Interleukin 2 Gene Transfer into Tumor Cells Abrogates Tumorigenicity and Induces Protective Immunity

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Summary

To study the effects of localized secretion of cytokines on tumor progression, the gene for human interleukin 2 (IL-2) was introduced via retroviral vectors into CMS-5 cells, a weakly immunogenic mouse fibrosarcoma cell line of BALB/c origin. Secretion of low levels of IL-2 from the tumor cells abrogated their tumorigenicity and induced a long-lasting protective immune response against a challenge with a tumorigenic dose of parental CMS-5 cells. Co-injection of IL-2–producing CMS-5 cells with unmodified tumor cells inhibited tumor formation even when highly tumorigenic doses of CMS-5 cells were used. Cytolytic activity in mice injected with parental CMS-5 cells was transient and was greatly diminished 3 wk after injection, as commonly observed in tumor-bearing animals. However, in mice injected with IL-2–producing cells, tumor-specific cytolytic activity persisted at high levels for the duration of the observation period (at least 75 d). High levels of tumor-specific cytolytic activity could also be detected in parental CMS-5 tumor-bearing animals 18 d after inoculation with tumor cells, if IL-2–producing CMS-5 cells but not unmodified parental tumor cells were used as targets. These studies highlight the potential advantages of localized secretion of cytokines mediated via gene transfer to induce potent anti-tumor immune responses.

Chemically induced tumors in animals possess tumor-specific antigens (TSA),[1] as demonstrated by the ability of animals transplanted with a syngeneic tumor to reject a second challenge of the same tumor (1–3). The relevance of these observations to human neoplasia has been questioned, since spontaneously arising tumors in mice are essentially nonimmunogenic, suggesting that the existence of highly immunogenic tumors in nature is quite rare (4). While it now appears that at least some spontaneous tumors can be recognized by the immune system, even these elicit a weak immune response of little or no protective value (5). In several instances, however, weak anti-tumor responses generated in animals bearing "nonimmunogenic" tumors could be potentiated, protecting the animal from a subsequent tumor challenge (5–7). The growth of some tumors in experimental animals, as well as in human patients, is often associated with the establishment of an active state of immune suppression (8–10). Studies by North and co-workers using chemically induced tumors in mice similar to the tumor system used in our studies, have shown that tumor progression in the animal induced a population of Lyt-1^+2^- T cells that suppressed the emerging anti-tumor response mediated by Lyt-1^-2^+ T cells (10).

IL-2 is a growth factor that stimulates the proliferation of cytotoxic T cells (11), helper T cells (12), NK cells (13), and LAK cells (14), all of which can participate in the anti-tumor response. In the mouse, systemic administration of IL-2 induces the expansion and long-term persistence of adoptively transferred tumor-specific T cells (15–18). To test whether "locally" secreted cytokines were able to influence the generation of anti-tumor immune responses we used retroviral vectors to introduce the gene for human IL-2 into CMS-5 cells. CMS-5, a methylcholanthrene-induced tumor in BALB/c mice, is a weakly immunogenic tumor devoid of viral antigens (19, 20). The effect of varying levels of cytokine expression on tumor growth in vivo and on the generation of a cellular immune response was then measured.

Our results demonstrated that local secretion of IL-2 abrogated the tumorigenicity of the cytokine-producing CMS-5 cells and induced a long-lasting protective immune response against a subsequent tumor graft. The IL-2–producing tumor cells abrogated growth of co-injected tumor cells that were

[1] *Abbreviation used in this paper:* TSA, tumor-specific antigens.

not transfected with cytokine genes and induced a long-lasting state of T cell immunity as measured by persistence of a specific cytotoxic activity. These results suggest that modification of tumor cells with cytokine genes is a potentially useful strategy to manipulate the host mediated anti-tumor immune response.

Materials and Methods

Retroviral Vector Design and Infection of Tumor Cells. A 528-bp long DNA fragment encoding the human IL-2 cDNA was obtained from the plasmid PBCII/RSV/ΔT (21) by digestion with restriction enzymes BamHI and HindIII. A 852-bp-long DNA fragment encoding the HSV thymidine kinase (TK) promoter was obtained from plasmid PHSV106 (22) by digestion with restriction enzymes BamHI and BglII. A 794-bp-long DNA fragment encoding the major immediate early human CMV promoter was obtained from plasmid PRR23 (23) by digestion with restriction enzymes BalI and SmaI. N2 is a retroviral vector derived from the genome of Moloney murine leukemia virus (MLV), containing the bacterial neomycin resistance (*neo*) gene which is used as a selectable marker (24). A schematic diagram presenting the various vector constructs used in these studies is shown in Fig. 1. Vector N2/IL2 was constructed by cloning the IL-2 cDNA into a unique BclI site present upstream from the initiation codon of the neo gene generating a bicistronic transcriptional unit. The TK and CMV promoter encoding DNA fragments were fused to the IL-2 cDNA fragments and cloned into a unique XhoI site present downstream to the *neo* gene coding sequences to generate vector constructs N2/TKIL2 and N2/CMVIL2. The TK-IL2 fusion product was cloned into the SnaBI site present in the polylinker in the 3′ LTR of a modified N2 vector as described by Hantzopoulos et al. (25), to generate vector constructs DC/TKIL2. The prefix DC is short for Double Copy to describe this vector design in which the foreign gene is inserted into the 3′ LTR of retroviral vector. For more details on DC vectors, see Hantzopoulos et al. (25). DCA is a previously described vector in which the human ADA minigene was cloned into the 3′ LTR of the N2 vector (25). Retroviral vector constructs were converted to corresponding virus using established procedures. Briefly, vector DNA was transfected into a helper-free, ecotropic (N2/IL2, N2/TKIL2, N2/CMVIL2, and DC/TKIL2) or amphotropic (DCA) packaging cell line (26, 27). Colonies were isolated by G418 selection, expanded to cell lines, and cell-free supernatant was tested for the presence of virus. Cell lines secreting high titer of virus (10^6–10^5/Neo CFU/ml) were used to infect CMS-5 cells. Clonal derivates of CMS-5 cells were isolated by G418 selection, expanded to cell lines, and secretion of IL-2 into the cell supernatant was measured. Absence of replication-competent virus in the cytokine-producing CMS-5 cells was shown by the inability to transfer G418 resistance to NIH 3T3 cells.

Human IL-2 Assay. Supernatant from 2×10^6 semiconfluent unmodified or IL-2 secreting CMS-5 cells was collected after 48 h and assayed for human IL-2. IL-2 activity was measured using IL-2–dependent human primary lymphoblasts in a proliferation assay as previously described (28), and results were confirmed by ELISA (Genzyme, Boston, MA). DMS-1, a specific mAb to human IL-2 (29), was used to inhibit IL-2 activity.

Tumor Cell Lines and Animal Studies. CMS-5 is a methylcholanthrene-induced fibrosarcoma of BALB/c origin, shown to be devoid of viral antigens (19) and weakly immunogenic when compared with CMS-13 or Meth A, two noncrossreacting methylcholanthrene-induced tumors of the same genetic background (Srivastava, P., personal communication; reference 20). CMS-5, CMS-13, and Meth A were adapted to culture and grown in DMEM medium supplemented with 10% FCS. BALB/c mice were obtained from a colony bred at Sloan-Kettering Institute, from which CMS-5, CMS-13, and Meth A tumors originated. Tumor cell injections were carried out using freshly prepared tumor cells that were removed from the culture plates by trypsinization and washed twice in PBS. Cells were injected intradermally in the dorsal root of the animal. Tumor growth was measured in millimeters using a caliper and was recorded as mean diameter (longest surface length (*a*) and width (*b*), $(a + b):2$).

^{51}Cr-release Assay. Cytotoxicity of spleen cells from tumor-bearing and tumor-free BALB/c mice was assayed on different target cells in an 18-h ^{51}Cr-release assay and carried out in U-bottomed, 96-well microtiter plates. CTL were generated by culturing in vivo immunized spleen cells for 6 d at a concentration of 10^6 cells/ml in RPMI medium, 10% FCS, penicillin, streptomycin, and 2 mM L-glutamine, together with 2.5×10^4 irradiated (6,000 rad) CMS-5 stimulator cells/ml. Cultures were harvested on day 6. Target cells were prepared by culturing cells for 1 h in the presence of 250 μCi of ^{51}Cr sodium chromate, washed twice, and counted. 10^4 labeled target cells per well were mixed with effector lymphocytes to yield several E/T cell ratios and were incubated for 18 h. Percent specific lysis was calculated as: $100 \times$ [(cpm experimental wells − cpm spontaneous release)/(cpm maximum release − cpm spontaneous release)]. Spontaneous release of all targets was ≤25%.

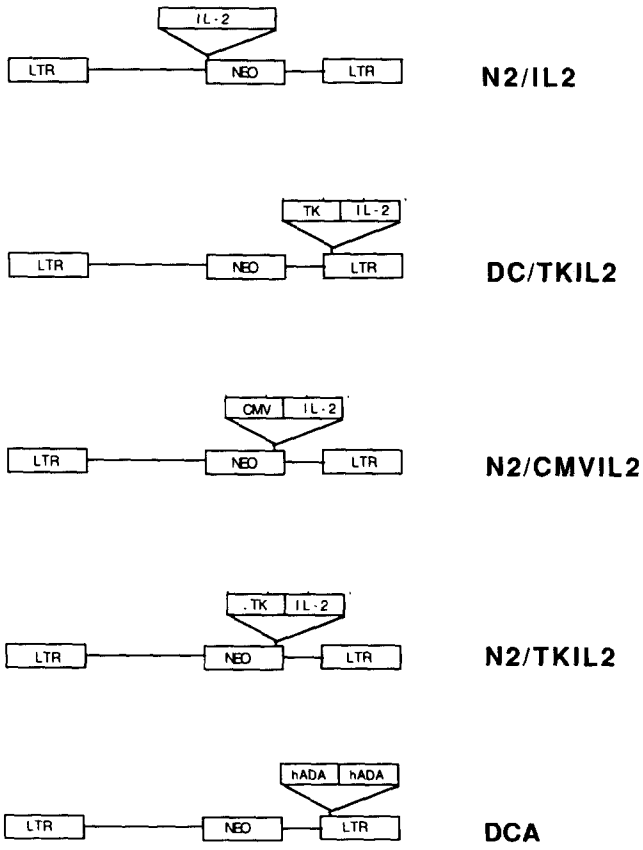

Figure 1. Structure of retroviral vectors containing the human IL-2 cDNAs (For additional details see Materials and Methods).

Results

Generation of Tumor Cell Lines Expressing IL-2. Fig. 1 shows the retroviral vector constructs that were used to introduce and express the human IL-2 gene in tumor cells. The retroviral vectors used in this study were derived from MLV and are based on the high titer N2 retroviral vector, which also contains the bacterial neomycin resistance (*neo*) selectable gene (24). For additional details see Materials and Methods. Vector DNA was converted to corresponding virus using established procedures. Briefly, vector DNA was transfected into a packaging cell line, and neomycin resistant colonies were selected with G418. Drug-resistant colonies were isolated and expanded to cell lines. Virus-containing cell-free supernatant was used to infect CMS-5 cells and clones were isolated. DNA blot analysis of G418-resistant CMS-5 cells was used to show that an intact provirus was present in the infected cells (data not shown).

Expression of the IL-2 gene in the CMS-5 cells transduced with the retroviral vectors was determined by measuring the secretion of the cytokine into the cell supernatant first using a bioassay and then confirming these results with an ELISA test (Table 1). Clonal isolates of CMS-5 cells were found to vary in their expression and secretion of IL-2 depending on the particular retroviral vector used (Table 1). In each case, human IL-2 activity could be neutralized by an appropriate mAb (data not shown). Secretion of human IL-2 had no discernible effect on cell morphology or on the growth rate in culture compared with parental CMS-5 cells or CMS-5 cells transduced with the control DCA vector (data not shown). These results underscored the importance of retroviral vector design and of the choice of expression system in these kinds of studies. Furthermore, they provided an opportunity to test the effects of varying levels of cytokine production on tumorigenesis.

Table 1. *IL-2 Secretion from Vector-transduced CMS-5 Cells*

Vector construct	IL-2/CMS-5 Clones			
	1*	2	3	4
	IL-2 U/ml			
N2/IL2/CMS5	30	29	25	23
N2/CMVIL2/CMS5	15	12	6	5
DC/TKIL2/CMS5	19	15	15	15
N2/TKIL2/CMS5	1	0.5	0.5	0.5
DCA/CMS5	0	0	0	0
CMS-5	0	0	0	0

* Used in subsequent experiments.

The Effect of IL-2 Secretion on Tumor Growth In Vivo. The main question addressed in this study was whether the local secretion of IL-2 from the genetically modified cells would

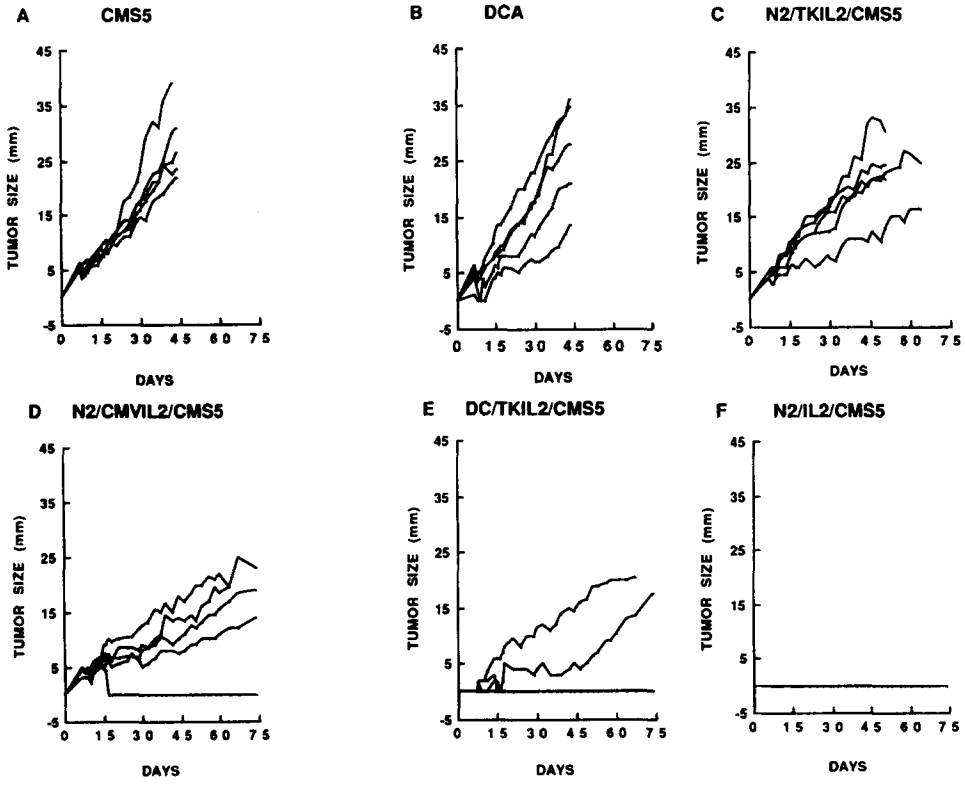

Figure 2. Growth of parental and cytokine producing CMS-5 cells in BALB/c mice. Groups of five mice in each category were injected with 2.5 × 10^5 tumor cells as indicated. Tumor growth was monitored as described in Materials and Methods.

affect their tumorigenicity in vivo. This was tested in two ways. First, equal numbers of unmodified or vector-modified CMS-5 cells were injected intradermally (i.d.) into the back of syngeneic BALB/c mice and the appearance and size of the resulting tumor were monitored over time. The results of the representative experiment shown indicated that mice injected with either 2.5 × 10^5 unmodified CMS-5 cells (Fig. 2 *A*) or with cells harboring the irrelevant vector DCA, which expresses the human ADA gene product (Fig. 2 *B*), developed tumors at the site of injection and had to be killed by day 40. On the other hand, in mice injected with various IL-2–producing CMS-5 cells, tumor growth correlated inversely with the amount of IL-2 secreted by the tumor cells. No tumors were induced in mice injected with high IL-2–producing cells (N2/IL2/CMS5, Fig. 2 *F*). In mice that received CMS-5 cells that secreted intermediate levels of IL-2, tumor growth was significantly retarded and mice survived for over 75 d (N2/CMVIL2/CMS5, DC/TKIL2/CMS5 Fig. 2, *D* and *E*, respectively). Tumor growth was minimally affected in mice injected with low IL-2–producing cells (N2/TKIL2/CMS5, Fig. 2 *C*).

The second approach used to measure the effect of cytokine production on tumorigenicity involved evaluating the numbers of IL-2–secreting tumor cells that could be injected without causing tumors. Whereas injection of only 10^5 CMS-5 cells into BALB/c mice caused the appearance of large tumors in five of six animals, injection of as many as 10^6 IL-2 producing CMS-5 cells (N2/IL2/CMS5) failed to result in tumors (Table 2). Thus, localized IL-2 secretion altered the ability of CMS-5 cells to cause tumors in vivo.

In Vivo Immune Response Generated by Cytokine-producing CMS-5 Cells. An association between cytokine secretion and growth of modified tumor cells, such as we observed, could be a result of altered growth properties of the tumor cells following the genetic manipulation they underwent or of an immune response triggered by the cytokines. We excluded the first possibility, since our results showed no change on cell morphology, viability, or growth rate in culture of vector modified tumor cells compared with parental CMS-5 cells (data not shown). To test the more intriguing possibility that increased amounts of cytokine secretion played a role in the reduced outgrowth of tumor cells an experiment was performed in which 10^6 N2/IL2/CMS5 cells were mixed with increasing numbers of unmodified tumor cells and injected into mice. Our results indicated that co-injection of 10^6 IL-2–producing cells was able to induce the rejection of up to 8 × 10^5 unmodified tumor cells (Fig. 3). Although 1.6 × 10^6 tumor cells were not rejected, the growth of the resulting tumor was considerably reduced. In fact, injection of 2 × 10^5 unmodified tumor cells alone generated a larger tumor than injection of 1.6 × 10^6 tumor cells mixed with the IL-2–producing cells. These observations demonstrated that local secretion of IL-2 influenced the growth of unmodified tumor cells and were consistent with the hypothesis that an immune response was induced that was responsible for the loss of tumorigenicity of CMS-5 cells.

Further support for this hypothesis was obtained when mice that had rejected an IL-2–producing CMS-5 tumor were challenged after 6 wk with 2 × 10^5 CMS-5 cells, a number that usually causes tumor formation. All mice failed to show tumors at the site of the challenge (Table 3). Furthermore, Meth A and CMS-13, two noncrossreactive methylcholan-

Table 2. *Tumorigenic Potential of Parental and Cytokine-producing CMS-5 Cells*

Tumor	Injection dose (Number of cells)	Number of mice with tumor per number mice injected
Parental CMS-5	50,000	0/3
	100,000	5/6
	150,000	8/9
	200,000	15/15
	250,000	15/15
N2/IL-2/CMS-5	125,000	0/3
	250,000	0/8
	500,000	0/3
	1,000,000	0/19
	2,000,000	2/2*

* Growing slowly and regressing after several weeks.

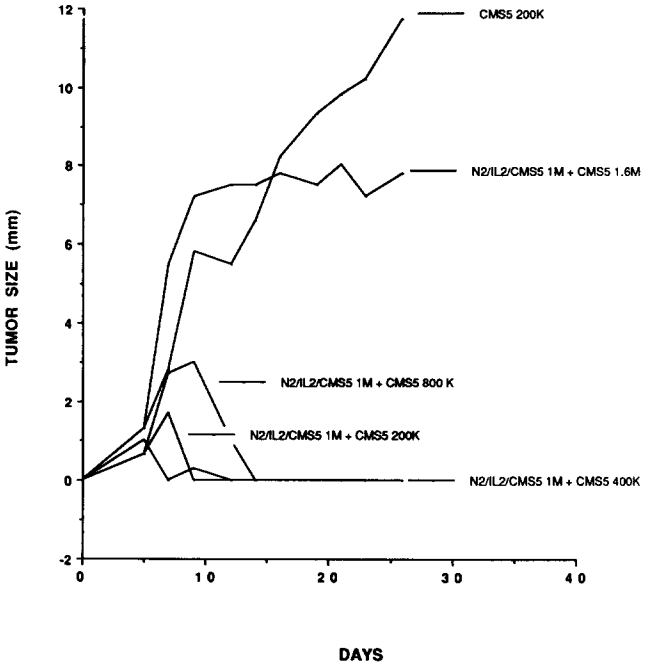

Figure 3. In vivo growth inhibition of parental CMS-5 cells mixed with IL-2-producing CMS-5 cells. 10^6 IL-2–producing CMS-5 cells, N2/IL2/CMS5 1M, were mixed with increasing numbers of parental CMS-5 cells as indicated, injected into the back of BALB/c mice and tumor growth monitored. Three mice were used in each group and the mean diameter for each group was used in the calculations.

Table 3. *Challenge of Mice that Rejected Cytokine-producing Tumor Cells with Parental CMS-5 Cells*

Rejected tumor	Number of mice with tumor* per number of mice injected
DC/TKIL2/CMS5	0/3
N2/IL2/CMS5	0/3
Control‡	5/5

* BALB/c mice were challenged with 2 × 10^5 CMS-5 cells in the left flank 6 wk after injection of 2.5 × 10^5 cytokine-producing tumor cells.
‡ Unimmunized BALB/c mice of the same age group.

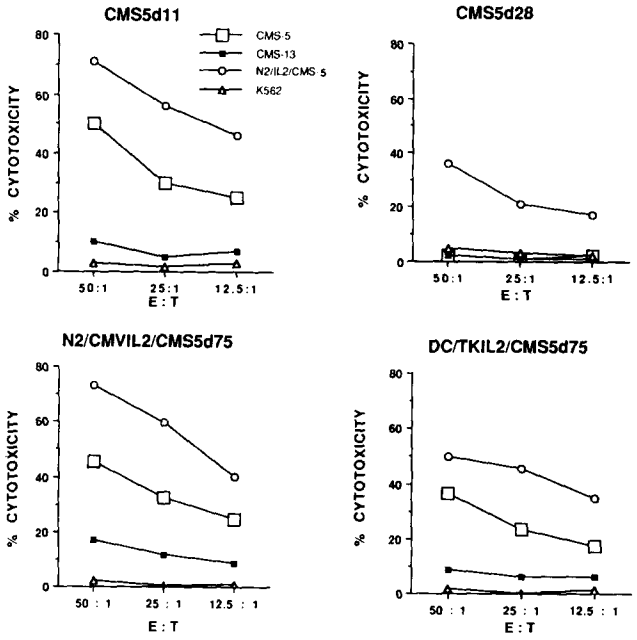

Figure 4. In vitro lytic activity in the spleens of animals injected with parental and cytokine-producing CMS-5 cells. BALB/c mice were injected with 2.5 × 10^5 tumor cells, spleens were isolated at specified time (see below), stimulated in vitro with irradiated parental CMS-5 cells and used as effectors in a ^{51}Cr-release assay against various targets shown in the legend of CMS5d11 in the upper left (see Materials and Methods for additional details). Mice were injected with: CMS5d11, unmodified CMS-5 cells, spleens isolated 11 d later; CMS5d28, unmodified CMS-5 cells, spleens isolated 28 d later; N2/CMVIL2/CMS5d75, N2/CMVIL2/CMS5 cells, spleens isolated 75 days later; DC/TKIL2/CMS5d75, DC/TKIL2/CMS5 cells, spleens isolated 75 d later.

trene-induced fibrosarcomas of the same genetic background, were not rejected (data not shown). Both of these cell lines had been grown in vitro in a fashion identical to CMS-5 cells, thereby also eliminating the possibility that rejection of CMS-5 cells was due to sensitization to serum proteins. Since all control mice developed tumors, protection was specific. Thus, the abrogation of tumorigenicity was characterized by a process that was CMS-5 specific, had memory, and was best explained by the existence of a protective immune response against the parental tumor.

In Vitro Lytic Activity of Spleen Cells Derived from Animals Injected with Parental and Cytokine-producing CMS-5 Cells. Next we tested whether a CTL response might be at least partially responsible for the failure of IL-2–producing tumor cells to grow. Since we reasoned that an ongoing anti-tumor immune response would best be detectable in animals exhibiting reduced tumor growth, spleens were taken from mice that had been inoculated with IL-2–producing tumor cells that grew at a significantly reduced rate, or from mice injected with unmodified CMS-5 cells. The nonadherent T cell–enriched populations were then cocultured in vitro with irradiated, parental CMS-5 cells for 6 d and tested in a standard ^{51}Cr-release assay for their ability to lyse various targets including parental CMS-5 cells, an IL-2–producing CMS-5 line (N2/IL2/CMS5), K562, an NK-sensitive cell line, and CMS-13, a methylcholanthrene induced noncrossreactive BALB/c fibrosarcoma (20).

Our results indicated that mice injected with unmodified CMS-5 cells were able to lyse parental CMS-5 cells and IL-2–producing CMS-5 cells, if effector spleen cells were taken at day 11 after injection of tumor cells (Fig. 4, *upper left*). Neither K562 nor CMS13 cells were lysed. In other experiments, YAC-1 cells, another NK sensitive cell line, were also shown to be resistant to lysis by the activated spleen cells (data not shown). On the other hand, if spleen cells were taken from mice inoculated with the unmodified tumor 28 d after the injection of CMS-5 cells, there was no longer detectable lysis of unmodified CMS-5 cells (Fig. 4, *upper right*). This observation is consistent with the transient nature of the CTL response against CMS-5 and several other mouse tumors, which is followed by the emergence of a state of immune suppression mediated by a population of tumor-specific T suppressor cells (10, 30, 31). In contrast, spleen cells derived as late as 75 d after injection of IL-2–producing CMS-5 cells still demonstrated a strong CTL response, similar to that observed with spleen cells taken 11 d after injection of unmodified CMS-5 cells (Fig. 4, *lower left and lower right*). Thus, the normal transient CTL response was not seen in the mice injected with IL-2–secreting tumors, but rather was replaced by one that was long lived.

We attempted to investigate in vitro whether secretion of IL-2 by the tumor cells could influence T cell activity by testing the ability of parental CMS-5 cells and their IL-2–producing counterparts to serve as targets for CTL. Effector cells were produced by inoculating mice with parental CMS-5 cells. After 18 d spleen cells were isolated, stimulated in vitro for 6 d with irradiated unmodified CMS-5 cells, and used as effectors in a CML assay against a panel of targets. As expected, given the immune suppression in mice inoculated with tumor cells close to 3 wk before the harvest of spleen cells (10), only low levels of lysis was observed when unmodified CMS-5 cells were used as targets (Fig. 5). In contrast, efficient lysis was observed when IL-2–producing CMS-5 cells were used as targets (Figs. 4 and 5). Moreover, the efficiency of lysis

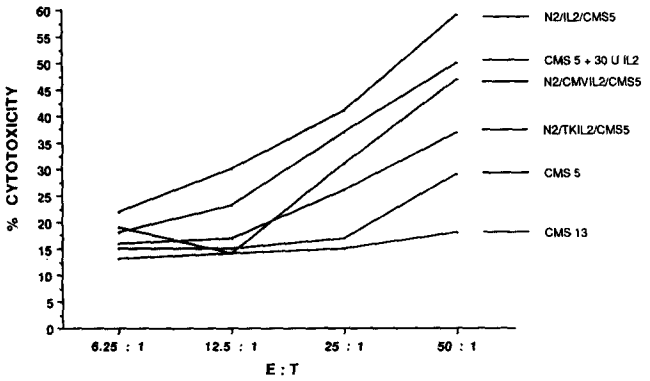

Figure 5. Cell-mediated lympholysis against different IL-2–secreting target cells. Mice were injected with 2.5 × 10^5 CMS-5 cells, spleens were isolated 18 d later, cultured in vitro for 6 d in the presence of irradiated CMS-5 cells, and used in a ^{51}Cr-release assay against targets, as indicated.

of various targets was directly proportional to the level of IL-2 secreted by the target cells. (N2/IL2/CMS5 > N2/CMVIL2/CMS5 > N2/TKIL2/CMS5, see Table 1). Even parental CMS-5 cells could be made susceptible to lysis by the addition of exogenous IL-2 during the 18 h of incubation with the activated CTL. It is estimated that 1–3 U/ml of IL-2 accumulate during the lysis reaction when 10,000 N2/IL2 vector–containing CMS-5 cells are used as targets, while 30 U/ml of exogenous IL-2 were used in the experiment shown in Fig. 5. In other experiments (data not shown), 5 and 2 U/ml of exogenous IL-2 were effective at inducing lysis of CMS-5 cells, indicating that IL-2 secretion from the tumor cells could indeed be responsible for the increase in lysis seen. Additional support was obtained in experiments in which the addition of an anti-IL-2, but not control, mAb to the overnight CML reaction inhibited the lysis of N2/IL-2/CMS-5 target cells by ~50% (data not shown). The possibility that the secreted IL-2 was responsible for the increased target cell destruction by reducing the viability of the target cells during the 18-h incubation period was excluded, since there was no lysis by lymphocytes that were taken directly from parental CMS-5 tumor-bearing mice and exposed to IL-2–secreting target cells without a 6-d period of cocultivation (data not shown).

Discussion

Systemic administration of cytokines into experimental animals is used with increased frequency to probe the functions of the immune system and to enhance the immunotherapeutic value of treatments for viral diseases and cancer. Transfer of cytokine genes into tumor cells approximates more closely the physiological mode of cytokine secretion in the course of an immune response, because it leads to the highly localized secretion of low levels of cytokines which ceases with the regression of the tumor. Retroviral gene transfer is a highly efficient method to introduce genes into mammalian cells, resulting in integration of cytokine genes into the cell chromosome and persistent expression of low levels of cytokine. In this study retroviral vectors were used to introduce and express the IL-2 gene in CMS-5 cells (Table 1).

Though we estimated that <50–400 U of IL-2 were secreted from the tumor cells injected in the back of the mouse (assuming that the injected tumor cells increase in number ~10-fold and persist in the mouse for 1 wk before complete regression), our results demonstrated that the modification of weakly immunogenic tumor cells to secrete IL-2 effectively abrogated their ability to form tumors in mice (Fig. 2 and Table 2). By comparison, only partial regression of tumor growth has been achieved via systemic administration of very high doses of IL-2 (32, 33). The potential advantages of localized secretion of cytokines was suggested by the studies of Forni et al. (34), who showed that local injection of low doses of several cytokines including IL-1, IL-2, or IFN-γ, enhanced the rejection of tumor cells admixed with tumor-specific T cells. More recently, other investigators have demonstrated the antitumor effect of cytokine gene transfer into tumor cells, using the IL-4 (35), IFN-γ (36), and the IL-2 genes (37). Our studies confirm the findings of Fearon et al. (37) that IL-2 secretion by tumor cells can abrogate the tumorigenicity of those cells and extend them by showing that it is a quantitative phenomenon, correlating with the amount of IL-2 secreted by the tumor cells. In addition we show that (*a*) the immune reaction induced prevents highly tumorigenic doses of coinjected unmodified tumor cells from establishing a tumor; (*b*) that IL-2 secretion by the tumor leads to a long-lasting state of T cell immunity as measured by persistence of a specific cytotoxic activity, not seen after injection of unmodified tumor cells; and (*c*) in vitro data suggest that IL-2 secretion into the immediate surrounding of the tumor cells leads to a much more efficient lysis of the cytokine secreting CMS-5 cells.

Abrogation of tumorigenicity of parental CMS-5 cells admixed with IL-2–producing counterparts suggests that the inhibition was host mediated (Fig. 3). Rejection of 8 × 10^5 tumor cells and significant inhibition of growth of 1.6 × 10^6 tumor cells admixed with IL-2 producers attested to the potency of the protective effect resulting from localized secretion of IL-2 in the vicinity of the emerging tumor. This was confirmed in the experiment described in Table 3 which shows that mice immunized with IL-2–producing tumor cells developed a specific and long-lasting immune response since they are fully protected from a challenge with a highly tumorigenic dose of CMS-5 cells. The ability to induce protective immunity using IL-2–producing tumor cells compares favorably with previous reports that have shown that BALB/c mice can be immunized against CMS-5 cells by two injections of 2.5 × 10^7 irradiated CMS-5 cells. The highest tumor challenge that could be rejected using this protocol was 75,000 CMS-5 cells (20).

The emergence of a protective immune response against the parental tumor cells correlated with the appearance of tumor-specific cytotoxic T cells in spleens derived from mice injected with IL-2–producing CMS-5 cells, implicating this effector arm of the immune system in their eradication (Fig. 4). Persistence of a long-lasting immune response (Table 3) and CTL activity in the spleens of mice injected with IL-2–producing cells, but not in the spleens of mice injected

with the parental CMS-5 cells (Fig. 4), suggested that secretion of IL-2 from the tumor cells led to the induction of a long-lasting state of T cell immunity.

As shown in Fig. 5 (and Fig. 4, *upper right*), IL-2–secreting CMS-5 cells, but not unmodified CMS-5 cells, were efficiently and specifically lysed by spleen cells taken from mice injected with unmodified CMS-5 cells at a time point when the animals were usually immunosuppressed, raising the possibility that (*a*) IL-2 secretion by the tumor cells makes it possible to circumvent an existing state of suppression and (*b*) in the spleen of an immunosuppressed tumor bearing animal, the suppressor cells coexist with primed CTL. These results can be also interpreted to suggest that a weak anti-tumor immune response induces T cell anergy that is overcome by IL-2.

Several observations described above rule out the possibility that the human IL-2 gene product is a target for an immune response that is responsible for the observed lack of tumorigenicity of the IL-2–secreting cells: (*a*) inhibition of tumor formation by parental CMS-5 cells admixed with IL-2–secreting counterparts (Fig. 3); (*b*) induction of protective immune response against unmodified CMS-5 cells in mice that rejected the IL-2–producing tumor cells (Table 3); and (*c*) spleen cells from mice injected with unmodified CMS-5 cells killed N2/IL2/CMS5 target cells as well as spleen cells from mice injected with IL-2–producing tumor cells (Fig. 4).

In conclusion, our data demonstrate that retroviral vectors can be used to introduce the IL-2 gene into tumor cells. Depending upon the construct chosen, the resulting persistent expression and secretion of low levels of cytokine are sufficient to have a decisive impact on the host anti-tumor response.

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References

1. Foley, E.J. 1953. Antigenic properties of methylcholanthrene-induced tumors in mice of the strain of origin. *Cancer Res.* 13:835.
2. Prehn, T.R., and J.M. Main. 1957. Immunity to methylcholanthrene-induced sarcomas. *J. Natl. Cancer Inst.* 18:769.
3. Old, L.J., E.A. Boyse, D.A. Clarke, and E.A. Carswell. 1962. Antigenic properties of chemically induced tumors. *Ann. NY Acad. Sci.* 101:80.
4. Hewitt, H.B., E.R. Blake, and A.S. Walder. 1976. A critique of the evidence for active host defence against cancer, based on personal studies of 27 murine tumors of spontaneous origin. *Br. J. Cancer.* 33:241.
5. Key, M.E., J.S. Brandhorst, and M.G. Hanna, Jr., 1984. More on the relevance of animal tumor models: immunogenicity of transplantable leukemias of recent origin in syngeneic strain 2 guinea pigs. *J. Biol. Response Modif.* 3:359.
6. Forni, G., and A. Santoni. 1984. Immunogenicity of "nonimmunogenic" tumors. *J. Biol. Response Modif.* 3:128.
7. Shu, S., T. Chou, and K. Sakai. 1989. Lymphocytes generated by in vivo priming and in vivo sensitization demonstrate therapeutic efficacy against a murine tumor that lacks apparent immunogenicity. *J. Immunol.* 143:740.
8. Kamo, I., and H. Friedman. 1977. Immunosuppression and the role of suppressive factors in cancer. *Adv. Cancer Res.* 25:271.
9. Naor, D. 1979. Suppressor cells: permitters and promoters of malignancy? *Adv. Cancer Res.* 29:45.
10. North, R.J. 1985. Down-regulation of the antitumor immune response. *Adv. Cancer Res.* 45:1.
11. Erard, F., P. Corthesy, M. Nabholz, J.W. Lowenthal, P. Zaech, G. Plaetinck, and H.R. MacDonald. 1985. Interleukin 2 is both necessary and sufficient for the growth and differentiation of lectin-stimulated cytolytic T lymphocyte precursors. *J. Immunol.* 134:1644.
12. Mosmann, T.R., and R.L. Coffman. 1987. Two types of mouse helper T-cell clone. *Immunol. Today.* 8:223.
13. Trinchieri, G. 1989. Biology of natural killer cells. *Adv. Immunol.* 47:187.
14. Rosenberg, S.A., and M.T. Lotze. 1986. Cancer immunotherapy using interleukin-2 and interleukin-2-activated lymphocytes. *Annu. Rev. Immunol.* 4:681.
15. Cheever, M.A., P.D. Greenberg, A. Fefer, S. Gillis. 1982. Augmentation of the anti-tumor therapeutic efficacy of long-term cultured T lymphocytes by in vivo administration of purified interleukin-2. *J. Exp. Med.* 155:968.
16. Chou, T., S. Bertera, A.E. Chang, and S. Shu. 1988. Adoptive immunotherapy of microscopic and advanced visceral metastases with in vitro sensitized lymphoid cells from mice bearing progressive tumors. *J. Immunol.* 141:1775.
17. Donohue, J.H., M. Rosenstein, A.E. Chang, M.T. Lotze, R.J. Robb, and S.A. Rosenberg. 1984. The systemic administration of purified interleukin 2 enhances the ability of sensitized murine lymphocytes to cure a disseminated syngeneic lymphoma. *J. Immunol.* 132:2123.
18. Cameron, R.B., P.J. Spiess, and S.A. Rosenberg. 1990. Syner-

gistic antitumor activity of tumor-infiltrating lymphocytes, interleukin 2, and local tumor irradiation. *J. Exp. Med.* 171:249.
19. DeLeo, A.B., H. Shiku, T. Takahashi, M. John, and L.J. Old. 1977. Cell surface antigens of chemically induced sarcomas of the mouse. I. Murine leukemia virus-related antigens and alloantigens on cultured fibroblasts and sarcoma cells: description of a unique antigen on BALB/c Meth A sarcoma. *J. Exp. Med.* 146:720.
20. Srivastava, P.K., A.B. DeLeo, and L.J. Old. 1986. Tumor rejection antigens of chemically induced sarcomas of inbred mice. *Proc. Natl. Acad. Sci. USA.* 83:3407.
21. Cullen, B.R. 1988. Expression of a cloned human interleukin-2 cDNA is enhanced by the substitution of a heterologous mRNA leader region. *DNA* (NY). 7:645.
22. McKnight, S. L. 1980. The nucleotide sequence and transcript map of the herpes simplex virus thymidine kinase gene. *Nucleic Acid Res.* 8:5949.
23. Wano, Y., B.R. Cullen, P.A. Svetlik, N.J. Pfeffer, and W.C. Greene. 1987. Reconstitution of high affinity IL-2 receptor expression in a human T-cell line using a retroviral cDNA expression vector. *Mol. Biol. Med.* 4:95.
24. Armentano, D., S.-F. Yu, P.W. Kantoff, T. von Ruden, W.F. Anderson, and E. Gilboa. 1987. Effect of internal viral sequences on the utility of retroviral vectors. *J. Virol.* 61:1647.
25. Hantzopoulos, P.A., B.A. Sullenger, G. Ungers, and E. Gilboa. 1989. Improved gene expression upon transfer of the adenosine deaminase minigene outside the transcriptional unit of a retroviral vector. *Proc. Natl. Acad. Sci. USA.* 86:3519.
26. Markowitz, D., S. Goff, and A. Bank. 1988. A safe packaging line for gene transfer: separating viral genes on two different plasmids. *J. Virol.* 62:1120.
27. Markowitz, D., S. Goff, and A. Bank. 1988. Construction and use of a safe and efficient amphotropic packaging cell line. *Virology.* 167:400.
28. Zier, K. 1982. Functional and antigenic properties of cultured T-cells in the cell mediated lympholysis (CML) assay. *Hum. Immunol.* 4:147.
29. Smith, K.A., M.F. Favata, and S. Oroszla. 1983. Production and characterization of monoclonal antibodies to human interleukin 2. Strategy and tactics. *J. Immunol.* 131:1808.
30. Takei, F., J.G. Levy, and D.G. Kilburn. 1976. In vitro induction of cytotoxicity against syngeneic mastocytoma and its suppression by spleen and thymus cells from tumor-bearing mice. *J. Immunol.* 116:288.
31. Takei, F., J.G. Levy, and D.G. Kilburn. 1977. Characterization of suppressor cells in mice bearing syngeneic mastocytoma. *J. Immunol.* 118:412.
32. Thompson, J.A., D.J. Peace, J.P. Klarnet, D.E. Kern, P.D. Greenberg, and M.A. Cheever. 1986. Eradication of disseminated murine leukemia by treatment with high-dose interleukin 2. *J. Immunol.* 137:3675.
33. Rosenberg, S.A., J.J. Mule, P.J. Spiess, C.M. Reichert, and S.L. Schwarz. 1985. Regression of established pulmonary metastases and subcutaneous tumors mediated by the systemic administration of high dose recombinant Interleukin 2. *J. Exp. Med.* 161:1169.
34. Forni, G., H. Fujiwara, F. Martino, T. Hamaoka, C. Jemma, P. Caretto, and M. Giovarelli. 1988. Helper strategy in tumor immunology: Expansion of helper lymphocytes and utilization of helper lymphokines for experimental and clinical immunotherapy. *Cancer Metastasis Rev.* 7:289.
35. Tepper, R.I., P.K. Pattengale, and P. Leder. 1989. Murine interleukin-4 displays potent anti-tumor activity in vivo. *Cell.* 57:503.
36. Watanabe, Y., K. Kuribayashi, S. Miyatake, K. Nishihara, E. Nakayama, T. Taniyama, and T. Sakata. 1989. Exogenous expression of mouse interferon-gamma cDNA in mouse neuroblastoma C1300 cells results in reduced tumorigenicity by augmented anti-tumor immunity. *Proc. Natl. Acad. Sci. USA.* 86:9456.
37. Fearon, E.R., D.M. Pardoll, T. Itaya, P. Golumbek, H.I. Levitsky, J.W. Simons, H. Karasuyama, B. Vogelstein, and P. Frost. 1990. Interleukin-2 production by tumor cells bypasses T helper function in the generation of an antitumor response. *Cell.* 60:397.