found with apparent molecular weights of 12,000 and 8000. The results described here support the hypothesis that at least some of the ISF obtained from the serum of tumor-bearing hosts may be released by the tumor cells themselves. In view of the potent *in vitro* activity of the murine sarcoma virus-transformed fibroblast-derived ISF, it is quite possible that ISF-like molecules may play a role in subverting *in vivo* tumor rejection processes involving the immune system.**

Murine sarcoma virus (MuSV)-transformed mouse fibroblasts produce polypeptide growth factors, termed sarcoma growth factors (SGF), that stimulate untransformed fibroblasts to proliferate in monolayer culture and also convert the indicator fibroblasts to anchorage-independent growth (1). These effects of SGF on fibroblasts are reversible and are characteristic of the transformed phenotype. SGF exhibits M_rs of 25,000, 10,000, and 6000 and is sensitive to trypsin and dithiothreitol treatments (1). During the initial phase of a study on the possible relationship between SGF and a macrophage-derived factor, lymphocyte activating factor [LAF; Interleukin 1 (IL 1)]‡ which stimulates thymocyte proliferation (2), we found that partially purified preparations of SGF were extremely suppressive for thymocyte proliferation. This observation prompted us to examine the possible immunosuppressive activity of peptides released from virally transformed cells on a spectrum of *in vitro* lymphocyte functions. Our results demonstrate that MuSV-transformed mouse fibroblasts produce very potent *in vitro* immunosuppressive factors (ISF) that interfere with a number of immune reactions. Although ISF is produced by the same transformed cell that produces SGF, these activities can be separated from one another.

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MATERIALS AND METHODS

Preparation of SGF and ISF. SGF and ISF were obtained from the culture fluid of Moloney MuSV-transformed 3T3 mouse fibroblasts maintained in serum-free medium (1). Both a virus-producing transformed cell line (3B11-IC) and a MuSV nonproducer cell line (3197.3) from Peter Fischinger (National Institutes of Health) were used. The SGF and ISF were partially purified by acetic acid extraction and Bio-Gel P-60 gel filtration chromatography (1). Unless otherwise indicated, the 8000–12,000 M_r pool of Bio-Gel P-60 fractions was used in these studies. The protein concentration of the Bio-Gel P-60 pool was approximately 50 μg/ml. These fractions contained both the ISF and SGF. Dithiotreitol-treated material was prepared by incubating the Bio-Gel P-60 pool with 65 mM dithiothreitol in 0.1 M $(NH_4)_2HCO_3$ for 1 hr at 25°C (1). Control and dithiothreitol-treated samples were lyophilized and reconstituted with assay medium.

Bioassay. SGF activity was assayed on normal rat kidney fibroblasts as described (1). The proliferation of C3H/HeJ mouse thymocytes was measured as described (2). The effect of ISF on thymocyte mitogenesis was tested in the presence of concanavalin A (Con A; 5 μg/ml) (Sigma) or phytohemagglutinin (PHA) (Burroughs-Wellcome Research, Triangle Park, NC) (1 μg/ml) plus LAF (IL 1) (100 units/ml) (2). To evaluate the relative potency of ISF in different assay systems, it was necessary to define a standard unit of ISF activity. One unit of ISF activity was defined as the amount (dilution) of ISF necessary for 50% inhibition of thymocyte proliferation in the presence of PHA plus LAF (IL 1). LAF (IL 1) was prepared from the culture fluid of the $P388D_1$ murine macrophage cell line and was partially purified by ammonium sulfate fractionation, DEAE-cellulose chromatography, and Sephacryl S-200 (superfine) chromatography (3).

The effect of ISF on lipopolysaccharide (LPS)-induced spleen cell mitogenesis was evaluated by incubating C57BL/6J mouse spleen cells (1×10^5 cells per 200-μl culture) with 10 μg of LPS (phenol/water extracted) per ml in the presence or absence of

Abbreviations: MuSV, murine sarcoma virus; SGF, sarcoma growth factor; LAF (IL 1), lymphocyte activating factor (Interleukin 1); Con A, concanavalin A; ISF, immunosuppressive factors; LPS, lipopolysaccharide; SRBC, sheep erythrocytes; PFC, plaque-forming cells; PHA, phytohemagglutinin.

‡ A revised nomenclature for LAF was proposed at the recent Second International Lymphokine Workshop (Ermatingen, Switzerland, May 27–31, 1979). The revised name for LAF is Interleukin 1. To avoid confusion, the term IL 1 will initially be assimilated into the literature by using both acronyms as follows: LAF (IL 1). With time, the term LAF will be deleted.

ISF for 72 hr at 37°C. During the last 4 hr of culture, the cells were pulsed with 0.5 μCi of [^{3}H]thymidine per well (1 Ci = 3.7 × 10^{10} becquerels). All samples were tested in triplicate.

The *in vitro* anti-sheep erythrocyte (SRBC) plaque-forming cell (PFC) response of *nude* mouse spleen cells was assayed as described by Farrar *et al.* (4). The spleen cells were stimulated with antigen in the presence of an aliquot of a culture supernatant obtained from Con A-activated spleen cells. This supernatant contains a number of soluble factors that are capable of enhancing the *in vitro* anti-SRBC PFC response of murine spleen cells (4). ISF was added in varying concentrations.

The effect of ISF on the induction of cytotoxic T cells was tested by incubating 2 × 10^5 C57BL/6J responder spleen cells with 2 × 10^5 BDF_1 stimulator cells in RPMI 1640 medium containing 10% fetal calf serum and 50 μM 2-mercaptoethanol with or without varying concentrations of ISF. After 5 days the cultures were tested for cytotoxicity on 1 × 10^4 ^{51}Cr-labeled P815 mastocytoma target cells (4 hr, 37°C) (5).

Interferon Assay. At a protein concentration of 25 μg/ml, the Bio-Gel P-60 pool did not exhibit any interferon activity on primary mouse embryo fibroblasts challenged with vesicular stomatitis virus.

Cell Viability. Cell viability was routinely measured by trypan blue exclusion.

RESULTS

Inhibition of Thymocyte Proliferation by ISF. The proliferation of mouse thymocytes is markedly enhanced by Con A or PHA in combination with the macrophage-derived thymocyte proliferation factor, LAF (IL 1). As shown in Fig. 1, a pool of ISF activity from the Bio-Gel P-60 column inhibited both Con A- and PHA plus LAF (IL 1)-induced thymocyte mitogenesis. Regardless of the mitogen(s) used to stimulate the cells, 12.5% ISF almost completely inhibited proliferation. This corresponds to a protein concentration of approximately 12.5 μg/ml. At ISF concentrations at or below 0.4%, Con A-induced proliferation was relatively unaffected. In contrast, PHA plus LAF (IL 1)-induced proliferation remained maximally inhibited at 0.4% ISF and was still inhibited by approximately 60% at 0.05% ISF (25 ng/ml of protein). The concentrated supernatants of both virus producer and nonproducer MuSV-transformed cells showed comparable ISF titers; in contrast, the untransformed central cells showed no detectable ISF activity.

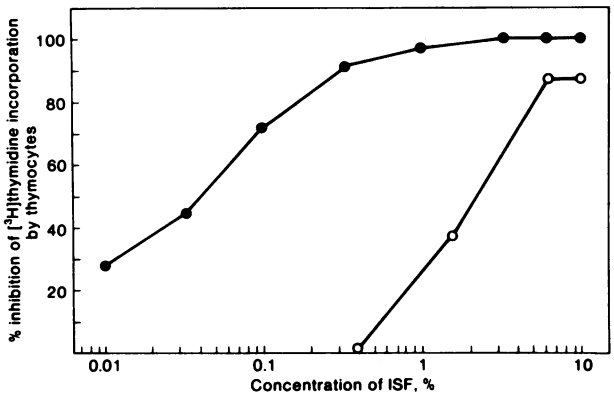

FIG. 1. Effect of ISF on Con A- and PHA plus LAF (IL 1)-induced C3H/HeJ mouse thymocyte proliferation. Thymocytes were incubated with either 5 μg of Con A (○) per ml or 1 μg of PHA per ml plus 100 units of LAF (IL 1) (●) per ml in the presence or absence of varying concentrations of ISF. In the absence of ISF, Con A-stimulated thymocytes incorporated 18,658 ± 100 cpm of [^{3}H]thymidine, whereas PHA plus LAF (IL 1)-stimulated cells incorporated 16,917 ± 1,272 cpm of [^{3}H]thymidine.

Having demonstrated the inhibitory effect of ISF on thymocyte proliferation, we next evaluated how long thymocytes must be exposed to ISF in order to inhibit their proliferation. PHA plus LAF (IL 1)-stimulated thymocytes were incubated with ISF for 1, 2, 4, or 24 hr, were washed three times to remove unbound ISF, and then were incubated for the remainder of the 72-hr assay in fresh medium containing PHA and LAF (IL 1) without added ISF. As shown in Table 1, proliferation was inhibited by almost 90% when cells were treated with ISF for 24 hr. A brief exposure to ISF of only 4 hr was sufficient to produce 60% inhibition of thymocyte proliferation. Thus, the effect of ISF on thymocyte proliferation required only a brief exposure and was not readily reversible. Because thymocyte proliferation was not significantly diminished after a 1-hr pulse with ISF, the inhibition detected in the cultures pulsed with ISF for ≥2 hr is most likely due to a prolonged effect of the inhibitor rather than to residual unbound ISF.

Inhibition of Spleen Cell Proliferation by ISF. We next tested whether ISF could inhibit the proliferation of B cell mitogen-stimulated spleen cells. ISF markedly inhibited LPS (B cell mitogen)-induced spleen cell proliferation. Maximal inhibition was observed with 625 units of ISF, 50% inhibition occuring with approximately 35 units of ISF. Significant inhibitory activity was still observed with 0.6 unit of ISF. However, in contrast to the ability of ISF to completely inhibit the proliferation of thymus-derived lymphocytes, the inhibition of LPS-induced spleen cell proliferation never exceeded 70%.

Inhibition of an *in Vitro* Spleen Cell Antibody Response by ISF. When *nude* mouse spleen cells were incubated with SRBC plus culture supernatant from Con A-stimulated spleen cells and subsequently challenged 5 days later with SRBC, there was a marked increase in the number of PFC per culture relative to control cultures initially incubated without SRBC and Con A-induced culture supernatant (4). However, when *nude* mouse spleen cells were incubated with SRBC and Con A supernatant in the presence of ISF for 5 days, the PFC response was almost completely suppressed (Fig. 2). Fifty percent inhibition of the anti-SRBC PFC response was obtained with approximately 4 units of ISF. ISF was ineffective at less than approximately 0.2 unit.

Inhibition of Alloantigen-Specific T Cell-Mediated Cytotoxicity by ISF. The effect of ISF on the anti-SRBC PFC response might be mediated via an inhibitory action on B cells as well as on T cells. To evaluate if ISF could affect a specific peripheral T cell function, we tested whether ISF could inhibit the activation of alloantigen-specific cytotoxic T cells. As shown in Table 2, ISF inhibited, in a concentration-dependent manner,

Table 1. Inhibition of thymocyte proliferation after treatment of cells with ISF

Time of incubation with ISF, hr	[^{3}H]TdR incorporation by thymocytes, cpm	% inhibition of [^{3}H]TdR incorporation
No ISF	43,809 ± 2,199	—
1	39,866 ± 3,971	9
2	32,966 ± 4,272	25
4	17,049 ± 761	61
24	5,170 ± 1,423	88
72	553 ± 104	99

Thymocytes were incubated with PHA plus LAF (IL 1). ISF (125 units/ml) was present for either 1, 2, 4, 24, or 72 hr prior to washing three times to remove unbound material. Washed cells were incubated in fresh medium containing PHA plus LAF (IL 1) for the remainder of the 72-hr assay period. ± indicates SEM.

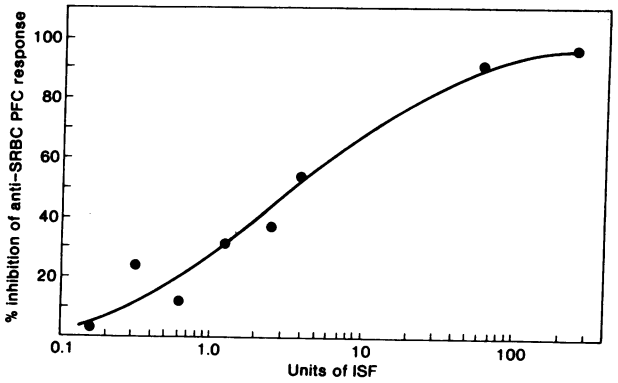

FIG. 2. Inhibition of the *in vitro* spleen cell anti-SRBC PFC response by ISF. *nude* mouse spleen cells (1×10^6) were incubated with SRBC in Mishell and Dutton medium containing 1% normal mouse serum, 50 μM 2-mercaptoethanol, 0.05 M α-methyl mannoside, and a 1:4 dilution of a Con A-stimulated spleen cell culture supernatant in the presence or absence of varying concentrations of ISF. After 5 days the number of PFC were enumerated by using a hemolytic PFC assay on slides (4). In the absence of ISF, there were approximately 350 PFC per culture.

the generation of alloantigen-specific cytotoxic T cells. This effect was observed with relatively pure responder T cell populations as well as with unfractionated responder cells. ISF appeared to have a slightly more potent effect on the cytotoxic response than on the PFC response (Fig. 2); 50% inhibition of cytotoxic T cell activation occurred with approximately 0.4 unit of ISF (Table 2) compared to 4 units of ISF for inhibition of the PFC response (Table 3). It should be noted that ISF had no effect on the cytotoxic reaction itself. Incubation of activated cytotoxic T cells with target cells in the presence of ISF did not block the killing effect of the T cells.

Nature of the Immunosuppressive Component of ISF and Mechanism of Action. In all of the preceding experiments, we used a Bio-Gel P-60 pool (M_r 8000–12,000) which contained not only ISF activity but also SGF activity (1). It was therefore necessary to determine whether ISF was chemically related to SGF, because these two activities were associated with factors that shared a number of biochemical properties. Initially, we tested whether an agent such as dithiothreitol that destroys SGF activity would also inactivate the ISF activity in the Bio-Gel P-60 pool. When fractions from the Bio-Gel P-60 pool were incubated in the presence of 65 mM dithiothreitol for 1 hr at 25°C, SGF and ISF activities were markedly depressed, both activities being decreased by >95%. Thus, the biologically active form of the proteins responsible for the SGF and ISF activities shared a common requirement for intact disulfide

Table 2. Inhibition of generation of alloantigen-specific cytotoxicity

Units of ISF	% specific cytotoxicity	% inhibition of cytotoxicity
0	32.6 ± 1.8	—
0.25	38.1 ± 4.7	0
0.5	12.4 ± 2.1	62
2.0	6.5 ± 1.3	80
7.8	1.0 ± 0.0	97
31	1.6 ± 0.5	95

C57Bl/6J responder spleen cells (2×10^5 cells) were incubated with 2×10^5 BDF_1 stimulator spleen cells in the presence or absence of varying concentrations of ISF. After 5 days the generation of cytotoxic T cells was evaluated by using ^{51}Cr-labeled P815 target cells (1×10^4). ± indicates SEM.

Table 3. Relative sensitivity of *in vitro* immune responses to ISF

Assay	Units of ISF required for 50% inhibition
Thymocyte proliferation	
PHA plus LAF (IL 1)	1
Con A	56
LPS-induced spleen cell proliferation	35
In vitro anti-SRBC PFC	4
Alloantigen-specific cytotoxicity	0.4

linkages. However, the two factors could be resolved on Bio-Gel P-60 by analysis of each of the fractions comprising the 8000–12,000 M_r pool used in the experiments described above. As shown in Fig. 3, ISF exhibited two M_rs, 8000 and 12,000, whereas SGF eluted with molecules of M_rs 10,000 and 6000.

The *in vitro* immunosuppression by ISF was not dependent on a cytotoxic effect. When spleen cells were incubated with ISF for at least 24 hr and then assessed for the number of viable cells by trypan blue exclusion, no decrease in viable cell number was detected relative to control cultures.

In the absence of a cytotoxic effect of ISF, we next examined whether ISF was actually generating suppressor cells, which would then diminish the various *in vitro* immune responses. Spleen cells were incubated with ISF for 24 hr, washed three times to remove the ISF, and then incubated in varying numbers with fresh spleen cells supplemented with LPS. The ISF-treated cells did not have any effect on the proliferation of the LPS-stimulated cells, thus ruling out a direct and independent effect of ISF on the generation of suppressor cells.

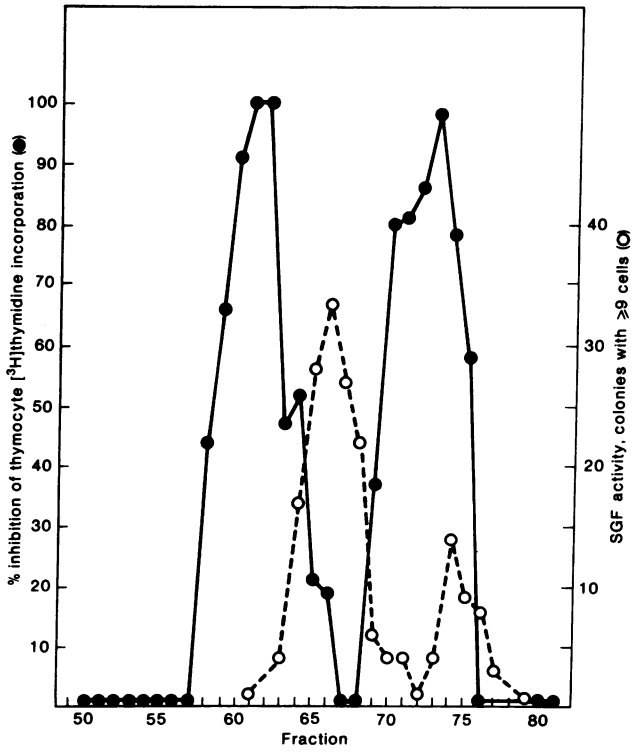

FIG. 3. Resolution of ISF and SGF on Bio-Gel P-60. Each column fraction was dialyzed against 1% acetic acid, lyophilized, and reconstituted for SGF (○) and ISF (●) assays. SGF and ISF activities were determined with a 1:10 dilution of the reconstituted fractions. ISF activity was assayed on thymocytes stimulated with PHA plus LAF (IL 1).

DISCUSSION

The results presented in this report demonstrate that MuSV-transformed mouse fibroblasts cultured in serum-free medium produce potent ISF that inhibit the *in vitro* initiation of T cell-dependent and T cell-independent immune responses. With the exception of Con A-induced thymocyte proliferation, the T cell-dependent responses [PHA plus LAF (IL 1)-induced thymocyte proliferation, anti-SRBC antibody synthesis, and alloantigen-specific T cell-mediated cytotoxicity] were markedly more sensitive to inhibition by ISF than was the T cell-independent process of LPS-stimulated splenic B cell proliferation (Table 3). The basis for the difference in ISF sensitivity between Con A- and PHA plus LAF (IL 1)-induced thymocyte proliferation is unclear. However, the evaluation of thymocyte subpopulations in terms of their sensitivity to ISF may provide a means to determine the cellular basis for the observed difference.

The primary cell target(s) and mechanism(s) by which ISF induces immunosuppression are not known. However, we have found no evidence for a direct cytotoxic effect of ISF or for the presence of interferon activity in the ISF preparations used in these studies. In addition, ISF did not appear to activate independently suppressor cell function. We have not, however, precluded the possibility that ISF may activate suppressor cells, possibly by a mechanism requiring a second signal such as antigen. Furthermore, we also cannot rule out the possibility that ISF may inhibit lymphocyte activation and proliferation by primarily affecting accessory cell function. In this regard, Pike and Snyderman (6) have found that murine tumors produce immunosuppressive factors *in vivo* that inhibit the accumulation of macrophages at sites of inflammation. Sephadex G-50 chromatography of the tumor cell extracts revealed that the molecular weight of the active material was in the same range (6000–12,000) as we have observed for the MuSV-transformed-cell-derived ISF, thus raising the possibility that these two activities are associated with similar polypeptides.

Recently, Cianciolo *et al.* (7) reported that the accumulation of macrophages at inflammatory sites could also be blocked by low molecular weight products of both murine leukemia virus and Rauscher leukemia virus, suggesting the possibility that the tumor cell-derived macrophage migration inhibitor(s) obtained by these investigators, as well as the ISF described in our studies, may also be of viral origin. With regard to ISF, it has been shown that attenuated MuSV possesses *in vivo* and *in vitro* immunosuppressive activity (8). It is quite possible, therefore, that specific viral structural components may serve a second function—*in vivo* immunosuppression at sites of infectivity and propagation. However, our results showing comparable levels of ISF production by producer and nonproducer MuSV-transformed cells demonstrate that ISF production by tumor cells is not dependent on production of virus particles.

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