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Transforming growth factor-beta inhibits the in vitro generation of lymphokine-activated killer cells and cytotoxic T cells

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Summary. The effect(s) of purified transforming growth factor-beta (TGF-beta) and platelet-derived growth factor (PDGF) on the induction and function of lymphokine-activated killer (LAK) cells and cytotoxic T lymphocytes (CTL) was examined. The addition of TGF-beta, but not PDGF, to cultures containing fresh C57BL/6 mouse splenocytes or human peripheral blood lymphocytes plus recombinant interleukin-2 markedly inhibited the development of mouse and human LAK cell activity (measured after 3 days for cytotoxicity against cultured or fresh tumor targets in 4-h ^{51}Cr release assays). The addition of TGF-beta, but not PDGF, to a one-way, C57BL/6 anti-DBA/2, mixed lymphocyte reaction effectively blocked the generation of allospecific CTL as well. However, TGF-beta did not inhibit the effector function of LAK cells or of allospecific CTL when added directly to the short-term cytolytic assay. A second form of homodimeric TGF-beta, type 2, was also found to be suppressive on the development of murine LAK cells and allospecific CTL. Collectively, these data demonstrate that the peptide TGF-beta is a potent inhibitor of LAK cell and CTL generation in vitro.

Introduction

The peptide transforming growth factor-beta (TGF-beta) was first described by its capacity to phenotypically transform rat fibroblasts [25, 26, 40], and is a homodimer with a molecular mass of 25×10^3 daltons and is synthesized by a variety of cell types. It has numerous regulatory actions in both normal (immune, connective tissue, and epithelial cells) and neoplastic cells. These include effects on cell proliferation and differentiation, and regulation of cellular responses to other signals (e.g., other peptide growth factors or hormones). Two forms of homodimeric TGF-beta have been described [5, 36]; type 1 is more abundant than type 2 in the sources examined [25]. The two peptides are indistinguishable on sodium dodecyl sulfate-polyacrylamide gels and have apparently identical biological activities. The TGF-beta type 1 and type 2 differ, however, in sequence and in the binding patterns of each to cellular receptors [25].

Recently, TGF-beta has been shown to strongly inhibit the mitogenic actions of interleukin-2 (IL-2) on T lymphocytes [13]. It also suppresses natural killer (NK) cell function and responsiveness to interferon-alpha as well as the ability of IL-2 to up-regulate its own receptor in activated T cells [29]. The synthesis of both IL-2 and TGF-beta are induced in activated T cells [13]. Further, B cells have also been shown to synthesize and secrete TGF-beta and to express receptors for TGF-beta. The addition of exogenous TGF-beta to cultures of stimulated B cells inhibits subsequent proliferation and immunoglobulin secretion [14].

We have shown that the systemic administration of IL-2 alone at high-dose or combined at lower doses with lymphokine-activated killer (LAK) cells mediates the regression of established pulmonary and hepatic metastases from a variety of tumors in both mice [17, 20, 32] and man [18, 31]. Animal studies have also evaluated the adoptive transfer of IL-2-dependent, expanded proliferative/helper and cytotoxic specific immune lymphocytes in graft rejection [16, 34] and in tumor therapy [4, 8, 10, 37]. The potent inhibitory activity of TGF-beta on cellular responses, particularly those involving IL-2, raises the possibility that this peptide may adversely affect LAK cells and specific cytotoxic T lymphocytes (CTL) which are dependent on IL-2 for both their generation and expansion. In the current study, we have evaluated the in vitro effect(s) of purified TGF-beta on the generation and effector function of LAK cells and CTL.

Materials and methods

Mice. C57BL/6 mice (B6), DBA/2, and BALB/c mice were 10 weeks or older when used in these experiments. They were obtained from The Jackson Laboratory, Bar Harbor, Me. Mice were caged in groups of six or fewer, fed NIH laboratory chow and given water ad libitum.

Tumors. Mouse: The MCA-102 is a 3-methylcholanthrene-induced fibrosarcoma of B6 origin and is resistant to lysis by NK cells in vitro. It was maintained in vivo in syngeneic mice by serial s.c. transplantation of cryopreserved tumor [21, 23]. Single cell suspensions were prepared as described elsewhere [21]. MCA-102 was used in the 3rd to 7th transplantation generation. The P815 mastocytoma, syngeneic to DBA/2 mice, and the EL-4 lymphoma, syngeneic to B6 mice, were maintained in vivo as ascites tumors. The YAC-1 lymphoma of A/Sn origin [38] was a gift from Dr. John Wunderlich (NIH) and was maintained in continuous in vitro culture.

Human: Single cell supensions of sarcoma 466 (Tu 466) were obtained by incubating the freshly resected tu-

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mor with an enzyme mixture as described previously [28], and were then cryopreserved until use. The NK-sensitive K562 erythroleukemia was maintained in continuous in vitro culture.

Mouse splenocytes. Spleens were removed aseptically and crushed with the hub of a syringe in complete medium (CM). The cell suspension was passed through 100-gauge nylon mesh (Nitex; Lawshe Instrument Co., Rockville, Md.), and the erythrocytes were lysed by hypotonic shock with buffered ammonium chloride solution at room temperature for 2 min. The cells were then centrifuged and washed twice in CM.

Human blood mononuclear cells. Heparinized venous blood was obtained from normal volunteers, and fractionation of peripheral blood lymphocytes (PBL) on LSM gradients (Litton Bionetics, Kensington, Md.) was performed as described previously [41].

Complete media. Mouse: RPMI 1640 (Biofluids, Rockville, Md.) contained 50 μg/ml gentamicin, 0.1 m*M* nonessential amino acids, 1 μm sodium pyruvate (all from M. A. Bioproducts, Walkersville, Md.) 5×10^{-5} *M* 2-mercaptoethanol (Aldrich Chemical Co., Inc., Milwaukee, Wis.), 100 μg/ml streptomycin, 100 units/ml penicillin, 0.03% fresh glutamine (all from NIH Media unit), 0.5 μg/ml Amphotericin B (Fungizone; Flow Laboratories, McLean, Va.), and 10% heat-inactivated fetal calf serum (Biofluids).

Human: RPMI 1640 contained 2% human AB serum, 2 m*M* glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin, and 50 μg/ml gentamicin (all from M. A. Bioproducts) with 250 ng/ml Amphotericin B (Aldrich).

Growth factors and interleukin-2. The TGF-beta was purified to homogeneity from human platelets as described elsewhere [2]. The material isolated from human platelets was exclusively type 1, and was used in all experiments unless noted otherwise. Two successive steps of gel filtration and HPLC yielded a protein of 25,000 daltons that was homogenous by analysis on sodium dodecyl sulfate-polyacrylamide gels and was quantitated by amino acid analysis. The bioactivity of TGF-beta was assessed as described previously [2]. The TGF-beta preparations contained 2 μg purified peptide in 40 μl of 4 m*M* HCl with 1 mg/ml bovine serum albumin; further dilutions were made in CM. Porcine platelet-derived growth factor (PDGF) was obtained from Bethesda Research Laboratories (Bethesda, Md.). It was diluted from a 1 mg/ml stock in 4 m*M* HCl containing 2 mg/ml bovine serum albumin. This PDGF was biologically active in a mitogenesis assay using either human or mouse cells. The TGF-beta type 2 was purified from porcine platelets and was obtained from R & D Systems, Minneapolis, Minn. Human recombinant IL-2 (rIL-2) was kindly supplied by the Cetus Corporation, Emeryville, Calif. The biological and biochemical activities of rIL-2 have been described previously [30]. Purified material had a specific activity of 4 to 8×10^6 units (U)/mg as measured in a standard bioassay [7]. The endotoxin level in the purified preparation was < 0.1 mg/10^6 U rIL-2 as measured by a standard limulus assay.

Generation of LAK cells. Mouse: 1 ml of CM containing 4×10^6 viable B6 splenocytes was added to each well of a 24-well Costar tissue culture plate (no. 3524; Costar, Cambridge, Mass.), and 1 ml of CM containing varying amounts of rIL-2 was also added to each well. The cultures were incubated for 3 days at 37° C with 5% CO_2. Alternatively, B6 splenocytes were activated in 175-cm^2 (750 ml) flasks (Falcon Labware, Oxnard, Calif.) as described previously [20].

Human: Fresh human PBL were cultured for 3 days in 25-cm^2 Costar flasks (no. 3050) containing 10 ml of CM with 10^6 cells/ml and varying amounts of rIL-2.

In vitro sensitization to allogeneic cells. Conditions for allogeneic in vitro sensitization have been described in detail elsewhere [34]. Briefly, 4×10^6 responder B6 splenocytes and 1×10^6 irradiated (3,000 rad) DBA/2 spleen stimulator cells were cultured in 2.0 ml of CM in individual wells of a 24-well tissue culture plate (Costar) for 4 days at 37° C at 5% CO_2. The resultant cells, allospecific CTL, were harvested and tested for cytotoxicity to P815 targets in a 4-h ^{51}Cr release assay. EL-4 targets served as the specificity control.

Cytotoxicity assay. A standard 4-h ^{51}Cr release assay was used to measure cytotoxicity against tumor cells (5×10^3/well) as described previously for the mouse [35] and human [41].

Results

Effect(s) of TGF-beta and PDGF on murine and human LAK cells

B6 splenocytes were cultured for a 3-day period in rIL-2 (1,000 U/ml) with and without TGF-beta or PDGF. The resulting LAK cells were then tested for cytolytic activity in a 4-h ^{51}Cr release assay against NK-sensitive, cultured YAC-1 and NK-resistant, fresh MCA-102 sarcoma. As shown in Fig. 1, the addition of 0.5 ng/ml or 5 ng/ml TGF-beta markedly suppressed the development of LAK cell activity. In contrast, the addition of 5 ng/ml PDGF, another dimeric peptide derived from platelets with a molecular weight and disulfide pattern comparable to TGF-beta, had little, if any, effect on LAK cell generation. Table 1 shows a similar suppressive effect of TGF-beta (5 ng/ml) on the development of LAK cells from cultures of B6 splenocytes incubated with increasing concentrations of rIL-2. The TGF-beta suppressive activity was observed in three additional experiments. Both TGF-beta (doses of 0.05, 0.5, and 5.0 ng/ml) and PDGF (dose of 5.0 mg/ml) did not affect the yields of total recoverable cells at 3 days of culture (not shown).

We next tested whether or not TGF-beta directly inhibited LAK effector function. Figure 2 shows that the direct addition of TGF-beta (at increasing concentrations) to the effector:target mixture during the 4-h incubation did not, even at the highest peptide dose of 5 ng/ml, suppress LAK cytotoxicity against the MCA-102 sarcoma.

Human PBL from a normal donor were used to generate LAK cells and the effect of TGF-beta on their development was similarly evaluated. The LAK cells from 3-day cultures of PBL with increasing concentrations of rIL-2 were tested against NK-sensitive, cultured K562 and NK-resistant, fresh human sarcoma 466 (Tu 466). As can be seen in Table 2, TGF-beta, but not PDGF, blocked the generation of human LAK cells as well.

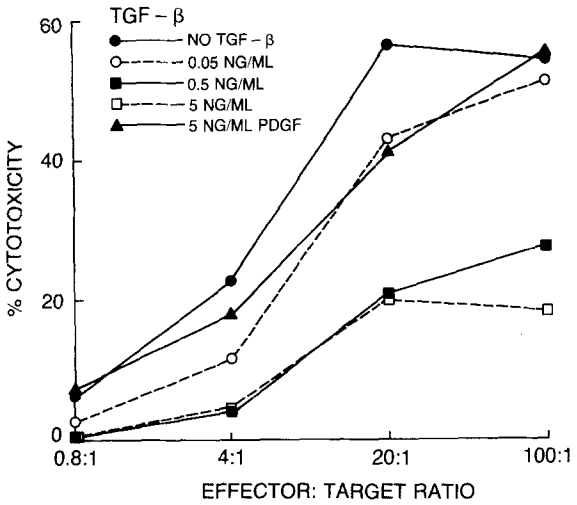

Fig. 1. Transforming growth factor-beta (TGF-beta) inhibits generation of murine lymphokine-activated killer (LAK) cells. Normal B6 splenocytes were cultured for 3 days in recombinant interleukin-2 (rIL-2) (1,000 U/ml) with or without TGF-beta. The LAK cells were then tested against fresh MCA-102 sarcoma cells in a 4-h ^{51}Cr release assay. TGF-beta (0.5 and 5.0 ng/ml), but not platelet-derived growth factor (PDGF), had a suppressive effect

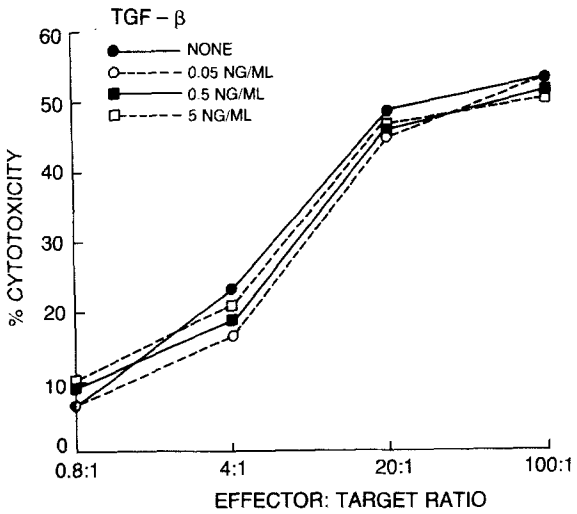

Fig. 2. TGF-beta does not suppress LAK effector function in vitro. The cytotoxicity of 3-day B6 LAK cells was tested against fresh MCA-102 sarcoma cells in a 4-h ^{51}Cr release assay. Addition of purified TGF-beta directly to the effector-target mixture had no effect

Table 1. TGF-beta inhibits the development of murine LAK cells

TGF-beta (5 ng/ml)	rIL-2 (U/ml)	L.U.[a]	
		MCA-102	YAC-1
–	0	<1	<1
+	0	<1	<1
–	10	2	290
+	10	<1	<1
–	100	50	217
+	100	<1	8
–	1,000	103	1,040
+	1,000	<1	66

[a] LAK cells were generated by culturing normal B6 splenocytes in increasing concentrations of rIL-2 for 3 days. L.U. = lytic units/10^7 cells at 30% specific lysis

Table 2. TGF-beta inhibits the development of human LAK cells

TGF-beta (ng/ml)	rIL-2 (U/ml) L.U.[a]			
	0	10	100	1,000
	Tu 466			
0	<1	3	18	23
0.05	<1	<1	22	29
0.5	<1	<1	3	<1
5	<1	<1	2	4
PDGF (5 ng/ml)	<1	4	26	31
	K562			
0	71	286	801	606
0.05	50	192	476	526
0.5	2	19	83	128
5	9	26	42	95
PDGF (5 ng/ml)	22	192	909	714

[a] Normal donor peripheral blood lymphocytes were cultured in increasing concentrations of rIL-2 with or without TGF-beta or PDGF for 3 days. L.U. = lytic units/10^7 cells at 20% specific lysis

Thus, these data showed that TGF-beta was a potent inhibitor in vitro of LAK cell development from rIL-2-activated murine and human precursors but not of LAK effector function.

Effect(s) of TGF-beta and PDGF on the allogeneic mixed lymphocyte reaction

B6 splenocytes were incubated with irradiated DBA/2 splenocytes for 4 days in the presence or absence of TGF-beta or PDGF. The resulting cells (allospecific CTL) were then tested for cytolytic activity against the P815 target in a 4-h ^{51}Cr release assay. Figure 3 shows that TGF-beta at doses of 0.5 ng/ml and 5 ng/ml significantly inhibited the generation of allogeneic CTL whereas PDGF at 5 ng/ml had no suppressive activity. The TGF-beta also reduced the cell yields obtained from the 4-day mixed lymphocyte culture. In two combined experiments cell yields were 106.2%±6.3%, 64.5%±2.1%, and 55.4%±7.1% of control PDGF (5 ng/ml) cultures for 0.05 ng/ml, 0.5 ng/ml, and 5.0 ng/ml TGF-beta, respectively. In contrast, the addition of TGF-beta directly to the short-term cytotoxicity assay did not affect the lytic function of these CTL at the effector level (Fig. 4). Thus, TGF-beta, but not PDGF, effectively blocked the generation of allospecific CTL in vitro.

TGF-beta type 2 inhibits the development of LAK cells and allospecific CTL

The effect(s) of a second form of homodimeric TGF-beta on the development of mouse LAK cells and on the allogeneic mixed lymphocyte reaction is shown in Table 3. The addition of TGF-beta type 2, particularly at the concentra-

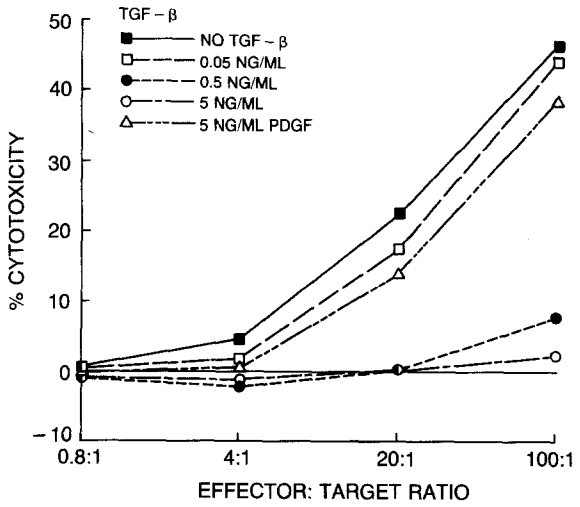

Fig. 3. TGF-beta inhibits the generation of allogeneic cytotoxic T lymphocytes (CTL) in a mixed lymphocyte culture. B6 anti-DBA/2 CTL were generated in the presence or absence of TGF-beta or PDGF as described in *Materials and methods.* After 4 days, effector cells were tested for cytolytic activity against fresh P815 in a 4-h ^{51}Cr release assay. TGF-beta, but not PDGF, blocked the generation of specific CTL. Control, syngeneic EL-4 target cells were not lysed in any group (not shown)

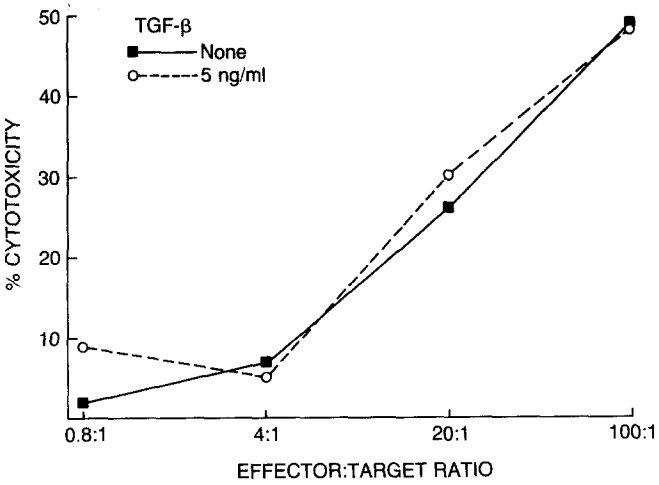

Fig. 4. TGF-beta does not inhibit allospecific CTL effector function in vitro. The cytotoxicity of cells from a 4-day C57BL/6 anti-DBA mixed lymphocyte reaction was tested against fresh P815 tumor targets in a 4-h ^{51}Cr release assay. Addition of purified TGF-beta (5 ng/ml) directly to the effector-target mixture had no effect

tion of 5 ng/ml, resulted in a profound inhibition of both LAK and allospecific CTL activity, with a >95% suppression of cytolytic function when compared to the control culture. Thus, the two forms of TGF-beta, type 1 and type 2, appeared to be equally inhibitory.

Discussion

TGF-beta has been shown to have inhibitory effects on T, B, and NK cells [13, 14, 29]. In the current study, we have shown that purified TGF-beta is a potent inhibitor of the in vitro development of both LAK cells and allospecific CTL. This peptide, however, had no effect on the cytolytic activity of either effector cell, once generated. Al-

Table 3. TGF-beta type 2 inhibits the development of murine LAK cells and allospecific CTL

TGF-beta (ng/ml)	L.U.[a]	
	LAK vs MCA-102	CTL vs P815
0	454	42
	TGF-beta	
0.05	154	30
0.5	15	<1
5	40	2
	TGF-beta type 2	
0.05	185	29
0.5	111	16
5	13	<1

[a] LAK cells were generated by culturing normal B6 splenocytes in 1,000 U/ml rIL-2 for 3 days. B6 anti-DBA/2 CTL were generated as described in Fig. 3 legend. L.U. = lytic units/10^7 cells at 30% specific lysis

though TGF-beta suppressed the development of LAK cytotoxicity, it did not affect the total cell recoveries of human and murine lymphocytes from these 3-day LAK cultures. In contrast, the cell recovery following a 4-day mixed lymphocyte reaction was substantially lowered by TGF-beta. It is conceivable that the TGF-beta effects on differentiation of LAK cells and CTL are more potent than those on mitosis. High concentrations of rIL-2 were used (1,000 U/ml) to generate LAK cells in comparison to the endogenous IL-2 levels produced during a mixed lymphocyte reaction; thus, the rIL-2 concentration was probably sufficient to overcome any antimitotic effect of TGF-beta in the former culture. In agreement, Kehrl et al. have demonstrated that increases in the concentration of IL-2 can overcome the suppressive activity of TGF-beta on IL-2-dependent proliferation of concanavalin A-stimulated lymphocytes [13] and B cells [14]. In the latter study, however, the inhibition of Ig secretion induced by TGF-beta could not be overcome by higher concentrations of the lymphokine, demonstrating that blocking of B cell differentiation by TGF-beta was not due solely to its effects on mitosis.

The mechanism(s) of TGF-beta inhibition of LAK cell generation remains to be determined. It is unclear whether or not TGF-beta blocks generation of LAK cells and allospecific CTL from precursors via inhibition of IL-2 binding, by down-regulation of IL-2 receptors (IL-2R) or by inhibiting a postreceptor binding differentiation step. TGF-beta has been shown to inhibit IL-2-induced up-regulation of the IL-2R (Tac^+ alpha chain) and transferrin receptor [13]. Further, TGF-beta binding to its own receptor may exert its suppressive effect by down-regulating receptors other than IL-2R. Rook et al. [29] have shown that TGF-beta markedly blunts the boosting of NK cytolysis by interferon-alpha, possibly by down-regulation of interferon-alpha receptors. In this same study, NK boosting by a 20-h incubation in rIL-2 was unaffected by TGF-beta. Thus, the effect(s) of TGF-beta on IL-2-augmented NK cells appears to be different from its effect(s) on LAK cells.

Alternatively, the mechanism(s) of TGF-beta inhibitory activities might follow the ligand-receptor binding event. For example, TGF-beta has been reported to inhibit

the mitogenic effects of other growth factors on various target cells in vitro, including epidermal growth factor, insulin, thrombin, and fibroblast growth factor [25]. The blocking effects of TGF-beta occur without interference with the generation of many of the early intracellular signals resulting from activation of receptors on target cells by binding mitogenic peptides; these include phospholipid turnover and activation of protein kinase C, Na^+/H^+ antiport activity, expression of *myc* and *fos* in the nucleus and ornithine decarboxylase activity (Chambard and Pouyssegur, personal communication). Thus, in these studies it is likely that TGF-beta blocks initiation of DNA synthesis at some step distal to these early signalling events.

Although certain virally or chemically induced tumors produce TGF-beta [1, 27], it is unknown what effect, if any, the peptide has in vivo on antitumor responses, particularly on those mediated by IL-2 and by transferred LAK cells. The amount of TGF-beta endogenously produced during normal physiologic immune or inflammatory responses is not yet known. However, it is conceivable that local concentrations of TGF-beta could attain levels that suppress IL-2 responses in vivo. Tumor-derived factors with potent immunosuppressive activities have been described [9, 11, 12, 24]. Tumor fluids of certain chemically induced murine sarcomas have been shown in vivo to both enhance tumor growth and block tumor inhibition by immune lymphocytes as measured in Winn-type neutralization assays [11]. In addition, Putnam and Roth [24] have identified a low molecular weight glycoprotein from a murine melanoma, which suppresses a variety of immunologic responses in vitro and in vivo. The relation of these tumor-derived factors to TGF-beta has not been determined. Immunosuppressive factors which inhibit mitogen- and antigen-induced proliferation of normal lymphocytes have been described in tumor cyst fluid of patients with glioblastoma [15] and in patient serum before but not after tumor removal [3]. Moreover, tumor-infiltrating lymphocytes harvested postsurgery from glioblastoma tissue show no response to T cell mitogens in vitro [19]. The T cell suppressive factor produced by human glioblastoma cells inhibits T cell proliferation in vitro; it particularly interferes with IL-2-dependent T cell growth. This factor has been purified and is TGF-beta type 2 [5, 42]. Whether or not tumor-derived TGF-beta is responsible for the failure in some instances both to generate and to expand tumor-infiltrating lymphocytes with antitumor reactivity in IL-2 [33, 39], or of the ability of high-dose IL-2 administration to reduce established metastases [22] remains to be determined. However, since large amounts of TGF-beta can be obtained from porcine platelets [5] and since cDNA clones have been obtained for this peptide [6], it is feasible to test the in vivo activities of systemically administered natural or recombinant TGF-beta in tumor-bearing animals receiving immunotherapy.

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