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Immunology

A highly immunogenic tumor transfected with a murine transforming growth factor type β_1 cDNA escapes immune surveillance

(tumor progression/immunosuppression)

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ABSTRACT **A highly immunogenic C3H-derived UV-induced tumor was cotransfected with a murine transforming growth factor type β_1 (TGF-β_1) cDNA and a neomycin-resistance gene. Stable clones were isolated and used *in vitro* and *in vivo* to determine the effects of endogenously produced TGF-β on cytolytic T-lymphocyte (CTL) responses. Tumor cells producing TGF-β, though retaining expression for class I major histocompatibility complex molecules and the tumor-specific antigen, did not stimulate primary CTL responses *in vitro* and were not effective *in vivo* for directly stimulating primary CTL or in priming for CTL responses. Furthermore, TGF-β-producing tumors grew progressively in transiently immunosuppressed mice without losing the tumor antigen; thus, TGF-β produced by tumors may promote escape from immune surveillance.**

Transforming growth factor type β (TGF-β) is secreted in an inactive or latent form; activated TGF-β powerfully suppresses T- and B-lymphocyte activation/differentiation but has little effect on lymphocyte proliferation once immune responses are initiated and does not suppress effector functions of activated lymphocytes such as cytolysis (1–5). The evidence for immunoregulation by TGF-β is based almost entirely on studies using TGF-β activated exogenously, usually by acidification at pH 1–2 for 1 hr. To determine whether TGF-β secreted in the latent form can be immunoregulatory, a TGF-β_1 cDNA driven by the simian virus 40 early promoter was transfected into a highly immunogenic, C3H-derived, UV-induced fibrosarcoma, designated 1591. The 1591 tumor is highly immunogenic and expresses three major histocompatibility complex class I antigens not found in the genome of the *k* haplotype (6–8). The tumors elicit a primary cytolytic T-lymphocyte (CTL) response *in vitro*, and tumor rejection requires $CD8^+$ T cells, which recognize the K^{216} gene product (9). Very rarely, the tumor grows progressively in normal mice but these "progressor" variants invariably have lost class I genes. A single class I molecule, K^{216}, is sufficient for the regressor phenotype of the tumor, and transfection of K^{216} into progressor variants causes the tumors to be rejected (10). Thus, the 1591 system provides a model for defining a specific CTL response against the target antigen K^{216} and also permits that analysis of tumor growth based on the expression of K^{216}. The transfected 1591 tumor cells secrete TGF-β in the latent form, continue to express the original surface antigens, and are as susceptible to lysis by specific cytolytic T cells as parental tumor cells. Thus, the transfected tumors provide a suitable model for testing the effects of tumor-produced TGF-β on host immune responses to tumor antigens.

MATERIALS AND METHODS

Mice. C3H female and C3H female nude mice were purchased from Frederick Cancer Research Institute (Frederick, MD) and were used between 7 and 10 weeks of age.

Reagents and Antibodies. McCoy's 5A medium and minimal essential medium (MEM) (GIBCO), porcine TGF-β_1 (pTGF-β_1) and ^{125}I-labeled pTGF-β_1 (^{125}I-pTGF-β_1) (R&D Systems, Minneapolis), low toxic rabbit complement (Accurate Chemicals, Westbury, NY), and a fluorescein isothiocyanate-labeled rabbit anti-mouse IgG (Sigma) were purchased. Recombinant murine interferon γ (IFN-γ) was purchased from Genentech. Purified hamster anti-murine CD3 monoclonal antibody (mAb), 145-2C11, was generously provided by Jeffrey Bluestone (The University of Chicago). A mouse mAb, CP28, that reacts with K^{216} and an antibody that reacts with the 1591 lineage, 154, were previously described (11, 12). An anti-K^k antibody and anti-Lyt-2 antibody were culture supernatants of 11-4.1 and 3.155 hybridomas (American Type Culture Collection). The murine TGF-β cDNA was kindly provided by Rik Derynck (Genentech), and the pKCR3 was provided by Lynn Matrisian (Vanderbilt University).

Tumors and Cell Lines. 1591, 1316, and 6134 are C3H-derived UV-induced fibrosarcomas. The tumor cells were grown in MEM supplemented with 10% (vol/vol) heat-inactivated fetal calf serum in 7.5% CO_2. For transplantation, 1-mm^3 fragments of solid tumors grown in C3H nude mice were implanted subcutaneously with a 13-gauge trocar in normal, irradiated, or anti-CD3 mAb-treated C3H recipients. Tumors that grew progressively were readapted to growth *in vitro* and were analyzed directly for the expression of surface antigens and TGF-β production. AKR-2B (clone 84A) grown in McCoy's 5A medium supplemented with 5% heat-inactivated fetal calf serum in 5% CO_2 is an indicator cell line in assays for TGF-β (13).

RNA Analysis by Northern Hybridization. RNA collected from subconfluent cells growing in a monolayer was extracted by the method of Schwab *et al.* (14). Oligo(dT)-selected polyadenylylated [poly(A)$^+$] RNA was separated by electrophoresis; Northern blotting was performed as described (15). The TGF-β_1 probe is a 974-base-pair *Sma* I insert of a mouse TGF-β_1 cDNA clone (16). The inserts were

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Abbreviations: CTL, cytolytic T lymphocyte; TGF-β, transforming growth factor type β; pTGF-β_1, porcine TGF-β_1; mAb, monoclonal antibody; BSA, bovine serum albumin; IFN-γ, interferon γ; MLTC, mixed-lymphocyte tumor cell.

labeled by a random primer method (17). Hybridization and posthybridization washes were performed as described (18).

Construction of pSVTGF-β_1 for Transfection. The pSVTGF-β_1 construct was prepared by introduction of the full-length TGF-β_1 mouse cDNA (16) into the *Eco*RI site of pKCR3 (19). The construct (see Fig. 1*A*) has the simian virus 40 early promoter and origin of replication, portions of rabbit β-globin exons 2 and 3 (which are not translated), a splice site, and a rabbit β-globin polyadenylylation signal.

Transfection and Selection of TGF-β-Producing Clones. The pSVTGF-β_1 and pZipNeo (which confers neomycin resistance; ref. 20) constructs were cotransfected by the calcium phosphate precipitation method (21) and selected in G418 (500 μg/ml; Gibco). G418-resistant colonies were assayed for TGF-β by using the radioreceptor assay.

Culture Supernatants. Tumor cells were grown to confluency in MEM containing 10% heat-inactivated fetal calf serum in 150-cm^3 tissue culture flasks (Corning). The attached tumor cells were washed three times in MEM alone and cultured for an additional 24 hr in MEM alone. Aliquots of culture supernatants were acidified with HCl to pH 2 for 1 hr. The samples were neutralized with NaOH.

Radioreceptor Assay for TGF-β. Culture supernatants were assayed for competitive binding activity by using the indicator cell line AKR-2B (13). Briefly, cells resuspended in McCoy's 5A medium with 5% heat-inactivated fetal calf serum were seeded in 6-well plates at a density of 2×10^5 cells per well and were incubated for 24 hr. The cells were washed three times in phosphate-buffered saline (PBS) containing 0.1% (wt/vol) bovine serum albumin (BSA) and incubated for 1 hr in binding buffer (128 mM NaCl/5 mM KCl/5 mM $MgSO_4$/1.2 mM $CaCl_2$/50 mM HEPES at pH 7.5 containing BSA at 2 mg/ml). A sample of culture supernatant and ^{125}I-pTGF-β_1 (0.25 ng) were added to each well. After 2 hr at room temperature, the wells were washed five times in PBS containing 0.1% BSA and then incubated with 1 ml of PBS containing 1.0% Triton X-100. After 15 min, the buffer was collected and counted in a γ counter. Total binding was measured in the absence of pTGF-β or culture supernatants. Nonspecific binding was determined by using a 150-fold excess of pTGF-β_1 and was always <30%.

Mixed-Lymphocyte Tumor Cell (MLTC) Cultures. Primary MLTC cultures were set up in 16- × 125-mm tissue culture tubes (Falcon), using 5×10^6 NH_4Cl-treated C3H spleen cells and 2×10^5 tumor cells. The tumor cells were either mitomycin C-treated or irradiated (10,000 R; 1 R = 0.258 mC/kg). Cells were harvested on day 6. For the secondary MLTC, C3H mice were primed with 10^7 tumor cells intraperitoneally; 12 days later, spleen cells were harvested and restimulated *in vitro* by using 8×10^6 C3H responder cells and 4×10^4 mitomycin C-treated tumor cells. The cultures were harvested 6 days later.

Sponge Matrix Allograft. Polyurethane sponges (0.5 cm^3) were treated as previously described (22) and implanted subcutaneously in the dorsum of C3H mice. Sponges were injected with 5×10^6 tumor cells in 400 μl of medium or with the same amount of medium alone. Sponges were removed 9–15 days later, and recovered cells were either treated with complement (final concentration 1/10) or with anti-Lyt-2 antibody (final concentration 1/10) and complement for 1 hr, washed, and assayed for cytolytic activity.

Flow Cytometric Analysis. Tumor cells (10^6) were cultured in 75-cm^3 tissue culture flasks (Corning) and treated with or without IFN-γ (30 units per ml of medium) for 48 hr. Harvested cells were washed in PBS containing 1% BSA and incubated with 25 μl of CP28, 11-4-1, or 154 antibodies for 30 min. The cells were washed three times in PBS containing 1% BSA and incubated with 25 μl of fluorescein isothiocyanate-labeled rabbit anti-mouse IgG (final concentration 1/10). The cells were washed twice, fixed with 1% paraformaldehyde, and analyzed in an EPICS 752 flow cytometer (Coulter).

Cytolytic Activity. Effector cell lysis of target cells was measured in a 4-hr ^{51}Cr release assay by using labeling procedures, culture conditions, and calculations of specific lysis as described (23).

RESULTS

Characterization of TGF-β_1 Transfectants. A murine TGF-β_1 minigene driven by the simian virus 40 early promoter (Fig. 1*A*) was introduced into 1591 tumor cells along with a plasmid carrying the gene for neomycin resistance. Drug-resistant

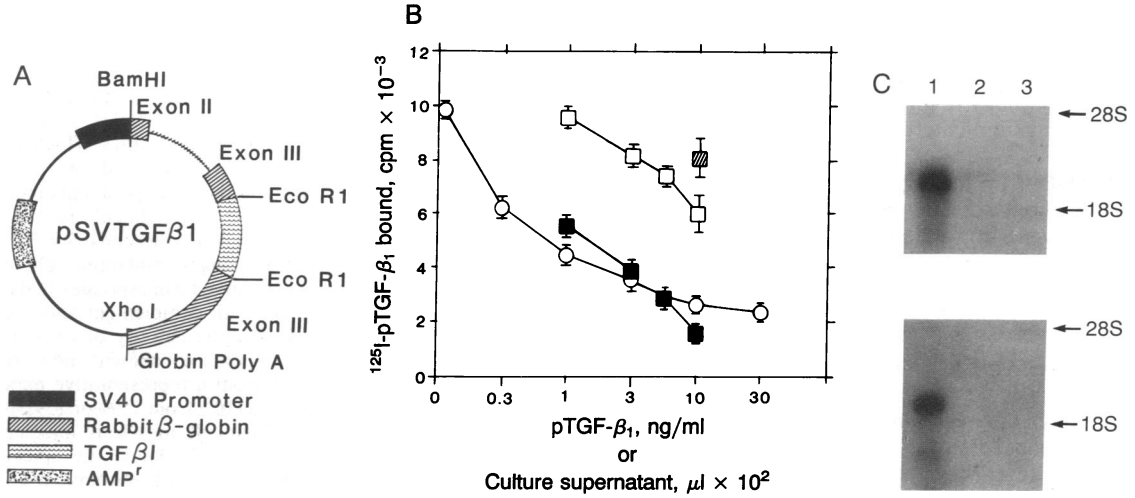

FIG. 1. Characterization of TGF-β-producing clones of 1591 tumor cells. (*A*) Genetic map of the pSVTGF-β_1 construct. The mouse TGF-β cDNA was ligated into the *Eco*RI site of pKCR3, which carries the ampicillin-resistance gene (AMP^r). (*B*) Acid-activated culture supernatant of 10^5 1591-N (□) or 1591-N-TGF-1 (■) cells per ml was assayed for competing activity for ^{125}I-pTGF-β_1. Unactivated samples were assayed and, because there were no differences between the control and the TGF-β-producing clone, only one point for the two is reported (▨). Competing activity of purified TGF-β_1 (○) is also shown. (*C*) Northern blot analysis of poly(A)$^+$ RNA (2 μg per lane) isolated from 1591-N-TGF-1 (lane 1), 1591-N (lane 2), and 1591 (lane 3). (*Upper*) The blot was probed with a ^{32}P-labeled mouse TGF-β_1 cDNA and exposed to Kodak XAR film for 9 hr at −70°C. The probe was stripped from the blot, and the blot was exposed to radiographic film for 72 hr, revealing no residual signal. (*Lower*) The blot was reprobed with the *Eco*RI/*Xho* I 527-base-pair insert from the pSVTGF-β_1 vector, which contains a portion of the rabbit β-globin sequence, and exposed to XAR film for 5 hr at −70°C. 28S and 18S ribosomal RNA sizes are given for reference.

colonies isolated from independent transfections were screened for TGF-β production. The higher producers of TGF-β were arbitrarily designated 1591-N-TGF-1, 1591-N-TGF-2 etc; clones that did not secrete TGF-β but were neomycin resistant were designated 1591-N. An assay for the amount of TGF-β released per ml during the 24 hr of culture of 10^5 1591-N or 1591-N-TGF-1 cells is shown in Fig. 1*B*. The nonacidified supernatant medium from 1591-N-TGF-1 contained the equivalent of <0.3 ng/ml, but after acidification it contained more than 10.0 ng of TGF-β per ml. In contrast, the supernatant from 1591-N cultures (or 1591; data not shown) grown identically had <0.3 ng of TGF-β per ml before or after acidification.

The increase in TGF-β production was the result of an increase in TGF-β_1 transcription as shown by Northern blot analysis (Fig. 1*C*). TGF-β_1 mRNA was present in 1591-N-TGF-1 but was not detected in 1591 or 1591-N. Because the rabbit β-globin exons in the pSVTGF-β_1 construct should be transcribed but not translated, we could rehybridize the blot with a β-globin probe to demonstrate that the TGF-β signal was the result of the transgene and not the result of an increase in endogenous TGF-β_1 transcription. Indeed, we found a strong signal for rabbit β-globin only in 1591-N-TGF-1 (Fig. 1*C*). These data demonstrate that the increase in TGF-β production by the 1591-N-TGF-1 clone was the result of increased transcription of the transfected pSVTGF-β_1. Also, we found that the 1591 tumor cells do not express TGF-β_2 or TGF-β_3 (data not shown), indicating that all TGF-β competing activity present in either 1591, 1591-N, or 1591-N-TGF cells was due to TGF-β_1.

Both the parental and TGF-β-producing clones attach to plastic and proliferate as monolayers. The 1591 tumor cells grow in a disorganized array, whereas the TGF-β-producing clones have a distinct morphology characterized by growth in organized swirls. 1591 cells grown for 3 days or more in medium containing activated pTGF-β_1 at 3 ng/ml (but not at 1 ng/ml) acquire this same morphology. Most interestingly, 1591 cells grown for 3 days in culture supernatant obtained from 1591-N-TGF-1 cells (containing $\approx$0.3 ng of active TGF-β per ml and 10 ng of TGF-β in the inactive form per ml) acquired the same morphology as the 1591-N-TGF-1 clone. (The same culture supernatant diluted 1:5 in fresh medium did not cause this change.) When the medium from 1591 cells, which were altered by growth in the presence of TGF-β in either the active or the latent form, was replaced by fresh medium, the morphology reverted to the original form in 2–3 days. These findings indicate that 1591 tumor cells activate latent TGF-β.

Failure of TGF-β-Producing Cells to Stimulate Primary CTL Responses. To assess the effect of TGF-β production, transfected cells were compared with parental 1591 cells for eliciting primary CTL responses *in vitro*. 1591-N cells stimulated a low but measurable CTL response, whereas 1591-N-TGF tumor cells stimulated no cytolytic responses *in vitro* (Fig. 2*A*).

Polyurethane sponges implanted subcutaneously and injected with 1591 cells accumulate cytolytic cells that, when removed 9–12 days after implantation, are cytolytic for 1591 and usually "nonspecifically" lyse unrelated tumor target cells at higher effector-to-target cell ratios. By 15 days, however, responses are higher and are almost exclusively for 1591; treatment of cells recovered at this time with anti-Lyt-2 antibody and complement eliminates cytotoxicity. In a series of experiments, sponges implanted in C3H mice were injected with 1591-N or 1591-N-TGF-1 cells. Cells recovered 9, 12, and 15 days later were assayed against 1591 and 1316 (another C3H UV-derived fibrosarcoma). 1591-N-TGF-1 failed to stimulate either nonspecific or 1591-specific responses; in contrast, 1591-N stimulated low specific and nonspecific responses at 9 and 12 days (data not shown) and specific $CD8^+$ anti-1591 CTL at day 15 (Fig. 2*B*).

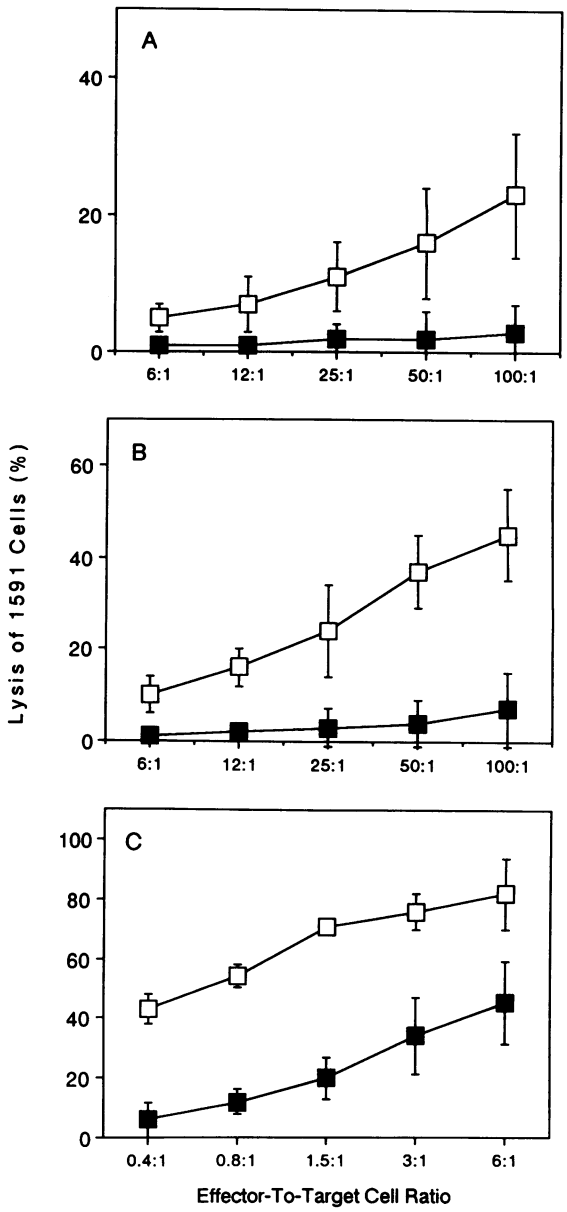

FIG. 2. TGF-β-producing cells fail to stimulate primary CTL responses. (*A*) Irradiated 1591 or 1591-N (□) or four different 1591-N-TGF (■) cell lines were cultured with C3H whole spleen cells for 6 days. The recovered cells were assayed for cytolytic activity. Data were pooled from three separate experiments including a total of five cultures for 1591 or 1591-N and eight cultures for 1591-N-TGF cells. (*B*) 1591-N (□) or 1591-N-TGF-1 (■) cells (5×10^6) in 400 μl of medium were injected into sponges implanted subcutaneously in C3H mice. Infiltrating cells removed from sponges 15 days later were treated with complement or with complement and anti-Lyt-2 antibodies (which eliminated cytolytic activity of cells obtained from sponges injected with 1591-N; data not shown) and were assayed for cytolytic activity. Data are from a representative experiment with two mice per group. (*C*) Cells (10^7) from 1591 or 1591-N (□) or from three different 1591-N-TGF (■) cell lines were injected intraperitoneally into C3H mice. Two weeks later, the spleen cells were harvested and stimulated *in vitro* with mitomycin C-treated 1591 cells. Cytolytic activity was assayed 6 days later. Data was pooled from two separate experiments with two mice for 1591 or 1591-N and four mice for 1591-N-TGF cells.

The relative failure of 1591-N-TGF-1 to stimulate CTL *in vivo* was confirmed in another way. Normal mice injected intraperitoneally with 10^7 1591 cells have no or very low

primary CTL responses, but spleen cells from mice primed 2 weeks previously with 1591 or 1591-N cells stimulated *in vitro* with mitomycin-treated 1591 cells give high specific CTL responses to 1591; in contrast, spleen cells from mice primed with 1591-N-TGF-1 cells gave very low CTL responses (Fig. 2*C*).

1591 TGF-β-Producing Cells Continue to Express a CTL-Defined Target Antigen. Activated TGF-β does not suppress proliferation of lymphocytes in ongoing immune responses; e.g., we found that spleen cells obtained from mice injected with 1591 cells 12 days previously continued to proliferate vigorously in the presence of 5 ng of activated pTGFβ1 per ml of medium and mitomycin-treated 1591 cells. If TGF-β secretion was the essential difference between 1591 and 1591-N-TGF, then TGF-β-producing cells should stimulate already sensitized spleen cells and be susceptible targets for CTL. As shown in Fig. 3*A*, 1591-N-TGF cells were as effective as 1591 or 1591-N cells in stimulating 1591-primed spleen cells; furthermore, TGF-β producing clones were susceptible to lysis by anti-1591 CTL (Fig. 3*B*). These findings were consistent with the observation that K^{216} and K^k molecules were equally expressed on 1591, 1591-N, and 1591-N-TGF cells as determined by staining and flow cytometric analysis (data not shown).

Absence of Selection for Variants and Progressive Growth of 1591-N-TGF-1. Cells from 1591, 1591-N, and 1591-N-TGF clones grew in C3H nude mice but did not grow in normal C3H mice. The 1591 tumor grows progressively in 30–40% of irradiated (400 R) mice, but progressively growing tumors invariably lost expression of the K^{216} antigen (G.T.A., unpublished results), an observation consistent with previous studies showing selection of tumor variants in mice partially immunosuppressed with UV radiation (24). Furthermore, selection for antigen loss variants in partially immunosuppressed mice is the result of specific anti-1591 CTL (24, 25). To determine the phenotype of 1591 and 1591-N-TGF-1 tumors reisolated from mildly irradiated mice, we confirmed that reisolated 1591 tumors were K^{216}-negative; in contrast, 1591-N-TGF-1 tumor reisolates continued to express K^{216} (data not shown). These findings suggested a failure of selection for antigen loss variants in the TGF-β-producing tumors, though under these experimental conditions no differences in growth in 1591-N and 1591-N-TGF-β_1 tumors were observed.

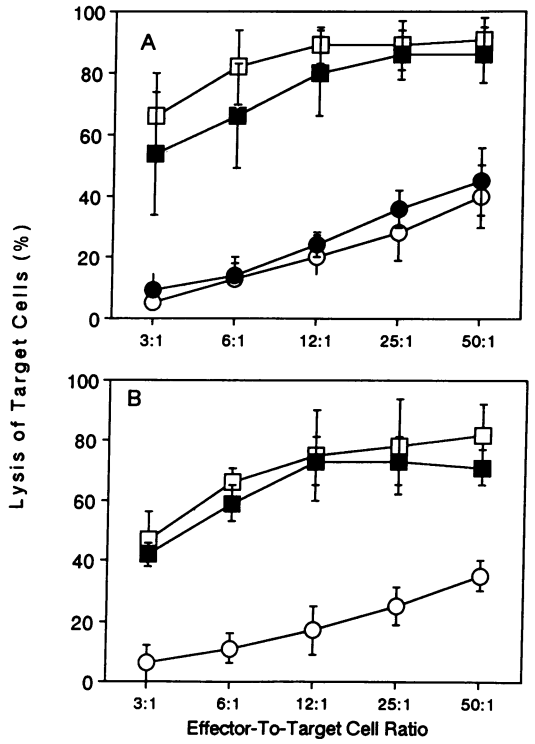

FIG. 3. TGF-β-producing clones retain the CTL-defined target antigen. (*A*) C3H mice were injected intraperitoneally with 10^7 1591 cells; 12 days later, the spleen cells were either restimulated *in vitro* with mitomycin C-treated 1591 or 1591-N (□) or with 1591-N-TGF (■) cells and 6 days later were assayed for cytolytic activity. Cytolytic activity for each culture was assayed against 1591 (□, ■) and 1316 (○, ●), a C3H-derived, UV-induced fibrosarcoma. Data were pooled from three separate experiments to include a total of eight separate cultures for 1591 or 1591-N and nine separate cultures for 1591-N-TGF cells. (*B*) 1591 or 1591-N cells (□), four different 1591-N-TGF cell lines (■), or 1316 tumor cells (○) were labeled with ^{51}Cr and used as targets for anti-1591-RE CTL generated by restimulating 1591-primed spleen cells with mitomycin C-treated 1591 cells. Data pooled from three separate experiments include a total of five different assays for 1591 or 1591-N cells, eight assays for 1591-N-TGF cells, and three assays for 1316.

These observations were extended by using another method of immunosuppression. High doses of anti-CD3 mAb in the mouse or in humans are immunosuppressive (26, 27); 400 μg of purified 145-2C11 mAb (hamster anti-mouse CD3) injected into mice partially depletes T cells from the periphery and prolonges skin allograft survival (26). To compare the growth and specific anti-tumor immunity against 1591-N and 1591-N-TGF-1, mice were transiently suppressed with 400 μg of 145-2C11 and anti-CD3 mAb. Tumor fragments of 1591-N and 1591-N-TGF-1 first grown in C3H nude mice were injected into normal immunocompetent C3H mice. Ten mice were recipients of 1591-N fragments and 10 were recipients of 1591-N-TGF-1 fragments; 12 hr after transplantation, 5 mice in each group were injected with saline and 5 were injected with the anti-CD3 mAb. Four weeks later, the tumors were measured and isolated in culture, spleen cells from the mice were used in MLTC cultures with mitomycin C-treated 1591 cells, and the cultures were incubated for 6 days and assayed for CTL against 1591 and an unrelated C3H UV-derived tumor.

For saline-injected mice, zero out of five transplanted with 1591-N and zero out of five transplanted with 1591-N-TGF-1 had tumors. Three out of four mice‡ transplanted with 1591-N and treated with anti-CD3 mAb had small detectable tumors, but all three tumors had lost expression of K^{216}, while expressing normal levels of K^k and 154. Furthermore, spleen cells from 1591-N tumor-bearing mice cultured for 6 days with 1591 tumor cells had high and specific CTL activity against 1591. In contrast, four out of four mice‡ that were recipients of 1591-N-TGF-1 tumors and were treated with anti-CD3 mAb had large tumors (mean, 16 cm^3); all tumor reisolates were K^{216}, K^k, and 154 positive. Furthermore, spleen cells from these mice cultured *in vitro* for 6 days with 1591 cells were not cytolytic for 1591 (Table 1). Thus, the continued growth of 1591-N-TGF-1, while retaining the tumor target antigen, suggests a major role for TGF-β in the escape from immune surveillance.

DISCUSSION

A possible role for TGF-β has been suggested for the local immune suppression of pregnancy (28) and in severe systemic immune suppression induced by staphylococcal cell wall (29). Though lymphocytes have receptors for active TGF-β and active TGF-β prevents interleukin 2-driven proliferation of T cells (1), TGF-β is secreted in an inactive or latent form that does not bind to receptors or suppress primary lymphocyte responses. Plasmin activates the small latent TGF-β

‡Two of the mice transplanted with 1591-N and one mouse transplanted with 1591-N-TGF-1 and treated with 400 μg of anti-CD3 mAb died within 1 week; these mice were not included.

Table 1. 1591-N-TGF-1 cells grow progressively in anti-CD3-treated mice; the tumor induces no or very low specific anti-tumor CTL and retains the tumor target antigen

No. mice	1591 tumor*	Tumors at 4 weeks† Size, cm^3	Tumors at 4 weeks† K^{216}, %	% lysis‡ 1591 cells 50:1	1591 cells 12:1	1591 cells 3:1	6134 cells 50:1	6134 cells 12:1	6134 cells 3:1
1	−N	2.4	40	86	46	15	7	2	0
2	−N	0.74	0	94	82	36	18	7	4
3	−N	0.03	0	100	79	34	11	2	0
1	−TGF-1	30.00	100	19	3	4	7	2	0
2	−TGF-1	19.60	100	26	6	2	14	4	0
3	−TGF-1	15.00	100	16	3	2	7	4	2
4	−TGF-1	2.20	100	9	4	1	9	2	1

*1591-N or 1591-N-TGF-1 cells were first grown in C3H nude mice; solid tumor fragments were transplanted into C3H mice. Twelve hours after transplantation, mice were injected intraperitoneally with saline or with 400 μg of anti-CD3 mAb. No tumors grew in mice receiving saline.

†Four weeks after transplantation, tumors were measured and readapted to growth *in vitro*. The reisolated tumors were stained for K^{216}, K^k, and 154 (an antibody that reacts with the 1591 lineage). Surface expression was also determined in the presence of IFN-γ at 30 units/ml; such treatment increased expression of K^{216}, K^k, and 154. Under these conditions the K^{216}-negative tumors remain negative. All tumors expressed similar levels of K^k and 154 (data not shown). Data represent percentage of cells positive for the K^{216} antigen in the presence of IFN-γ.

‡Spleen cells from tumor-bearing mice were harvested and cultured with mitomycin-treated 1591 cells for 6 days; the recovered cells were assayed for cytolytic activity. Values are percent lysis of target cells (1591 or 6134 cells) at the indicated effector-to-target ratios, which were measured for serial 2-fold dilutions but for convenience are reported only for 4-fold dilutions.

complex *in vitro* (30), and many other proteolytic agents are potential activators. Quite possibly, the activation of TGF-β and the inhibition of responses may be the result of complex interactions involving not only TGF-β-producing cells and lymphocytes but also other cells. Activated macrophages and monocytes are potential sources of both TGF-β and activators, and TGF-β may indirectly suppress immune responses by inhibiting tumor necrosis factor α production (2) and causing decreased expression of class II major histocompatibility complex molecules (31).

The 1591 tumor transfected to produce TGF-β_1 failed to stimulate CTL *in vitro* and *in vivo*. The lack of response was not the result of decreased expression of endogenous class I genes or due to loss of the tumor antigen, indicating that the failure to induce primary responses was the result of TGF-β-mediated effects on the responder cells. Almost all TGF-β recovered from cultured 1591-N-TGF cells was in the latent form, but presumably the 1591 tumor (and the 1591-N and 1591-N-TGF tumors as well) activates TGF-β, since the tumor reversibly acquires the same altered morphology whether cocultured with latent TGF-β or with activated TGF-β. The putative activator may be membrane bound, and possibly the failure to detect active TGF-β in culture supernatant of 1591-N-TGF cells occurs because almost all activated TGF-β is receptor bound.

Because TGF-β is so powerfully immunosuppressive, tumors producing/activating TGF-β might be expected to escape immune surveillance. 1591-N-TGF cells, however, did not grow progressively in normal recipients, presumably because a fraction of CTL specific for K^{216} antigen escaped initial suppression by TGF-β and expanded to become sufficient to cause tumor rejection. The tumor, nevertheless, did not grow progressively in transiently immunosuppressed mice and escaped selection for antigen loss variants, indicating that TGF-β production by a tumor may be important in determining whether the tumor disappears or grows progressively.

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