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Interleukin-2 Production by Tumor Cells Bypasses T Helper Function in the Generation of an Antitumor Response

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Summary

A poorly immunogenic murine colon cancer was used to investigate mechanisms of antitumor immunity. Injection of tumor cells engineered by gene transfection to secrete IL-2 stimulated an MHC class I–restricted cytolytic T lymphocyte (CTL) response against the parental tumor. The tumor cells secreting IL-2 produced an antitumor response in vivo, even in the absence of $CD4^+$ T cells. Animals immunized with the engineered cells were protected against subsequent challenge with the parental tumor cell line. Similar findings were demonstrated for other tumor types. Thus, provision of a helper lymphokine in a paracrine fashion induced a tumor-specific immune response involving activation of endogenous CTLs and other immune effector cells. These findings demonstrate that the failure of an effective antitumor immune response may be primarily due to a helper arm deficiency of the immune system rather than a paucity of tumor-specific cytotoxic effector cells. Furthermore, they outline a novel strategy for augmenting tumor immunity.

Introduction

One of the most critical questions in cancer immunology is why the immune system fails to eliminate tumors arising de novo. In the 1970s, Hewitt articulated the notion that most tumors did not express any tumor-specific or neoantigens and thus could not be recognized as "foreign" by the immune system. Indeed, virtually no tumor cell surface antigens recognized by antibodies were found to be tumor specific, and furthermore, most spontaneous murine tumors were considered "poorly immunogenic" as defined by their failure to be eliminated when transferred into syngeneic hosts (Hewitt et al., 1976). While these same tumors could be rendered "immunogenic" by mutagenesis (Van Pel and Boon, 1982), the mechanisms of this conversion remain unclear.

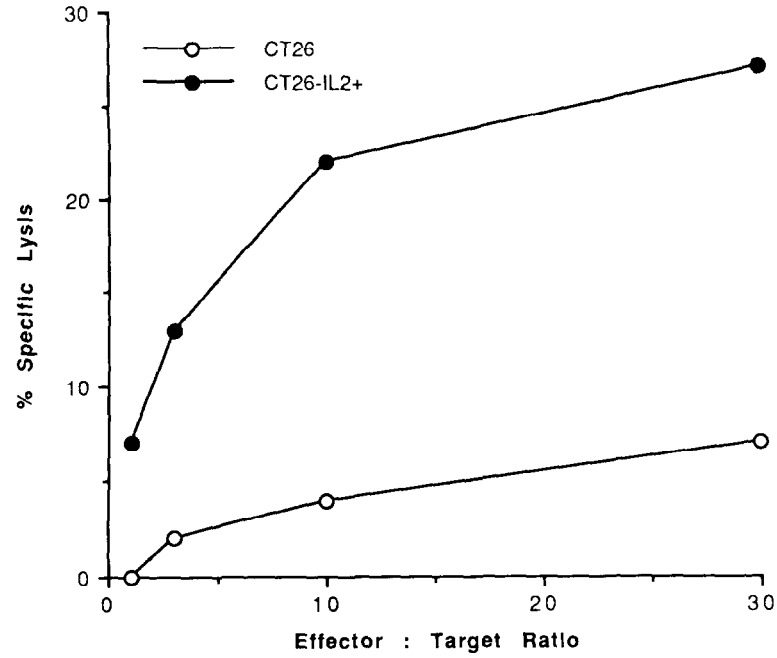
Recent insights into the nature of antigen recognition by cytotoxic T lymphocytes (CTLs) warrant a reevaluation of the neoantigen concept. Classic CTLs recognize peptides derived from endogenously synthesized proteins and presented on the cell surface together with major histocompatibility (MHC) class I molecules (Townsend et al., 1986; Maryanski et al., 1986; Moore et al., 1988). Consequently, the universe of potential neoantigens includes any primary amino acid sequence in any cellular proteins, either membrane bound or intracellular. The large number of genetic alterations found in advanced cancers could give rise to peptide "neoepitopes" capable of being recognized with MHC class I molecules by CTLs (Lurquin et al., 1989). In addition to MHC-restricted CTLs other cytotoxic cell types, such as natural killer (NK) and lymphokine-activated killer (LAK) cells, appear to demonstrate specificity for tumor cells relative to nontumor cells, although the nature of their ligand remains undefined (Herberman, 1982; Grimm et al., 1982). Hence, it is possible that the immune system fails to eliminate tumors not because neoantigens are absent, but rather because the response to these neoantigens is inadequate.

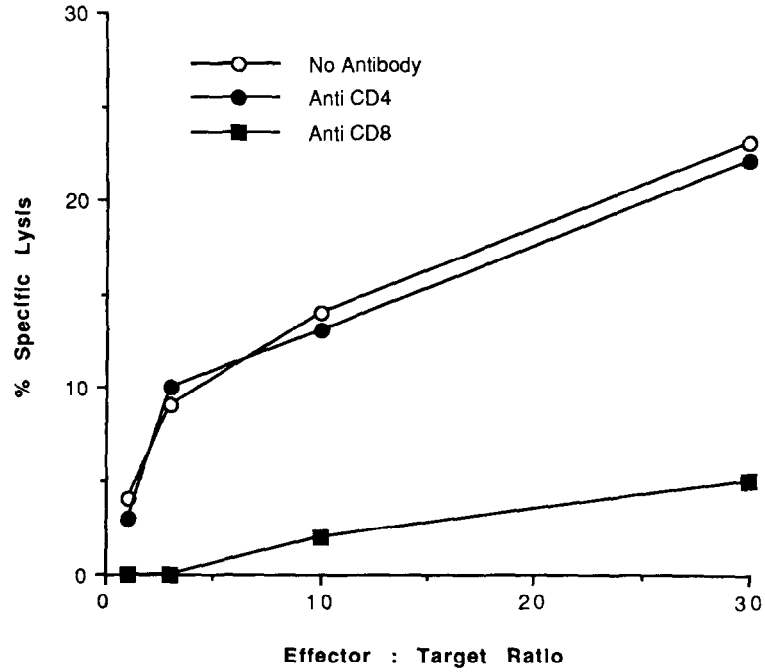
We consider here the hypothesis that this failure to respond to tumor neoantigens is due, at least in part, to a failure of T cell help. That most CTL responses require T helper (Th) cells has long been appreciated (Cantor and Boyse, 1975; Zinkernagel et al., 1978; vonBoehmer and Haas, 1979; Keene and Forman, 1982). The molecular basis for Th function is the local secretion of lymphokines such as interleukin-2 (IL-2) that act upon CTLs whose T cell receptors have first been engaged by the appropriate antigen–MHC complex (reviewed in Moller, 1980). The cytotoxic potential of NK and LAK cells is also enhanced by IL-2 (Grimm et al., 1982; Phillips and Lanier, 1986; Ortaldo et al., 1986).

To experimentally test the possibility of failure of adequate T cell help, we designed a system in which IL-2 is provided locally by the tumor cell target, thus bypassing Th in vivo.

Results

The N-nitroso-N-methylurethane–induced murine colon tumor line CT26 chosen for study is poorly immunogenic; a small number of cells (1×10^3–1×10^4) injected into syngeneic (BALB/c) mice cause a lethal tumor and do not induce detectable tumor-specific CTLs (Fearon et al., 1988). CT26 cells were transfected with a bovine papilloma virus (BPV) vector containing a neomycin-resistance gene and a murine IL-2 cDNA. Transfectants were selected in the neomycin analog G418, and a G418-resistant line ($CT26\text{-}IL\text{-}2^+$) prepared from more than 50 pooled clones of approximately equal size was chosen for further





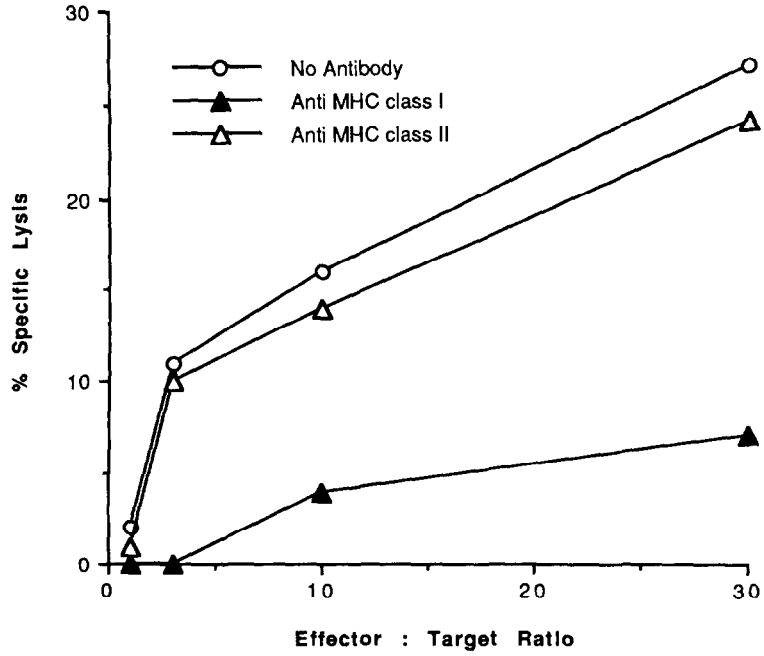


Figure 1. Induction of CTLs after In Vivo Injection of CT26 Cells and IL-2–Transfected CT26-IL-2^+ Cells

Cells (1×10^6) of either the CT26 or CT26-IL-2^+ lines were injected subcutaneously into the left flank of BALB/c mice. After 2 weeks, splenocytes were removed and cultured 5 days with mitomycin C–treated CT26 cells in the presence of IL-2. At the end of culture, live cells were mixed with ^{51}Cr-labeled CT26 targets at different effector to target ratios in a 4 hr ^{51}Cr release assay. (a) CTLs generated from CT26 vs. CT26-IL-2^+ cells. (b) Anti-CD4 and anti-CD8 blocking of CT26 lysis by splenocytes from mice immunized with CT26-IL-2^+ cells. (c) Anti-MHC class I and class II blocking of CT26 lysis by splenocytes from mice immunized with CT26-IL-2^+ cells.

study. To assay for IL-2 production, the CT26-IL-2$^+$ line was plated at 5 × 10^4 cells per well in a 24-well plate, and after 3 days, supernatants (1.5 ml per well) were removed and transferred in serial dilutions to the IL-2–dependent CTLL-2 cell line. They were found to contain 40 U of IL-2 activity (1 U defined as induction of 50% maximal CTLL stimulation in a 24 hr assay), indicating that CT26-IL-2$^+$ cells secreted significant quantities of IL-2. No detectable IL-2 activity was found in supernatants from either the parental CT26 cells or CT26 cells transfected with the BCMG vector that had the IL-2 cDNA insert cut out.

CTL Generation Induced by CT26-IL-2$^+$ Cells

Subcutaneous injection of CT26 cells has been shown previously to elicit little if any detectable systemic CTL activity, even after a secondary in vitro stimulation in the presence of IL-2 (Fearon et al., 1988). However, after subcutaneous injection of the IL-2–producing CT26-IL-2$^+$ cells, significant anti-CT26 CTL activity was detected in the spleen after secondary in vitro stimulation (Figure 1a). The majority of in vitro CTL activity was blocked by antibodies to CD8 and to MHC class I, but not by antibodies to CD4 or to MHC class II (Figures 1b and 1c). This suggested that CT26I-IL-2$^+$ cells indeed activated endogenous, MHC class I–restricted CD8$^+$ CTLs. Virtually no cytolysis was observed against another BALB/c-derived tumor target (SS-5) or the MHC class I$^-$ NK target, YAC-1 (<5% specific lysis at an effector to target ratio of 100:1). These results demonstrate that most of the effector cells induced by CT26-IL-2$^+$ immunization were antigen- and MHC class I–specific and that NK and LAK cells represent a minority of the activity measured in vitro.

In Vivo Immune Response Induced by CT26-IL-2$^+$ Cells

We next sought to determine whether the CTL response elicited by CT26-IL-2$^+$ cells correlated with in vivo immunity. Following even the largest subcutaneous injection of up to 1 × 10^6 CT26-IL-2$^+$ cells, no tumors were seen up to 8 weeks after injection. In contrast, tumors were present in all animals following injection of the parental CT26 cells by 2 weeks (Table 1). Thus, BALB/c mice were capable of rejecting 3–4 orders of magnitude greater numbers of CT26-IL-2$^+$ cells than the parental CT26 cells. To demonstrate that rejection of CT26-IL-2$^+$ cells is due to activation of CT26-specific effector cells by the locally produced IL-2, CT26 cells were transfected with the entire BPV vector sequences alone with the IL-2 insert removed. These transfectants (CT26-neo-IL-2$^-$), which were confirmed not to secrete IL-2 by CTLL functional assay, produced tumors in BALB/c mice at a rate indistinguishable from the nontransfected CT26 cells (Table 1). In addition, we observed that injection of 1 × 10^6 CT26-IL-2$^+$ cells on the left flank did not inhibit the growth of 1 × 10^5 CT26 cells on the opposite flank. Thus, the effect of IL-2 production by CT26-IL-2$^+$ cells appeared initially to stimulate a local, rather than systemic, immune response.

Table 1. Growth of CT26, CT26-IL-2$^+$, and CT26-neo-Il-2$^-$ in BALB/c Mice

Tumor	Injection Dose[a]	# Mice with Tumor/ # Mice Injected
CT26	1 × 10^3 cells	9/10[b]
	1 × 10^4 cells	20/20[c]
	1 × 10^5 cells	25/25[c]
	1 × 10^6 cells	20/20[c]
CT26-IL-2$^+$	1 × 10^4 cells	0/20[e]
	1 × 10^5 cells	0/35[e]
	1 × 10^6 cells	2/55[e]
	1 × 10^7 cells	4/15[e]
CT26-neo-IL-2$^-$	1 × 10^4 cells	10/10[b]
	1 × 10^5 cells	20/20[d]
	1 × 10^6 cells	10/10[d]

[a] BALB/c mice were injected subcutaneously on the left hind leg, and tumor growth was observed.
[b] All mice had tumors by 3 weeks.
[c] All mice had tumors by 2 weeks.
[d] Most mice had tumors by 2 weeks, all had tumors by 3 weeks.
[e] Tumor-free mice were observed from 6–12 weeks.

Given the result that immunization with CT26-IL-2$^+$ cells induced systemic CTLs after two weeks, as measured in vitro, we sought to determine whether this correlated with an in vivo antitumor response against the parental CT26 cells. Indeed, injection of CT26-IL-2$^+$ cells completely protected mice against a challenge with 1 × 10^5 CT26 cells 2 weeks later (Figure 2). This protection

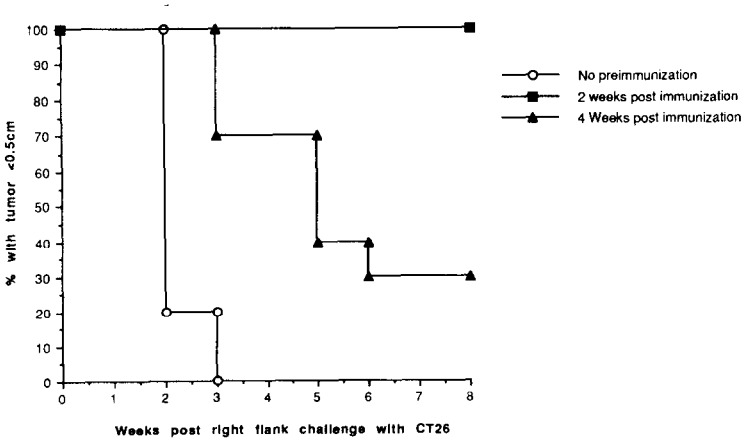


Figure 2. Protective Immunity against CT26 Cells Induced by Injection of CT26-IL-2$^+$ Cells

BALB/c mice were injected subcutaneously in the left flank with 1 × 10^6 CT26-IL-2$^+$ cells followed by subcutaneous challenge in the right flank with 1 × 10^5 CT26 cells either 2 weeks or 4 weeks later. Also shown is CT26 growth without CT26-IL-2$^+$ preimmunization. Tumor growth was assessed every week by palpation and measurement. The pooled results of 20 mice per group are shown. Data is presented as a Kaplan-Meier plot.

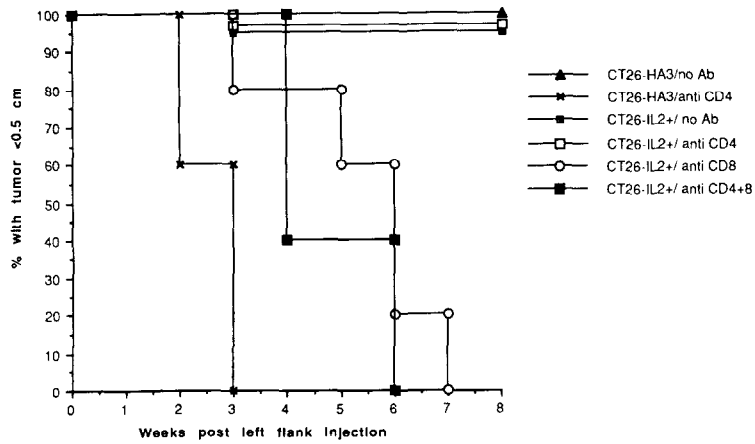


Figure 3. Effects of T Cell Subset Depletion on the In Vivo Response to CT26-IL-2$^+$ and CT26-HA$^+$ Cells

BALB/c mice were depleted in vivo of either CD4$^+$, CD8$^+$, or both CD4$^+$ and CD8$^+$ T cells by intraperitoneal injection of purified anti-CD4 or anti-CD8. They were then injected subcutaneously in the left flank with 1 × 10^6 CT26-neo-IL-2$^+$ cells. Also shown is the growth in normal and CD4-depleted BALB/c mice of a CT26-HA$^+$ line rendered immunogenic by transfection with the influenza hemagglutinin gene. Tumor growth was measured every week and is presented as in Figure 2. Specific depletion of T cell subsets was documented as described in Experimental Procedures.

was not long-lived, as roughly 50% of mice challenged 4 weeks after immunization developed tumors (Figure 2). The protection was tumor specific in that other BALB/c tumors (SS-5, RENCA) grew normally when injected 2 weeks after CT26-IL-2$^+$ immunization (data not shown).

CT26-IL-2$^+$ Cells Bypass CD4$^+$ T Helper Function

Because most Th function is performed by the CD4$^+$8$^-$ subset and most MHC-restricted CTL function is performed by the CD4$^-$8$^+$ subset, we next investigated the effect of selective in vivo depletion of these subsets on the rejection of CT26-IL-2$^+$ cells. Figure 3 shows that mice depleted of CD4$^+$8$^-$ T cells were fully capable of rejecting CT26-IL-2$^+$ cells. Previous studies have shown that CT26 cells could be rendered immunogenic by transfection with a foreign gene such as influenza hemagglutinin (CT26-HA$^+$; Fearon et al., 1988). The "failure of help" hypothesis suggested that the enhanced immune response induced by CT26-HA$^+$ cells might be mediated, at least in part, by CD4$^+$ helper T cells responding to MHC class II–restricted epitopes on the exogenously introduced hemagglutinin gene product. In support of this hypothesis, CT26-HA$^+$ cells failed to be rejected by CD4-depleted mice (Figure 3). Taken together, these results support the concept that CT26-IL-2$^+$ immunization effectively bypasses Th function in the generation of an antitumor CTL response.

CD8$^+$ Cells Are Required In Vivo for Tumor Rejection

Mice depleted of CD4$^-$8$^+$ cells were incapable of rejecting CT26-IL-2$^+$ cells, indicating that this T cell subset is involved in the antitumor response in vivo (Figure 3). It is noteworthy, however, that there was a significant lag in the tumor's growth kinetics relative to the kinetics of CT26 growth in normal BALB/c mice. While this partial response could be due to the action of residual CD4$^-$8$^+$ cells, none were detected in lymph nodes by flow cytometric analysis either at the time of initial tumor injection or during the course of in vivo antibody treatment. A similar delay in tumor growth was seen in the presence of both CD4 and CD8 depletion. It is therefore likely that an IL-2–responsive, CD4$^-$8$^-$ effector cell population was additionally involved in the in vivo antitumor response. The identity of this additional effector population is currently being investigated.

CTL Generation and In Vivo Immunity Induced by IL-2–Producing Melanoma Cells

To assess whether the induction of systemic immunity was a general feature of tumors engineered to secrete IL-2, we transfected a second poorly immunogenic tumor, B16 melanoma, with the BCMG-IL-2 vector. The B16 melanoma is a highly aggressive melanocyte tumor of C57BL/6 origin. After transfection, G418-resistant clones were chosen (B16-IL-2$^+$) that produced quantities of bioactive IL-2 equal to or greater than CT26-IL-2$^+$ cells, when assayed under identical conditions on CTLL cells. Table 2 shows the results of a representative clone. While small numbers of B16 melanoma cells caused tumors in syngeneic C57BL/6 mice, B16-IL-2$^+$ cells were completely rejected. As with the CT26 tumor, greater CTL activity was generated against the parental B16 tumor in mice injected with B16-IL-2$^+$ cells. C57BL/6 mice injected with B16-IL-2$^+$ cells developed protective immunity in vivo against challenge with B16 cells in a dose-dependent manner. The protection was not as great as seen in the CT26 system, as roughly half the mice immunized with 1 × 10^6 B16-IL-2$^+$ cells did eventually develop tumors after challenge with 1 × 10^5 parental B16 cells (Table 2).

Discussion

We have shown that transfection of poorly immunogenic murine tumors with the gene for a murine helper lymphokine, IL-2, elicits a strong systemic antitumor immune response. In addition to the colon tumor and melanoma presented here, similar results have been recently observed with an IL-2–transfected murine CBA-SP1 sarcoma and the rat Dunning prostate carcinoma (data not shown). The fact that similar effects were seen with two tumors of widely differing cellular origins suggests that the principles outlined here may be generalizable to a variety of cancers.

IL-2–transfected CT26 cells and B16 melanoma cells in-

Table 2. In Vivo Growth, Protective Immunity, and CTLs Generated by IL-2–Transfected B16 Melanoma Cells

	# Cells Injected[b]	Tumor Growth[c]	Protection against Challenge with B16[d]	Lysis of B16[e]
B16	1×10^4	4/5	–	–
	1×10^5	5/5	–	16%
	1×10^6	5/5	–	–
B16-IL-2$^+$[a]	1×10^4	0/9	9/9	–
	1×10^5	0/8	6/8	26%
	1×10^6	0/9	5/9	–

[a] B16-IL-2$^+$ transfectant was produced by transfection with pBCMG-neo-mIL-2 as described in Experimental Procedures.
[b] Cells were injected subcutaneously into the left flank as in Table 1.
[c] Tumor growth was measured weekly. Animals were observed for 7 weeks. All animals that developed tumors did so by 2 weeks after injection.
[d] Challenge with the 1×10^5 parental B16 cells was done in the right flank 2 weeks after immunization with B16-IL-2$^+$ cells as in Figure 3. Animals were observed for 7 weeks after challenge. Numbers represent animals that acquired tumors. These tumors were observed to arise between 3 and 5 weeks after challenge. Tumor-free animals were observed for 7 weeks.
[e] CTL assays were performed after a 5 day stimulation with mitomycin C–treated B16 cells as in Figure 1a. Numbers represent % specific lysis after an 8 hr ^{51}Cr release assay at 100:1 effector to target ratio.

jected into syngeneic mice induced greater CTL activity against the parental tumor than nontransfected cells. The tumors were rejected in vivo and induced protective immunity against the parental, nontransfected cells. These effects appeared to be dependent on IL-2 secretion by the tumor rather than other components of the vector. This induction is not immediate, as systemic CTLs were not detected 1 week after injection, and CT26 cells injected at the same time as CT26-IL-2$^+$ cells failed to be rejected.

The abrogation of a significant portion of the antitumor response by anti-CD8 both in vitro and in vivo emphasizes an important role for T cell receptor–mediated, MHC class I–restricted recognition by the effector cells in this system (Tanaka et al., 1984; Hui et al., 1984; Wallich et al., 1985). It is noteworthy that MHC class I expression on B16 melanoma cells is extremely low—roughly 5- to 10-fold lower than on CT26 cells (data not shown). This may account for the apparently lower efficiency of protection against challenge with parental tumor in the B16 than in the CT26 system, although the faster growth rate of B16 melanoma cells may be an additional contributing factor.

The lag in growth of the CT26-IL-2$^+$ cells in the CD8-depleted mice indicates there is an additional CD8$^-$ IL-2–responsive effector cell population active in the antitumor response. Three IL-2–responsive candidates are NK cells, LAK cells, and T cells bearing the γδ T cell receptor (reviewed in Pardoll et al., 1987; Brenner et al., 1988; Raulet, 1989). As most of the cells in these populations are CD4$^-$8$^-$, they would still be present in CD8-depleted mice. Other potential effector cells involved in the antitumor response, based on histologic analysis of regressing tumors, include macrophages and mast cells (data not shown).

Recently, Tepper et al. (1989) demonstrated that murine tumors transfected with the IL-4 gene were rejected, although no evidence for either induction of tumor-reactive CTLs or systemic immunity was presented. While these differences may reflect greater potency of IL-2 as a helper lymphokine for CTLs, it will be of interest to investigate the induction of systemic antitumor CTLs by combined transfection with both IL-2 and IL-4, as these lymphokines demonstrate synergistic activity for CTL activation (Widmer et al., 1987; Trenn et al., 1988).

These studies outline two important principles. First, they emphasize the concept that the absence of an effective in vivo immune response to a tumor may be largely due to a failure of Th cells to effectively provide lymphokines, rather than an absence of CTLs capable of recognizing tumor-specific antigens. Indeed, in vivo and in vitro studies suggest that antigen-specific Th cells might be paralyzed in their capacity to produce lymphokines by their encounter with antigen–MHC complexes on presenting cells that are incapable of providing the necessary costimulatory signals (Jenkins et al., 1987a, 1987b; Lo et al., 1988; Markman et al., 1988). A second principle illustrated by these studies is the effectiveness of "local help" in inducing antitumor CTLs. The design of the system described here is based on the production of helper lymphokine by the same cell presenting antigen to CTLs, i.e., the tumor cell. Because the normal physiology of lymphokines is paracrine (local action) and not endocrine (Sitkovsky and Paul, 1988), the strategy described here may have certain advantages over high-dose systemic lymphokine injections.

Experimental Procedures

Cells

CT26 cells were obtained from M. Brattain (Brattain et al., 1980). The F10 subline of B16 melanoma cells (Fidler, 1975) was obtained from the NIH DCT tumor repository. RENCA is a murine renal cell carcinoma originally described by Murphy and Haushesky (1973). SS-5 is a spontaneous mammary adenocanthoma induced by methylcholanthene in one of our laboratories (P. F.). YAC-1, an MHC$^-$ NK target cell line (Kiessling et al., 1975), was kindly provided by J. Wagner, Johns Hopkins University.

Transfections

DNA was introduced into cells as a coprecipitate with calcium phosphate (Graham and van der Eb, 1973; Wigler et al., 1979). The CT26-IL-2$^+$ cell line was obtained by transfection with 5 μg of the plasmid vector pBCMG-neo-mIL-2, a BPV expression vector containing a murine IL-2 cDNA clone under the transcriptional control of a cytomegalovirus promoter with a rabbit β-globin intron, splice, and poly(A) addition signals; it also contains the Tn5 neomycin-resistance gene (Karasuyama and Melchers, 1988; Karasuyama et al., 1989). Cells were exposed to the precipitate for 14–16 hr, washed once with Hanks' balanced salt solution without Ca^{2+} or Mg^{2+}, refed with Dulbecco's modified Eagle's medium with 10% FCS, and incubated at 37°C. Selection in G418 at 400 μg/ml was begun 48 hr after cells were exposed to precipitate. The CT26-neo-IL-2$^-$ line was produced by transfection of CT26 cells with a plasmid derived from pBCMG-neo-IL-2 by removing the cytomegalovirus early promoter, rabbit β-globin intron, and mIL-2 sequences. The hemagglutinin-expressing CT26-HA$^+$ cell line (described in Fearon et al., 1988) was produced by cotransfection of CT26 with 5 μg of the plasmid vector pBV1-MTHA and pSV2-neo followed by G418 selection. FACS 3, clone 5 was used in the studies here. The B16-IL-2$^+$ transfectant was generated similarly to the CT26-IL-2$^+$ transfectant but was cloned by limiting dilution after G418 selection.

IL-2 Assays

Supernatants of transfected cells were assayed for IL-2 as previously described (Janis et al., 1989) by transferring dilutions to 96-well microtiter plates containing 3000 CTLL-2 cells per well. After 24 hr, [^{3}H]thymidine was added for 12 hr, after which incorporation was assessed with a PHD cell harvester. Units per milliliter IL-2 was calculated as the reciprocal of the supernatant dilution giving half-maximal proliferation of CTLL-2.

CTL Assays

For CTL assays, spleens were removed from BALB/c mice 2 weeks after subcutaneous injection in the left flank of 1×10^6 CT26 or CT26-IL-2$^+$ cells. Mitomycin C treatment of CT26 cells was performed by incubating them in 50 μg/ml mitomycin C for 45 min at 37°C followed by three washes with RPMI–10% FCS. In vitro stimulations were performed in 24-well plates for 5 days with 2×10^5 CT26 stimulators and 6×10^6 splenocyte responders per well plus 20–50 U/ml recombinant murine IL-2. ^{51}Cr release assays were performed by mixing various numbers of effector cells with 5000 ^{51}Cr-labeled targets per well in 96-well V-bottom plates. After 4 hr at 37°C, 100 μl per well was removed and counted in a gamma counter. Percent specific lysis ($[cpm_{exp} - cpm_{min}]/[cpm_{max} - cpm_{min}] \times 100$) is plotted on the y-axis for various effector to target ratios. CTL assays for the B16 melanoma system were performed in a similar fashion, except that incubations with ^{51}Cr-labeled B16 targets were performed for 8 hr. For antibody blocking, a 1:100 dilution of ammonium sulfate–purified preparations of the following antibodies were added to microwells at the start of ^{51}Cr release assays: GK1.5, monoclonal antibody (MAb) to CD4 (Dialynis et al., 1983); 2.43, MAb to CD8.2 (Sarmiento et al., 1980); M5-114, MAb to I-A^d + I-E^d (Battacharya et al., 1981); 28-14-8, MAb to L^d (Ozato et al., 1980); 34-1-2, MAb to K^d + D^d (Ozato et al., 1982). All blocking antibodies were carefully titered such that the concentrations used were 5–10 times the minimal concentration that yielded saturation binding to splenic lymphocytes as assayed by flow cytometric analysis of serially diluted antibody. Prior to use, blocking specificity was assessed as follows: anti-CD4 and anti-MHC class II antibodies inhibited in vitro secondary PPD-proliferative responses by more than 80% and allo-CTL lysis (B6 anti-BALB/c) by more than 5%; anti-CD8 and anti-MHC class I antibodies inhibited secondary PPD-proliferative responses by more than 5% and allo-CTL lysis by more than 80%.

In Vivo Antibody Depletions

In vivo antibody depletions were started 1–2 days prior to injection of the tumor. MAb GK1.5 was used for CD4 depletions and MAb 2.43 was used for CD8 depletions. Ammonium sulfate–purified ascites fluid (titered at >1:2000 by staining of thymocytes on the FACS) was injected intraperitoneally (0.1 ml per mouse) every other day for the first 3 weeks and then once per week afterward. Depletion of T cell subsets was assessed on the day of tumor injections, 3 weeks, and 5 weeks after tumor injection by flow cytometric analysis of lymph node cells stained with 2.43 or GK1.5 followed by fluorescein isothiocyanate-labeled goat antibody to rat IgG (Kirkegaard and Perry). For each time point of analysis, >99% depletion of the appropriate subset was achieved with normal levels of the opposite subset present (in the case of the single depletions).

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