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Adenoviral Gene Transfer of Interleukin 12 into Tumors Synergizes with Adoptive T Cell Therapy Both at the Induction and Effector Level

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ABSTRACT

Tumors infected with a recombinant defective adenovirus expressing interleukin 12 (IL-12) undergo regression, associated with a cytotoxic T lymphocyte (CTL)-mediated antitumor immune response. In the present study we generated anti-CT26 CTLs by short-term coculture of CT26 cells and lymph node cells obtained from mice harboring subcutaneous CT26 tumors injected with an adenoviral vector expressing IL-12 (AdCMVIL-12), control adenovirus (AdCMVlacZ), or saline. Regression of small intrahepatic CT26 tumors in unrelated syngeneic animals was achieved with CTLs derived from mice whose subcutaneous tumors had been injected with AdCMVIL-12 but not with CTLs from the other two control groups. The necessary and sufficient effector cell population for adoptive transfer consisted of $CD8^+$ T cells that showed anti-CT26 specificity partly directed against the AH1 epitope presented by $H\text{-}2L^d$. Interestingly, treatment of a subcutaneous tumor nodule with AdCMVIL-12, combined with intravenous adoptive T cell therapy with short-term CTL cultures, had a marked synergistic effect against large, concomitant live tumors. Expression of IL-12 in the liver in the vicinity of the hepatic tumor nodules, owing to spillover of the vector into the systemic circulation, appeared to be involved in the increased *in vivo* antitumor activity of injected CTLs. In addition, adoptive T cell therapy improved the outcome of tumor nodules transduced with suboptimal doses of AdCMVIL-12. Our data provide evidence of a strong synergy between gene transfer of IL-12 and adoptive T cell therapy. This synergy operates both at the induction and effector phases of the CTL response, thus providing a rationale for combined therapeutic strategies for human malignancies.

OVERVIEW SUMMARY

Evidence that the IL-12 gene, transferred into tumors by adenovirus, displays antitumor activity is well confirmed, and this activity has been shown to involve immune and nonimmune mechanisms. IL-12 bioactivity results in a potent induction of tumor-specific CTLs that can be used for adoptive T cell therapy. This study demonstrates that IL-12 intratumor gene transfer and adoptive T cell therapy are synergistic to the treatment of experimental transplantable colon cancer. Synergy includes not only the facilitation of CTL obtention but also mechanisms of cooperation at the effector phase of the combined therapy. This synergy seems to be highly beneficial in the treatment of established metastatic disease.

INTRODUCTION

Adoptive cellular therapy is defined as the infusion of immune effector cells for the treatment and/or prevention of a disease (Riddell and Greenberg, 1995; Yee *et al.*, 1997). Different types of T cell populations have been used to treat viral infections and tumors in murine models (Kast *et al.*, 1989; Lynch and Miller, 1991), leading to their use in the clinic (Riddell *et al.*, 1992; Rossemberg *et al.*, 1994; Henslop *et al.*, 1996).

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A major hurdle for the feasibility of T cell adoptive therapy in oncology is the availability of tumor-specific T cells for *in vitro* expansion and differentiation into effector populations (Kast *et al.*, 1989; Lynch and Miller, 1991; Riddell *et al.*, 1992; Rossemberg *et al.*, 1994; Riddell and Greenberg, 1995; Henslop *et al.*, 1996; Yee *et al.*, 1997). Passive immunotherapy using T cells relies on the culture of autologous lymphocytes in the presence of a source of tumor antigens, professional antigen-presenting cells (APCs) and growth factors (Lynch and Miller, 1991; Yee *et al.*, 1997). Low numbers of T cell precursors in tumor hosts could be simply a consequence of the lack of an ongoing antitumor response (Melero *et al.*, 1997b; Wick *et al.*, 1997) or the result of immune-suppressive mechanisms, displayed by the malignancy, that could lead to antigen-specific tolerance (Speiser *et al.*, 1997; Chen, 1998).

The endogenous cellular immune response against tumor antigens can be boosted by a number of strategies, including vaccination with different formulations containing tumor antigens or by the administration of certain cytokines (Chen *et al.*, 1993). The most successful procedures of active immunotherapy include: artificial presentation of tumor antigens on professional APCs or genetic modifications of tumor cells to mimic the functions of professional antigen-presenting cells when producing cytokines and/or when expressing membrane-bound costimulatory molecules (cytokines) (Chen *et al.*, 1993; Mayordomo *et al.*, 1995).

Interleukin 12 (IL-12) is a cytokine with potent antitumor effects that result from its ability to stimulate cytotoxic T cells (CTLs), type 1 helper T cells (Th1 cells), and natural killer (NK) cells (Shurin *et al.*, 1997) and to trigger a cascade of mediators that ultimately impair tumor angiogenesis (Siders *et al.*, 1998; Tannenbaum *et al.*, 1998). IL-12 has been delivered *in vivo* into the tumor environment by different recombinant viral vectors (Caruso *et al.*, 1996; Toda *et al.*, 1998), achieving tumor regressions and upregulation of the cellular immune response against malignant cells (Caruso *et al.*, 1996; Toda *et al.*, 1998). The intimate mechanisms behind IL-12 antitumor activity involve immune and nonimmune phenomena. Experimentation has shown that interferon γ (IFN-γ), a major downstream mediator of IL-12 antitumor activity, is able to inhibit angiogenesis in malignant lesions through the induction of chemokines such as IFN-γ inducible protein 10 (IP-10) (Siders *et al.*, 1998). In some cases these mechanisms seem to be operational in the absence of conventional T cells (Boggio *et al.*, 1998; Siders *et al.*, 1998; Tannenbaum *et al.*, 1998).

Recombinant defective adenoviruses have been shown to be an excellent vehicle to deliver IL-12 to experimental tumors including a poorly immunogenic colon carcinoma (Caruso *et al.*, 1996; Mazzolini *et al.*, 1999). In our hands, high levels of IL-12 are produced on intratumoral injection of AdCMVIL-12 in CT26-derived tumors, leading to complete tumor regressions in 60–80% of cases. In such cases, a potent antitumor CTL response was detected, depletion of $CD8^+$ cells abrogated the therapeutic effect, and a lymphocyte infiltrate accompanied tumor regressions (Mazzolini *et al.*, 1999).

These findings provided the rationale for protocols of combination immunotherapy in which gene transfer with AdCMVIL-12 promotes the *in vivo* expansion of antitumor CTLs, thereby facilitating their subsequent *in vitro* culture for adoptive transfer. Our data demonstrate that active immunotherapy with AdCMVIL-12 not only permits CTL adoptive transfer by simplifying the obtention of the effector cells, but also synergizes with adoptive T cell therapy at the effector phase when both therapeutic strategies are used together to treat metastatic disease.

MATERIALS AND METHODS

Animals, cell culture, and reagents

Five- to 8-week-old BALB/c female mice were purchased from Charles River (Barcelona, Spain) and were housed according to institutional guidelines.

The 293 cell line (adenoviral E1-transformed human embryonic kidney cells) was obtained from American Type Culture Collection (ATCC, Rockville, MD). The BALB/c (H-2^d) mouse-derived CT26 tumor cell line is an undifferentiated murine colorectal adenocarcinoma (Brattain *et al.*, 1980) that was established from an *N*-nitroso-*N*-methylurethane-induced transplantable tumor (Corbett *et al.*, 1975), obtained from K. Brand (Max-Planck-Institut fur Biochemie, Munich, Germany). P815 and YAC-1 cells were obtained through the ATCC. The 293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 m*M* L-glutamine, streptomycin (100 U/ml), and penicillin (100 μg/ml), CT26 cells were maintained in RPMI 1640 medium identically supplemented. Cell culture reagents were from GIBCO (Basel, Switzerland).

Construction of adenovirus

Recombinant adenovirus carrying IL-12 (AdCMVIL-12) has been previously described (Mazzolini *et al.*, 1999). Briefly, an expression cassette of IL-12 under the control of the cytomegalovirus (CMV) promoter was constructed encompassing IL-12 p35 cDNA, an internal ribosomal entry site (IRES), IL-12 p40 cDNA, and a polyadenylation signal. Recombinant adenovirus encoding the IL-12 cassette of expression was generated by cotransfection of 293 cells according to standard procedures (Qian *et al.*, 1995). Adenovirus carrying the *lacZ* reporter gene under the control of the CMV promoter (AdCMVlacZ) was produced similarly. Recombinant adenoviruses were isolated from a single plaque, expanded in 293 cells, and purified by double cesium chloride ultracentrifugation (Qian *et al.*, 1995). Purified virus was extensively dialyzed against 10 m*M* Tris–1 m*M* $MgCl_2$ and stored in aliquots at $-80°C$, and it was carefully titrated by plaque assay.

Peptides

The H2-L^d-restricted peptides AH1 (SPSYVYHQF) (Huang *et al.*, 1996) and P815AB (LPYLGWLVF) (Van den Eynde *et al.*, 1991) were synthesized by 9-fluorenylmethoxycarbonyl (Fmoc) chemistry as described (Prieto *et al.*, 1995) and their purity confirmed by high-performance liquid chromatography (HPLC).

T cell culture

Mice carrying bilateral 5- to 8-mm (diameter) subcutaneous CT26 tumors were treated by intratumor injections of 10^8 PFU

of AdCMVIL-12 or control adenovirus (AdCMVlacZ), or were left untreated. Draining lymph nodes were removed aseptically 5 days later and single-cell suspensions were obtained by pressing the lymph nodes mechanically through mesh screens. Lymph node cells were cultured in 24-well plates (Greiner Labortechnik, Frickenhausen, Germany) for 7 days at 5×10^6 cells/well with 2×10^5 CT26 tumor cells/well pretreated for 1 hr at 37°C with mitomycin C (150 μg/ml; Sigma, Madrid, Spain), a reagent that was extensively washed. Culture medium was complete RPMI 1640 supplemented on day 5 with murine IL-2 (mIL-2, 8–10 IU/ml; Peprotech, London, UK).

^{51}Cr release assay

Cytotoxicity was analyzed in conventional 5-hr ^{51}Cr-release assays as described (Melero *et al.*, 1997a). Briefly, ^{51}Cr-loaded CT26, P815, and YAC-1 cells were incubated with effector cells at different effector-to-target (*E*:*T*) ratios in triplicate wells and ^{51}Cr release (cpm) into the supernatants was measured in a γ counter to calculate percent specific release as described (Melero *et al.*, 1997b). In some experiments, P815 cells were incubated during the assay with various concentrations of AH1 or P815AB peptide.

Immunofluorescence and flow cytometry

Double immunofluorescence staining with fluorescein isothiocyanate (FITC)-conjugated anti-CD8 and phycoerythrin (PE)-conjugated anti-CD4 (PharMingen-Becton Dickinson, San Diego, CA) was carried out as previously described (Melero *et al.*, 1997b). Electronically gated lymphocytes were analyzed on a FACscan (Becton Dickinson, Mountain View, CA).

In vivo *treatment of CT26 tumors with recombinant adenovirus and adoptive transfer of lymphocytes*

BALB/c mice, in groups of seven or eight, were injected subcutaneously in the right hand flank with 5×10^5 CT26 cells in 25 μl of phosphate-buffered saline (PBS). In some experiments, mice received in addition an identical dose of tumor cells injected surgically in the midlobe of the liver under general anesthesia. Subcutaneous tumors were injected with 10^8 PFU of recombinant adenovirus in 50 μl of PBS. For cellular adoptive therapy, mice were injected intravenously with 5×10^6 cells from short-term CTL lines and given three intraperitoneal injections of 2×10^4 human recombinant IL-2 (Chiron, Emeryville, CA) in PBS on alternate days. Tumor size (mean diameter) was assessed by laparotomy, using a precision caliper. Statistical significance of the differences among groups was evaluated by Mann–Whitney tests for tumor sizes and log-rank test for survival.

Immunomagnetic selection of CD8$^+$ T cells and negative selection with MAb plus complement

Cells from short-term CTL cultures were incubated with anti-CD8-coated magnetic beads according to manufacturer instructions (Miltenyi Biotec, Bergisch Gladbach, Germany) and purified on a mini-MACS column (Miltenyi). Purity of CD8$^+$ cells was 98% as shown by fluorescence activated cell sorting (FACS) analysis. Depletion of CD4$^+$ or CD8$^+$ cells was achieved by treatment with culture supernatants from the hybridomas GK1.5 and H35.17.2 (ATCC) and rabbit complement (Sigma). Effectiveness of the procedure was routinely checked by FACS.

IL-12 p70 quantitative assay

To evaluate the production of IL-12 by the liver parenchyma and subcutaneous tumor, we injected AdCMVIL-12, AdCMVlacZ, or saline into subcutaneous tumors. Three days later, tissues were collected for determination of IL-12 p70 concentration by enzyme-linked immunosorbent assay (ELISA). To estimate the *in vivo* production of cytokines 3 days after treatment of the tumors with adenoviruses, liver and subcutaneous tumors were removed and frozen in liquid nitrogen. Frozen tissue was homogenized in 0.5 ml of PBS containing 100 μM phenylmethylsulfonyl fluoride (PMSF) and aprotinin (10 μg/ml; ICN Biomedicals) by Ultra-Turrax (Labortechnik, Staufen, Germany). The homogenate was then sonicated for 10 sec and cleared of debris by centrifugation in a microcentrifuge for 5 min at 4°C. Samples were stored at −20°C.

The ELISA for IL-12 p70 was performed with a commercial kit (Endogen, Woburn, MA) according to manufacturer instructions, using superblocking buffer (Pierce, Rockford, IL).

X-Gal histochemical staining

Mice bearing both a subcutaneous and intrahepatic tumors were anesthetized with a mixture of ketamine and xylazine. AdCMVlacZ (5×10^8 PFU) in 50 μl of saline was injected into the subcutaneous tumor. Two days later, animals were sacrificed and the livers were excised and immediately embedded in O.C.T. compound (Tissue Tek, Zoeterwoude, The Netherlands) and frozen in liquid nitrogen. Sections of 6-μm thickness were fixed with glutaraldehyde (0.5%) and stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) as described previously (Qian *et al.*, 1997).

RESULTS

AdCMVIL-12 induces tumor regression and raises CTLs for adoptive therapy

rIL-12 delivered by gene transfer procedures into tumors has been shown to inhibit tumor growth and to induce tumor regressions (Caruso *et al.*, 1996; Toda *et al.*, 1998). In our hands (Mazzolini *et al.*, 1999), locally delivered AdCMVIL-12 induced the production of significant levels of IL-12 and displayed a potent effect against the CT26 colon carcinoma cell line forming either subcutaneous or intraheptic tumor nodules. In 60–80% of cases complete regressions were observed that were associated with an increase in tumor-specific CTL activity. Typically, a 5- to 8-mm (diameter) CT26 subcutaneous nodule treated with 10^8 PFU of AdCMVIL-12 started to regress during the next 4–6 days, unless the mouse had been treated with a depleting anti-CD8 monoclonal antibody (MAb), conditions under which the antitumor effect was completely abrogated. Surgical and pathological examination revealed enlarged draining lymph nodes and an infiltrate of lymphocytes into the tumor. Accordingly, it was hypothesized that the obtention of CTL cultures aimed for adoptive therapy would be efficiently simplified in such animals.

Since the CT26 cell line was derived from an adenocarcinoma arising from the colon, we established models in which 5×10^5 tumor cells were directly injected into the midlobe of the liver to give rise to intrahepatic tumors. These malignant nodules resemble those frequently observed in advanced human colon cancers. This model was employed to test whether treatment of CT26 tumors with AdCMVIL-12 would allow the generation of CTL cultures to be used in simple adoptive therapy protocols. Groups of four mice were injected subcutaneously and bilaterally with 5×10^5 CT26, and after 10 days tumors (4–8 mm in diameter) were injected with AdCMVIL-12, a control recombinant adenovirus encoding β-galactosidase (AdCMVlacZ), or saline. Seven days later, mice were killed and cell suspensions of their draining lymph nodes were cocultured for 7 days with mitomicin C-treated CT26 cells. Such cell cultures were subsequently injected intravenously (5×10^6 cells/mouse) into mice hosting CT26 tumor cells inside their livers for 4 days prior to adoptive transfer. The adoptive trans-

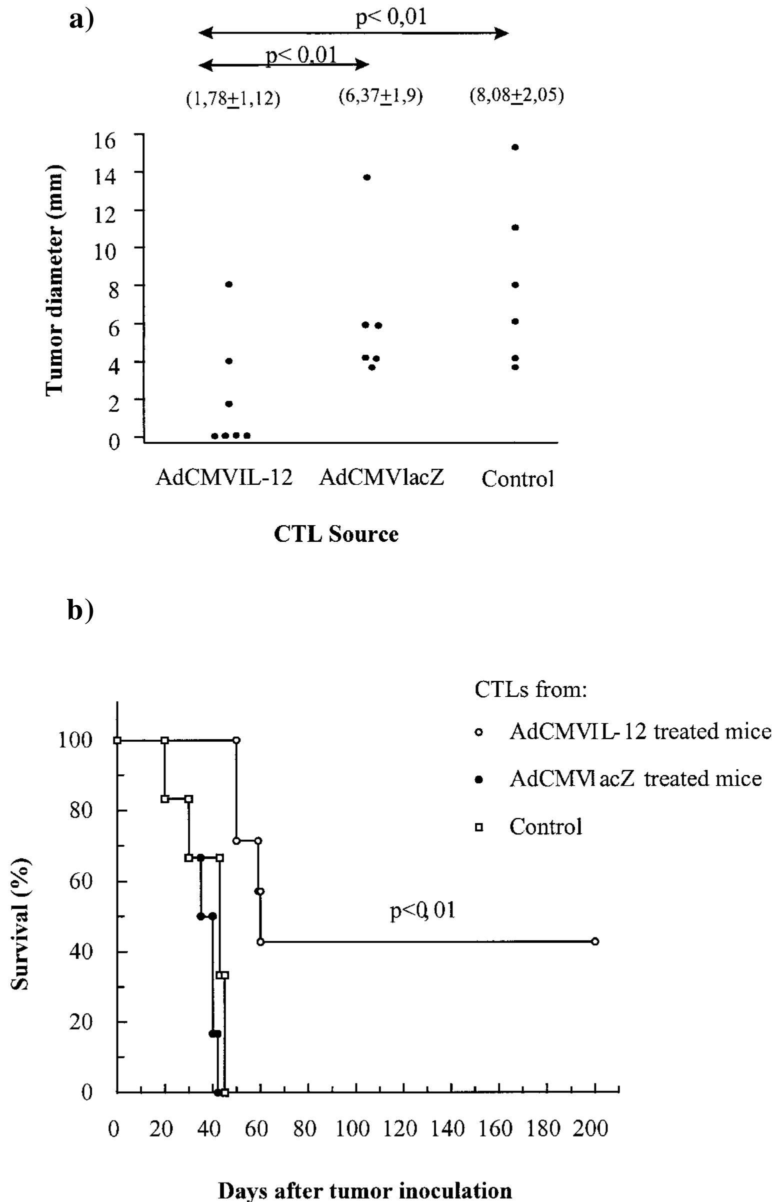

FIG. 1. Intratumoral injection of AdCMVIL-12 raises CTL cultures effective for adoptive therapy. CTL cultures were set up by coculturing mitomycin C-treated CT26 cells with mononuclear cells from draining lymph nodes of mice bearing 4- to 8-mm subcutaneous CT26 tumors previously treated by intratumoral injections of 10^8 PFU of AdCMVIL-12, 10^8 PFU of AdCMVlacZ, or saline buffer. Three groups of mice, which had been injected with 5×10^5 viable CT26 cells in the midlobe of the liver on day 0, were given on day 4 5×10^6 cells from each of the corresponding CTL cultures. Treatment was completed in every group by three doses (on alternate days) of 2×10^4 IU of hrIL-2. Tumor size **(a)** was assessed by surgical examination on day 14 and survival was monitored thereafter **(b).** Results were reproducible in three different experiments similarly performed (data not shown).

fer of cells was supported in every case by the intraperitoneal injection of three doses of 2×10^4 IU of rIL-2 every 2 days to support the effect of injected T cells, as formerly described for these types of therapeutic regimens (Kast *et al.*, 1989; Lynch and Miller, 1991). As shown in Fig. 1, only those mice into which were transferred CTL cultures obtained from mice treated with AdCMVIL-12 showed a marked decrease in tumor growth as assessed 14 days later by surgical examination. Follow-up survival studies of such animals showed slower tumor progression, in most instances with 40–60% tumor eradications depending on the experiment (Fig. 1b). In some cases, the liver tumors observed in mice treated with CTLs obtained from the AdCMVlacZ groups were slightly smaller than those of mice treated with CTLs from the saline-treated groups, but complete regressions did not occur, nor was a clear extension of survival observed. It is worth mentioning that mice treated with CTL cultures from the lymph nodes of the saline control groups developed multiple peritoneal tumors that were not observed in the other groups, indicating that adoptive transfer had had a therapeutic effect to prevent or control these kinds of metastases (data not shown).

In conclusion, delivery of IL-12 by recombinant adenovirus allowed the induction of effective CTL populations for adoptive therapy.

$CD8^+$ *cells mediate the antitumor effect*

Our 7-days cocultures of lymph node cells from mice whose tumors had been treated with AdCMVIL-12 and nonproliferating CT26 cells contained cellular mixtures with 20–50% $CD8^+$

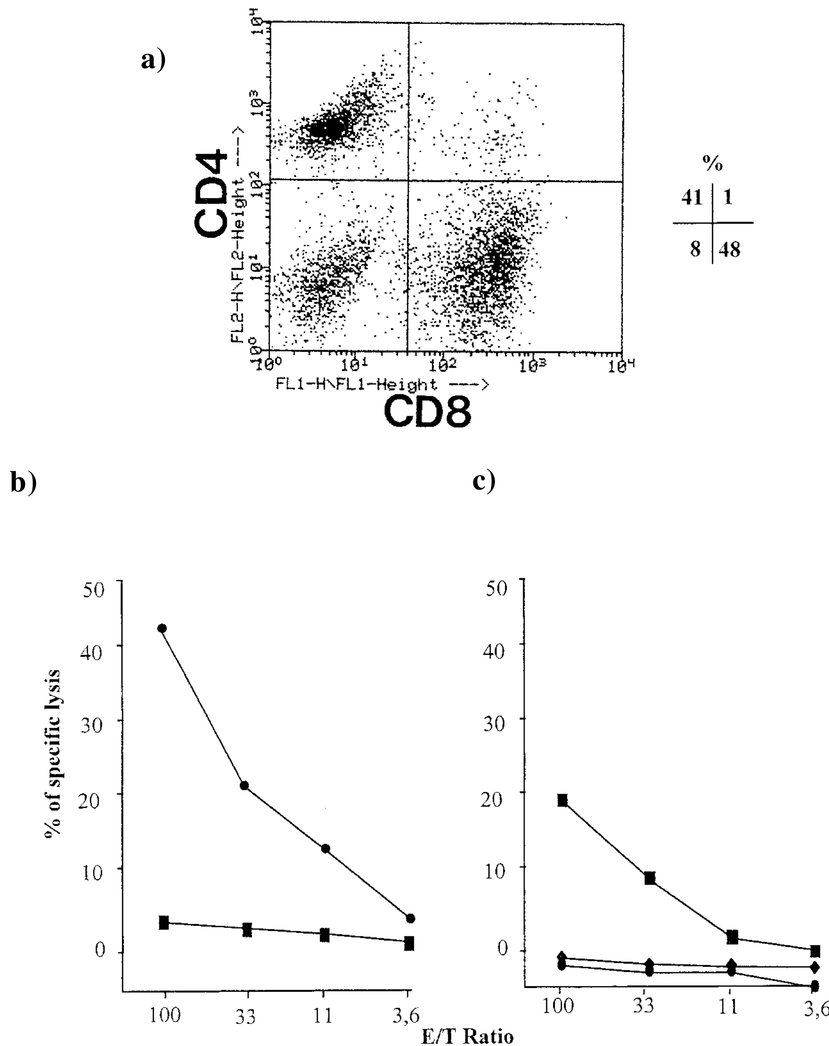

FIG. 2. Adoptively transferred T cell cultures contain anti-CT26-specific CTL activity. (**a**) FACS analysis after double staining by immunofluorescence with anti-CD4/anti-CD8 MAbs of a CTL culture obtained from mice whose subcutaneous CT26 tumors had been treated with AdCMVIL-12 as in Fig. 1. (**b**) Specific lysis against CT26 measured by standard ^{51}Cr release assays of CTL cultures obtained from mice bearing subcutaneous CT26 tumors injected with 10^8 PFU of AdCMVIL-12 (circles) or AdCMVlacZ (squares). (**c**) Specific lysis of P815 cells pulsed during a ^{51}Cr release assay with 1 μM AH1 peptide (squares) or 1 μM control irrelevant peptide (circles), or left unpulsed (diamonds), mediated by CTL cultures derived from mice whose tumors had been injected with 10^8 PFU of AdCMVIL-12.

T lymphocytes (Fig. 2a). Such lymphocyte populations displayed specific cytolytic activity against CT26, in contrast with cultures derived from mice bearing AdCMVlacZ- or saline-treated tumors. Indeed, CT26 cells were efficiently killed *in vitro* while the P815 mastocytoma cell line bearing identical MHC molecules was not (Fig. 2b). None of the cultures significantly lysed YAC-1 cells, indicating that NK activity was low in those lymphocyte cultures (data not shown). CTL clones obtained from mice immunized with CT26 granulocyte-macrophage colony-stimulating factor (GM-CSF) transfectants have been used to identify an immunodominant antigenic peptide of the tumor presented by H-2L^d. This peptide, termed AH1, was shown to be encoded by the *env* gene of the endogenous retrovirus Moloney murine leukemia virus (MoMuLV) (Huang *et al.*, 1996). CTLs directed against this antigen were also present in the spleens of animals treated with a recombinant herpes viral vector encoding IL-12 (Toda *et al.*, 1998). Results shown here (Fig. 2c) confirm that at least part of the CT26-specific CTL activity detected in our cultures was against this antigen when using P815 cells as targets pulsed with AH1 synthetic peptide.

The identity of the effector cells in the *in vivo* antitumor activity was addressed by negative and positive selection methods. Figure 3 displays results obtained in an experimental setting identical to the setting used in Fig. 1, but in this case CTL cultures obtained from mice whose subcutaneous tumors had been treated with AdCMVIL-12 were selectively depleted of CD4$^+$ or CD8$^+$ cells by incubation *in vitro* with specific MAb plus rabbit complement before being used for adoptive therapy. Results show a critical involvement for CD8$^+$ but not CD4$^+$ T cells.

TABLE 1. DOSE-DEPENDENT ANTITUMOR EFFECT OF CD8$^+$ CELLS

	Tumor size[b]	
CTL dose[a]	*Individual sizes*	*Mean*
0	10, 9, 20	13
2×10^4	3, 8, 8.5	6.5
2×10^5	0, 5.5, 8	4.5
2×10^6	0, 3, 2	1.7

[a]Number of CD8$^+$ cells purified from CTL cultures by immunoselection with magnetic beads, adoptively transferred intravenously to mice hosting liver CT26 tumors 4 days prior to adoptive therapy. Purity of CD8$^+$ cell suspensions was found to be above 98% by FACS analysis.

[b]Individual and mean tumor diameters, in millimeters. Size was measured by surgical inspection of the liver on day 12 after adoptive transfer.

In addition, when CD8$^+$ cells were purified from the cultures by magnetic immunoselection yielding 98% purity, selected cells displayed a dose-dependent antitumor effect, indicating that CD8$^+$ T cells were the necessary and sufficient populations for adoptive therapy (Table 1). Moreover, a lymphoid infiltrate was also observed in established CT26 tumors implanted in the liver of athymic BALB/c$^{nude/nude}$ mice that were treated intravenously with this CTL culture, thus clearly suggesting that adoptively transferred cells could home into the tumor tissue (data not shown).

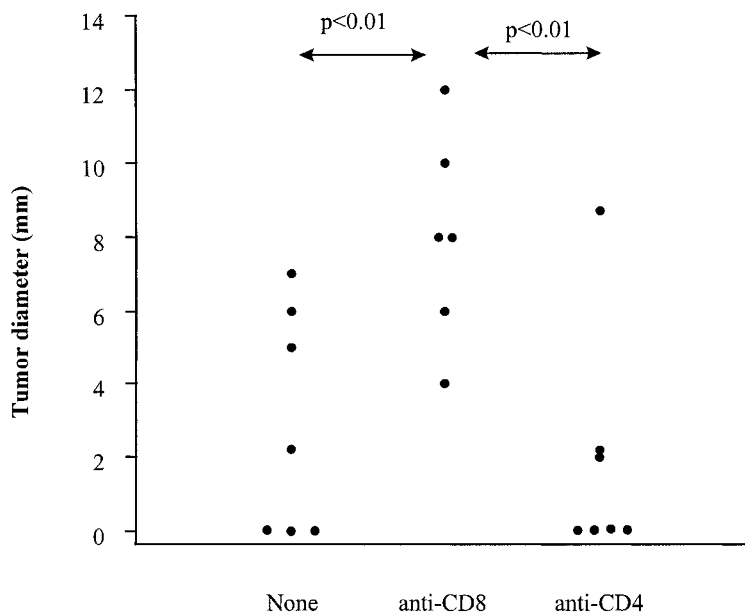

FIG. 3. CD8$^+$ T cells are required for the antitumor effect of adoptive therapy. Cells (5×10^6) from the CTL cultures generated from draining lymph nodes of mice with subcutaneous CT26 tumors treated with AdCMVIL-12, under conditions identical to those in Fig. 1, were adoptively transferred intravenously to mice that harbored CT26 tumors implanted 4 days previously in the liver. In this case, prior to adoptive transfer, cultures were depleted of CD4$^+$ or CD8$^+$ T cells by *in vitro* treatment with anti-CD4 MAb, anti-CD8 MAb, or medium and subsequently treated with rabbit complement. Tumor sizes were monitored 10 days after adoptive therapy.

Adenoviral transfer of IL-12 into CT26 tumors displays a synergistic effect with adoptive transfer of antitumor T lymphocytes

The preceding data show that AdCMVIL-12 treatment benefits T cell adoptive therapy through its effect on the induction of a stronger CTL response, easing the *in vitro* obtention of effective T cell cultures. We then explored the possibility that AdCMVIL-12 and CTL cultures were also synergistic at the antitumor effector phase. In this case, the CTLs (obtained under culture conditions similar to those described above) were given 5 days after the intratumoral injection of AdCMVIL-12. A tumor model was established by injection of 5×10^5 CT26 cells, both subcutaneously in the left flank and inside the midlobe of the liver. In these animals, both tumors progressed and killed the animals if left untreated. This experiment was carried out to mimic the frequent condition of having a local colon carcinoma with the suspicion of or evidence that the malignancy has spread into the liver. In a first set of experiments, tumors were allowed to grow for 10 days to reach a size of 4–8 mm in both sites. Under such conditions, we verified that the formation of the subcutaneous and intrahepatic tumors did not in-

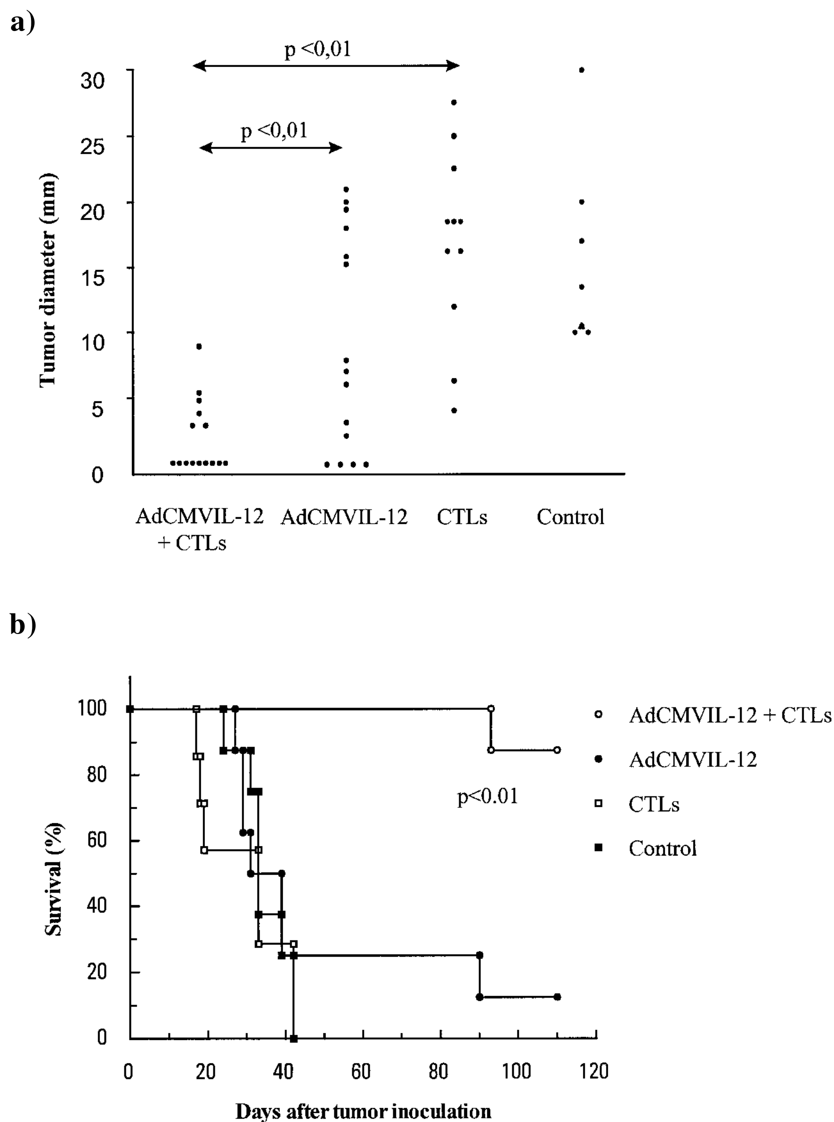

FIG. 4. Synergy of AdCMVIL-12 and T cell adoptive therapy in the treatment of distantly spread CT26 tumors. Mice hosting two CT26 tumors, one implanted subcutaneously and the other inside the midlobe of the liver, were divided in four groups that received the following injections: 10^8 PFU of AdCMVIL-12 in the subcutaneous tumor, 5×10^6 cells from the CTL cultures (intravenous), both treatments, or none. AdCMVIL-12 on day 10 and/or CTLs on day 15 after tumor inoculation were given to the indicated groups. The outcome was monitored by measuring the size of the liver on day 21 after tumor engraftment (**a**) or by following the survival of the different groups of mice (**b**). Every group received doses of 2×10^4 IU of rIL-2 intraperitoneally on three alternate days after the onset of treatment. Results represent pooled data from two different experiments sequentially performed.

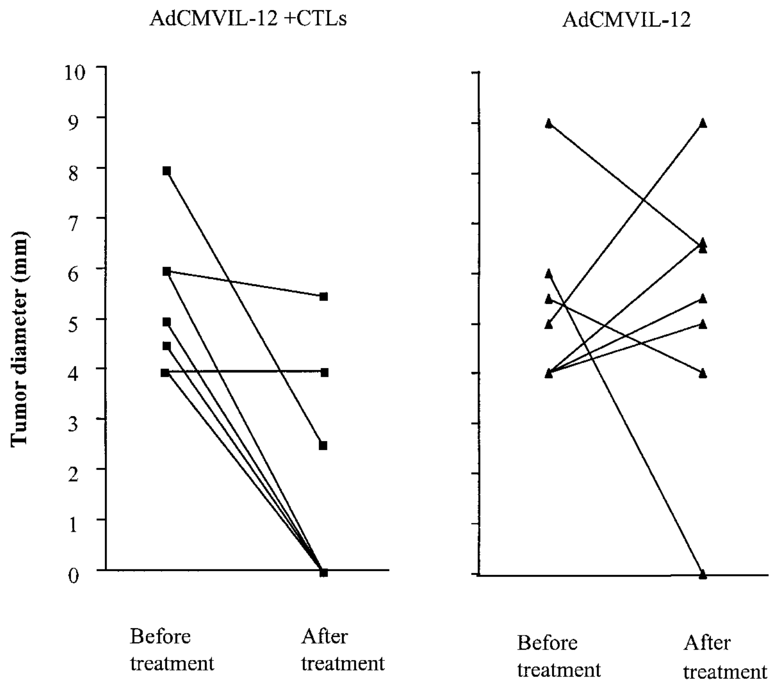

FIG. 5. Local injection of suboptimal doses of AdCMVIL-12 into a CT26 tumor nodule inside the liver synergizes with systemic administration of anti-CT26 CTLs. Mice bearing a single CT26 tumor nodule in the midlobe of the liver were injected on day 10 after tumor implantation with 10^7 PFU of AdCMVIL-12 with (*left*) or without (*right*) subsequent intravenous treatment by adoptive transfer of 5×10^6 cells from the CTL cultures. Individual size of tumor nodules was assessed before AdCMVIL-12 injection as well as 8 days after injection. Both groups received a similar regimen of treatment with IL-2.

terfere with each other without treatment (data not shown). Then, as shown in Fig. 4, four groups of mice were studied. The first group received the intravenous injection of CTLs in addition to the injection of AdCMVIL-12 into the subcutaneous tumor nodule. The other three groups received only one of these treatments or none. When the liver tumors were surgically inspected 10 days after the onset of treatment, a marked antitumor effect was observed in the CTL + AdCMVIL-12 group, leading to the complete regression of the hepatic tumors in 9 of 16 cases and with a clear decrease in the remaining tumor sizes. Single inoculation of AdCMVIL-12 into the subcutaneous site, but without CTL transfer, displayed only limited antitumor activity at the liver site. In the case of mice receiving only CTLs, no activity was detected against these relatively large nodules. Survival data confirmed these observations (Fig. 4b) and suggested that some tumors still detected on day 10 posttherapy eventually regressed. It is noteworthy that CTLs by themselves were efficacious against small tumor burdens (Fig. 1) but were unable to treat larger tumor nodules (Fig. 4).

It is interesting that combined therapy also improved the effect of AdCMVIL-12 injection at the local dermal site. In the follow-up of the subcutaneous tumor nodules, 14 of 15 cases receiving combined therapy completely regressed whereas only 10 of 15 regressed in those mice receiving AdCMVIL-12 only (data not shown). In the group receiving the infusion of CTLs but without AdCMVIL-12 only 1 regression of the subcutaneous nodule was observed of 10 mice, in comparison with none in the control group (data not shown). These data suggested that there was also synergy between local treatment by AdCMVIL-12 and the systemic administration of CTLs. To further confirm these observations, hepatic tumors were given an intratumoral injection of 10^7 PFU of AdCMVIL-12, a dose that had been found to be suboptimal for liver tumors implanted 10 days before treatment (G. Mazzolini, unpublished observations, 1998). Under these suboptimal conditions the intravenous infusion of CTLs on day 15 after injection of tumor cells was responsible for a marked improvement in efficacy in reducing the size of the tumors on day 21 (Fig. 5). Such data confirm the synergy observed at the effector phase of AdCMVIL-12 + CTLs combined therapy when studied locally at the adenovirally transduced tumor site.

Recombinant adenovirus injected in the subcutaneous tumor nodule gains access to liver parenchyma

To address the mechanisms underlying the effects on the liver tumors induced by injecting AdCMVIL-12 into a concurrent subcutaneous tumor nodule, several experiments were carried out. In a group of mice carrying concomitantly a subcutaneous CT26 tumor and intrahepatic tumor nodules, the subcutaneous tumor was treated with 5×10^8 PFU of AdCMVlacZ. Frozen sections of the midlobe of the liver of such animals harvested 2 days later were stained for β-galactosidase activity. On microscopic examination, scattered strongly positive cells were detected in the healthy liver parenchyma (Fig. 6B). It is interesting that an intense positive staining was detected in the liver tissue surrounding tumor nodules, thus de-

picting a rim of transduced cells. Also, some cells stained positive within such malignant lesions (Fig. 6A).

In a similar setting IL-12 p70 was detected by ELISA in homogenates of liver tissue when the subcutaneous tumor had been treated with AdCMVIL-12 (5×10^8 PFU) (Fig. 6C). Liver IL-12 concentrations per 100 mg of tissue were at least 10 times lower than that observed in the subcutaneous malignant nodule, which received the AdCMVIL-12 dose directly (Fig. 6D).

Our data strongly suggest a role for "leaky" adenovirus reaching the systemic circulation and eventually infecting the liver tissue in the observed therapeutic synergy. Such a phenomenon establishes a link between treatment of the subcutaneous tumor and the effects on the experimental metastasis in the liver.

DISCUSSION

The present study provides evidence that the efficacy of adoptive therapy with CTLs can be enhanced by active im-

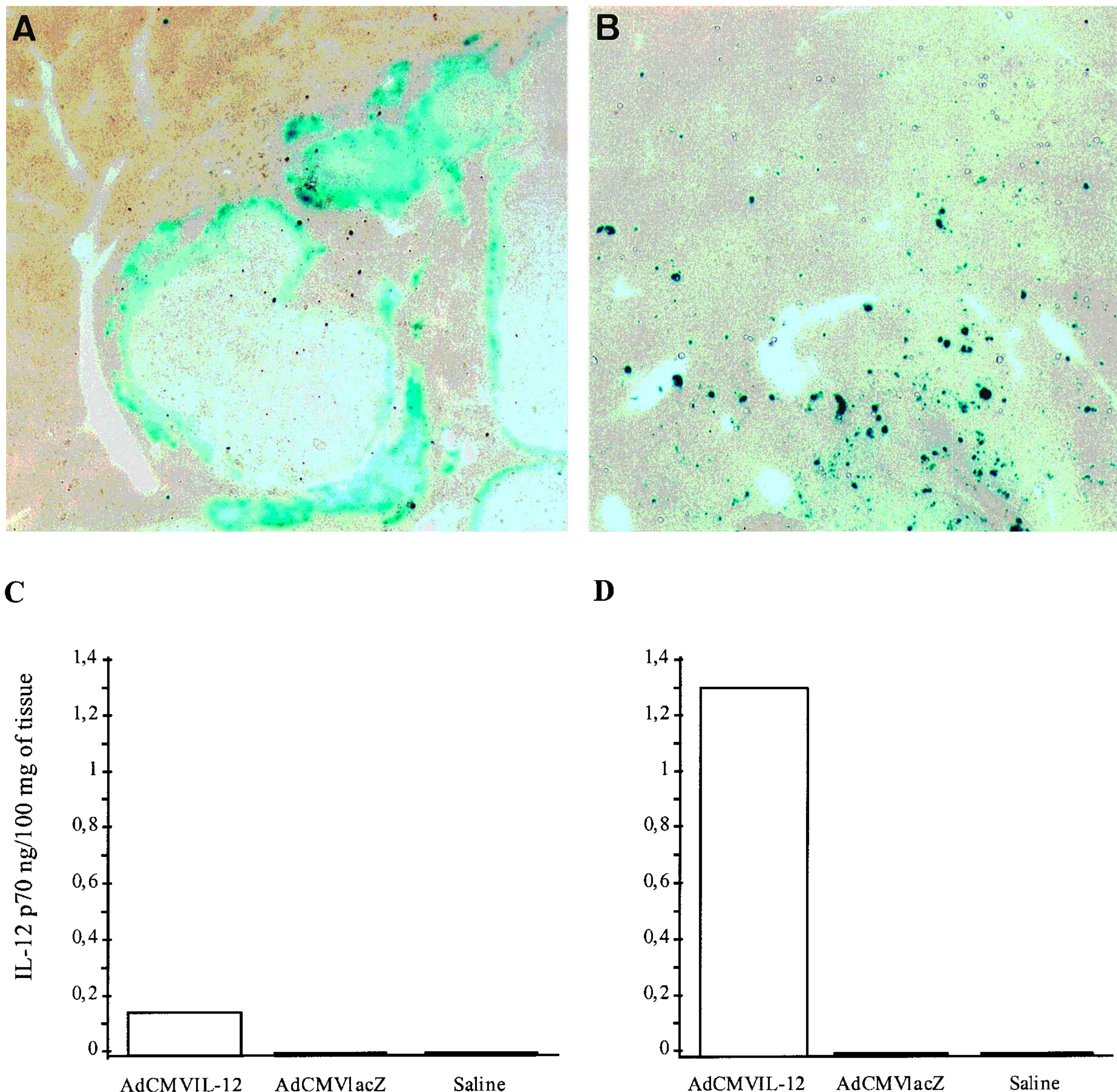

FIG. 6. Liver cells express the transgene encoded by a recombinant adenovirus injected into a subcutaneous tumor nodule. (**A** and **B**) Frozen sections of livers carrying CT26 tumor nodules stained by X-Gal that had been obtained from mice carrying a concomitant subcutaneous tumor treated with 5×10^8 PFU of AdCMVlacZ 2 days prior to liver harvest. In (**A**) healthy liver and tumor nodules are shown ($\times$ 10) in (**B**) healthy liver parenchyma is shown with greater magnification ($\times$ 20). (**C** and **D**) IL-12 p70 concentration per 100 mg of tissue obtained by ELISA in homogenates of (**C**) liver tissue and (**D**) concomitant subcutaneous tumor nodules injected with the indicated adenovirus or saline buffer 3 days prior to tissue collection. The concentration of IL-12 in tissue samples treated with AdCMVlacZ or saline was under the detection threshold of the ELISA (15 pg/ml). Data are representative of two different experiments.

munotherapy, as provided by an IL-12-encoding adenoviral vector. Thus, gene transfer of IL-12 into CT26 tumors improves the yield of tumor-specific CTLs in short-term cultures. Moreover, systemic treatment with those CTL cultures and infection of the tumor tissue with AdCMVIL-12 show synergy not only against tumor nodules transduced with the recombinant adenovirus but also against distant, nonadenovirally transduced tumor sites.

IL-12 has been shown to promote CTL precursor expansion and differentiation both *in vitro* and *in vivo* (Trinchieri, 1998). Since functional IL-12 receptors (IL-12Rs) have been detected on $CD8^+$ T cells, upregulation of CTL activity could result in part from direct effects of the cytokine on this T cell subset (Trinchieri, 1998). However, IL-12 also activates IFN-γ secretion and other functions from other cell types such as helper T lymphocytes and NK cells that can subsequently promote CTL expansion and activation (Topalian, 1994; Kos and Engleman, 1996). The importance and need of helper T populations for the IL-12-boosted antitumor response vary with the different tumor cell lines and experimental systems. Using IL-12 transfectants in CT26, it has been reported that $CD4^+$ T cells have a deleterious effect on the antitumor response otherwise mediated by $CD8^+$ T cells and NK cells (Martinotti *et al.*, 1995). However, it is conceivable that CTL generation, even in the CT26 setting, can be favored by Th1 cells that secrete IL-2 and IFN-γ (Trinchieri, 1998) and that are able to license professional APCs for activation of CTL precursors (Lanzavecchia, 1998; Ridge *et al.*, 1998). In fact, antitumor effects mediated by $CD4^+$ cells have been reported by the same group when the IL-12 transfectants are injected into IFN-γ receptor knockout mice (Zilocchi *et al.*, 1998). In this regard, too-high doses of IL-12 have been reported to downregulate the T cell response, acting through an excess of IFN-γ (Kurzawa Koblish *et al.*, 1998; Lasarte *et al.*, 1999).

Regardless of the complexity of the mechanisms whereby IL-12 augments the CTL response against CT26, we have observed that anti-CT26 CTL activity could be detected only after gene transfer of IL-12 into the tumors. Our short-term CTL lines were, at least in part, directed to the AH1 tumor antigen encoded by the *env* gene of an endogenous retrovirus, present in the genome of normal BALB/c mice, which is expressed by the CT26 tumor cell line (Huang *et al.*, 1996). Similar results of specificity had been obtained using a herpesvirus-based vector to transfer IL-12 expression into CT26 tumors *in vivo* (Toda *et al.*, 1998). Our CTL cultures did not lyse unpulsed P815 cells or YAC-1 cells, thus excluding lack of specificity. Even though CTL clones, rather than short-term CTL lines are usually preferred for T cell adoptive therapy, an early-passaged CTL line may be advantageous in preventing tumor antigen escape variants, because they are less selected by immunodominant antigens and can recognize more than one epitope. Experimentation in progress in our laboratory is comparatively testing the use of long-term versus short-term CTL lines in various *in vivo* tumor models.

Adoptive T cell therapy (Ridell and Greenberg, 1995; Yee *et al.*, 1997) against tumor antigens has been successfully used in a number of cases (Kast *et al.*, 1989; Lynch and Miller, 1991; Ridell and Greenberg, 1995; Yee *et al.*, 1997). We show that short-term CTL cultures from mice whose tumors were undergoing AdCMVIL-12-induced regression displayed a potent effect against small CT26 tumors implanted as cell suspensions in the liver, 4 days prior to adoptive therapy. Such an effect was not observed in identical lymphocyte cultures obtained from CT26 tumor-bearing animals treated with a control adenovirus or left untreated, indicating the dependence of CTL activity on the expression of the IL-12 transgene. These data imply that intratumoral treatment with AdCMVIL-12 simplifies the subsequent *in vitro* growth of effective CTLs. Although adoptively transferred $CD4^+$ cells have displayed antitumor activity in some systems (Hu *et al.*, 1993), the presence of $CD4^+$ T cells in our adoptively transferred cultures is not a requirement and only the depletion of $CD8^+$ cells abrogated their *in vitro* and *in vivo* activity. Moreover, purified $CD8^+$ cells from those cultures were sufficient to execute, in a dose-dependent manner, the antitumor effect on adoptive transfer. These results exclude a relevant role for other minor cell populations such as dendritic cells that could be present in our short-term T cell cultures. As a broader interpretation of these experiments, we postulate that protocols of adoptive immunotherapy will benefit from previous active immunotherapy approaches performed on the same patient, which thereby augment the number of responding T cells available for their subsequent *in vitro* expansion. Among the candidates for those active therapy procedures are the gene transfer of cytokines or costimulatory molecules to the tumor mass (Chen *et al.*, 1993) and/or vaccination with tumor antigens presented by dendritic cells (Mayordomo *et al.*, 1995). However, we cannot confirm that adoptively transferred antitumor CTLs carry out their effects only the basis of their tumor-specific cytolytic potential since their ability to secrete different cytokines can also be involved.

Intratumoral injection of AdCMVIL-12 into CT26 tumors causes tumor regressions in 60–80% of cases (Mazzolini *et al.*, 1999). In several systems it has been shown that IFN-γ is the main downstream mediator of the IL-12 antitumor effects. In the liver, a key role for NK T cells as a source of IFN-γ in the antitumor response induced by IL-12 has been identified using gene-targeted mice (Cui *et al.*, 1997). IFN-γ in turn induces a plethora of effects not only on immune system cells but also on tumor cells, making them prone to be lysed by CTLs (Coughlin *et al.*, 1998). Evidence shows that the chemokines IP-10 and Mig (monokine induced by IFN-γ) are induced by IFN-γ, on administration of IL-12, and mediate a potent tumor angiogenesis inhibition, while theoretically they also drive activated T cells to the tumor tissue.

Experiments carried out in mice bearing two distant tumor nodules, subcutaneous and intrahepatic, have found that injection of AdCMVIL-12 in one tumor site (subcutaneous nodule) has therapeutic effects on the second nodule, but only in a minority of cases. In contrast with the timid therapeutic effect on the liver tumor induced by AdCMVIL-12 injected into the subcutaneous nodule, combined administration of intravenously, adoptively transferred CTLs and injection of AdCMVIL-12 in the subcutaneous tumor nodule causes a dramatic antitumor effect against relatively large, well-established intrahepatic metastasis and further improved the outcome of subcutaneous tumor nodules themselves. Our current research is trying to disentangle the main molecular mechanisms behind the observation of the synergy phenomenon. A major question is whether IL-12 and/or IFN-γ can act at the distant, nonadenovirally transduced tumor site. In some reported models (Bramson *et al.*, 1997) and in our hands, a fraction of the adenovirus injected

into the tumor gains access to the systemic circulation and infects the healthy liver parenchyma, giving rise to expression of the transgene. Secretion of IL-12 in the liver can set in motion a cascade of proinflammatory cytokines potentially cooperative with adoptively transferred CTLs and with direct antitumor activity. The above-mentioned studies of NK T cell-deficient mice (Cui *et al*., 1997) predict a role for these liver-located lymphocytes in the overall process that is being addressed. If this scenario of local cytokine secretion inside the liver is important for the observed synergistic effect, then it might be advantageous to treat liver metastasis but perhaps not as powerful to treat the extension of the disease to other organs. Nonetheless, liver metastases are frequent, often fatal, complications of colon cancer. It is interesting that transgene-expressing cells were much more frequent in the liver tissue located in the immediate neighborhood of the malignant nodules. Such transduced cells form a sort of rim around the tumor nodules and could be involved in the observed therapeutic effects. Our current research is dealing with the molecular mechanisms explaining the selectivity of infection around the malignant tissue.

IL-2 and other cytokines are routinely used to potentiate adoptive therapy. In our protocols we injected rIL-2 intraperitoneally in all the experimental groups because of the belief that fully differentiated CTLs cannot produce IL-2 (Kast *et al*., 1989; Lynch and Miller, 1991; Ridell and Greenberg, 1995; Yee *et al*., 1997). Along this line, data show that coinjection into tumor tissue of two different recombinant adenoviruses encoding IL-12 and IL-2 had a marked synergistic effect (Addison *et al*., 1998), which likewise had been observed with the recombinant cytokines. However, the IL-2 doses given in our adoptive transfer protocols did not display measurable antitumor activity by themselves.

Our preliminary data indicate that AdCMVIL-12 injection into a subcutaneous CT26 tumor enhances the expression of vascular cell adhesion molecule 1 (VCAM-1) on the endothelial cells present within concomitant liver tumor nodules. We are exploring whether this phenomenon favors the homing of antitumor lymphocytes into malignant tissue as has been suggested (Ogawa *et al*., 1997), thus providing a mechanistic view of the observed effector-phase synergy (G. Mazzolini, unpublished observations, 1998). In light of the data showing expression of the transgene in the liver, the IL-12-triggered cascade of cytokines might be responsible for this local upregulation of VCAM-1.

Taken together, our data provide evidence of a potent synergy between T cell adoptive therapy and gene transfer of IL-12 into tumor tissue. Both therapies cooperate at least at two different levels: (1) AdCMVIL-12 treatment enhances CTL responses and facilitates their *in vitro* culture; and (2) systemic administration of CTLs and local treatment with AdCMVIL-12 display a potent effect against metastatic disease. Such a combined therapeutic strategy, rather than having an additive effect, shows a powerful synergy that should not be overlooked in the design of future clinical applications.

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