Interleukin-7/B7.1-encoding Adenoviruses Induce Rejection of Transplanted but not Nontransplanted Tumors

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ABSTRACT

Most cancer vaccine trials are based on efficacy studies against transplanted mouse tumors that poorly reflect the clinical situation. We constructed adenoviruses expressing interleukin-7 and B7.1 and tested their therapeutic efficacy after transfer into established transplanted and nontransplanted 3-methylcholanthrene-induced tumors. The adenoviruses efficiently induced rejection of transplanted tumors, leaving behind systemic immunity. Against nontransplanted tumors of similar size, there were almost no therapeutic effects. This result was not due to the site of tumor development, tumor type, general immune suppression, or differences in transduction efficacy. Adenoviral expression of β-galactosidase as a surrogate antigen in nontransplanted tumors induced cytotoxic T cells that were unable to quantitatively reach the tumor site. Based on rigorous mouse models and an effective in situ immunization procedure, it is suggested that cancer vaccines can be effective, if at all, against “minimal residual disease”; additional experimental procedures must be found against established nontransplanted tumors.

INTRODUCTION

Many tumor cells express antigens against which an immune response can be induced. In mice, such antigens can serve as rejection antigens for transplanted tumors (1). For example, 3-MC-induced tumors vary in inherent immunogenicity and often bear rejection antigens that are usually unique to the individual tumor (2–6). Rejection of transplanted tumors in immunized mice usually requires T cells, often CD8+ CTLs. The existence of associated or specific antigens has stimulated a variety of vaccine strategies. These include vaccination with tumor antigens as whole protein or peptide with or without adjuvant (7, 8), tumor antigen-presenting dendritic cells (9), tumor antigen-encoding viruses (10) or genetically modified tumor cells (11). A common denominator of these vaccines is that tumor antigens are provided in an immunogenic form at a neutral site to induce T cells that are subsequently expected to migrate to and eradicate the tumor. Whereas all these vaccines have shown some efficacy when given prophylactically or shortly after tumor challenge, the results were essentially obtained in tumor transplantation experiments. Transplanted and nontransplanted (autochthonous) tumors differ in several regards: nontransplanted tumors originate from one cell that has to acquire malignancy in a stepwise process by accumulation of somatic mutations. Therefore, tumor growth is slow (in mice, the time is in the range of several months), subject to evolutionary processes, and orthothropic. The nontransplanted tumor has more time and can use its natural environment to manipulate the host, e.g., to recruit host cells or use immune escape strategies (12). In contrast, tumor transplantation usually requires a high initial tumor cell inoculum. Then, however, the tumor grows much more rapidly (in the range of few weeks), because the cells have become malignant already in their initial host. One reason why successful tumor transplantation requires quite a large number of cells to be injected is that the cultured cells are usually transferred as a cell suspension (13). On one hand, this may allow some tumor cells to detach more easily from the injection site, which could facilitate the induction of an immune response (14, 15). On the other hand, the tumor might be more accessible for infiltrating immune cells (16). For example, implantation of tumor fragments requires fewer cells for tumor take in comparison to the same tumor injected as a cell suspension (13). An additional problem with transplanted tumors is that growth is most often studied at a site different from its origin, and it is very difficult to exclude phenotypic changes occurring during culture.

It has been known for a long time that the autochthonous host can be immunized against its own tumor (17). Therefore, tumors were induced by 3-MC, surgically removed after approximately 3 months at a size of 1 cm in diameter, established in culture, and used for immunization with irradiated cells and subsequent challenge with viable cells of the original host. It showed that the mice developed immunity to the transplanted tumor to a similar degree compared to naive syngeneic control animals. Whereas this experimental setting clearly indicated that tolerance (at least in the absence of the tumor) was not responsible for the failure to reject the nontransplanted tumor, it did not allow the determination of whether immunity induced by vaccination would have been operative against the primary, established tumor. Based on some transplanted tumor models, this is questionable. Transplantation of a tumor and a skin graft bearing the same defined antigen resulted in rejection of the skin but not the tumor graft (18). Half of the mice immunized by skin graft rejection rejected a subsequent tumor challenge through the shared antigen, but no tumor rejection was observed when skin and tumor grafts were given simultaneously (18). The unfavorable kinetic of T-cell activation compared to the rapid growth of the transplanted tumor may be responsible for this effect. This does not exclude the possibility that the established transplanted tumor can be made to be rejected by an in situ increase of its immunogenicity through a transfer of genes that encode immunostimulatory activity, as has been shown in other tumor models (19, 20). Therefore, the purpose of this study was to establish a system that allowed us to demonstrate rejection of established transplanted tumors at a time when they resist several of the above-mentioned vaccination strategies (21, 22) and then to ask how these results compare to those obtained with nontransplanted tumors.

This experimental setting required an efficient in vivo gene delivery system such as recombinant adenoviruses (23). We showed that a tumor cell vaccine coexpressing IL-7 and B7.1 compares well with a classical adjuvant admixed to tumor cells (24). Similar to others (25, 26), we found that the mode of CTL induction by the vaccine cells is both direct by the engineered tumor cells (in the case of B7.1) and indirect by antigen-presenting cells of the host (in the case of IL-7 and B7.1; Ref. 27). Here we describe the construction of adenoviruses encoding IL-7 and B7.1 that are used to deliver the genes in vivo into established tumors. The results show that the virus effectively induces rejection of established transplanted tumors but not nontransplanted...
tumors, probably because T cells inefficiently infiltrate the nontransplanted tumor.

**MATERIALS AND METHODS**

**Construction and Preparation of Recombinant Replication-defective Adenoviruses.** The CMV promoter-driven expression cassette allowing co-expression of the murine IL-7 and B7.1 genes was constructed in pcDNA3 (Invitrogen, Groningen, the Netherlands). Therefore, the IL-7 cDNA from plasmid pRML-IL-7 (28) was inserted into pcDNA3 as a BamHI fragment yielding pcDNA3.mIL-7. B7.1 cDNA was released from plasmid pHYTKCMV-mB7.1 (24) as a XbaI-ClaI fragment and cloned behind the poliovirus IRES of HncoII-cleaved plasmid pPBS. The resulting plasmid pPBS.mB7.1 was cut with XbaI and Apal to recover the IRES-B7.1 fragment, which was then cloned behind the IL-7 cDNA of XbaI and Apal digested pcDNA3.mIL-7. The resulting plasmid, pcDNA3.mIL-7/IRES/mB7.1, was digested with NotI and Smal, and a 3.8-kb fragment harboring the complete expression unit was then cleaved with PvuI to remove plasmid backbone sequences. This 3015-bp fragment was finally ligated into EcoRV-cut adenovirus shuttle plasmid pAdE1spA1a (29). Recombinant virus was generated by cotransfection of the shuttle plasmid with pMM17 (30) in subconfluent cultures of 293 cells using a CaPO4 transfection kit (Mammalian Transfection Kit; Stratagene, Heidelberg, Germany). Resulting plaques were picked, amplified once on 293 cells, and tested by PCR analysis using the following oligonucleotide primers: (a) sense strand IL-7, 5'-TGGAAATTCTCCACTGATCCT-3'; (b) antisense strand IL-7, 5'-GTGCTTTGTGATACTGTTAGTAAAGTGACACA-3'; (c) sense strand B7.1, 5'-CAATCGATCTGAGCTATGCGCTAATGGTC-3'; (d) antisense strand B7.1, 5'-GAATCGATCTAAGGAAGCGCTTCTGTACGTC-3'; and (e) adenovirus E4 primers as described previously (31). Furthermore, mB7.1 expression of the plaque isolates in day 1 infected 293 cells was determined by FACSscan analysis (see below). Positive isolates were finally plaque-purified twice. The adenovirus expressing the nuclear-targeted β-gal protein driven by the CMV promoter (Ad.βgal) was kindly provided by Ronald G. Crystal (The New York Hospital-Cornell Medical Center, New York, NY). For preparation of purified virus stocks, 293 cells were infected at a m.o.i. 5 and harvested after cytopathic effect became visible (48 h). A crude virus lysate was obtained by three rounds of freezing (−196°C) and thawing (37°C) of the collected cells and subsequent removing of the cell debris. The virus suspension was subjected to two rounds of CsCl step gradient centrifugation (32). CsCl was removed by gel filtration using Sephadex G25 columns (PD25; Pharmacia, Freiburg, Germany), and virus aliquots were stored at −80°C in storage buffer containing 150 mM NaCl, 3 mM KCl, 1 mM MgCl2, 10 mM Tris (pH 7.4), and 10% glycerol. Titer were determined by plaque assay or limiting dilution assay on 293 cells, and only adenovirus preparations with a particle:pfu ratio < 100 were used.

**Cell Culture.** The 293 cell line (ATCC CRL 1573; human transformed primary embryonic kidney cells) was maintained in DMEM supplemented with either 10% FCS or 5% horse serum (after adenoviral infection). Tumor cell lines syngeneic to BALB/c were as follows: (a) spontaneous mammary adenocarcinoma cell line TS/A (33); (b) the subline TS/A-IL-7/B7.1, which stably expresses IL-7 (400 units/ml) and B7.1 after retroviral transduction (24); (c) N-nitroso-N-methylurethane-induced colon carcinoma CT26 (34); and (d) Ψ2-pMFγnlslacZ retrovirally infected subline CT26-βgal (27, 35). Yac-1 is a MHC-deficient natural killer cell target cell line (36). MC51-9 is a 3-MC-induced tumor syngeneic to 129SvEi mice and will be described in detail elsewhere. All murine tumor cell lines were grown in RPMI 1640 plus 10% FCS. Cell line IxN/2b (37) for IL-7 bioassay was cultured in RPMI 1640/10% FCS supplemented with supernatant from TS/A-IL-7 cells (28).

**Mice.** Six-week-old female BALB/c mice or BALB/c nude mice and C57BL/6 mice were obtained from Bomholtgaard Breeding & Research Center (Ry, Denmark). 129SvEi mice were purchased from Taconic.

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Fig. 1. IL-7 and B7.1 expression of Ad.IL-7/B7.1-infected TS/A cells. The amount of transgene expression was analyzed before and 2 and 5 days after infection with Ad. IL-7/B7.1 (m.o.i. = 100). IL-7 expression was analyzed by the induction of proliferation of the IL-7-dependent growing cell line 1xN2b and is expressed as units (ng) per 10⁶ cells and 24 h. B7.1 expression was determined by fluorescence-activated cell-sorting analysis of TS/A cells. Cells were stained with PE-labeled rat antimouse B7.1 antibody (black peaks) and isotype-matched control (white peaks).
Adenoviruses Ad.IL-7/B7.1 (f; 6 weeks later by topical application of 1.8 nmol of TPA twice weekly for 6 weeks. Tumors were measured with a caliper determining two perpendicular diameters, and mean tumor size is expressed as the mean of the largest diameter and the diameter at a right angle (39, 40). Mice were scored as tumor bearing when the tumor size was ≥ 1.0 cm.

Adenoviral Gene Transfer. For adenoviral infection in vitro, 1 × 10^5 cells per 3-cm dish plated 1 day before were washed once with Dulbecco’s PBS, incubated with adenoviruses in Dulbecco’s PBS at the indicated m.o.i. for 45 min, and incubated at 37°C in the recommended medium plus 5% horse serum. Cells were either analyzed for β-gal expression with X-gal (Sigma; Ad.βgal) or for IL-7 and B7.1 expression (Ad.IL-7/B7.1) by bioassay of supernatants as described previously (24) and FACScan analysis of cells, respectively. For in vivo gene transfer experiments, 2.5 × 10^6 T5A or MC51-9 cells were injected s.c. into syngeneic mice. Seven and 10 days later, respectively, when tumors of an average tumor size of 3 mm had developed, tumors were injected with 1 × 10^9 pfu of adenoviruses in 50 μl of Dulbecco’s PBS with a 30-gauge hypodermic needle. To minimize leakage and to optimize the penetration of adenoviruses into the tumor tissue, injections were performed very slowly, and the needle was removed after a delay of 1 min. For treatment of tumors in the autochthonous host, adenoviruses were injected into the tumor 10 weeks (for fibrosarcomas) and 12 weeks (for skin tumors) after induction with 3-MC. Injections were performed as described above, except that injections of fibrosarcomas were repeated once a week for three times.

Analysis of Adenoviral Gene Expression. Biological activity of IL-7 was tested by assaying the proliferation of the IL-7-dependent cell line IxN/2b. Briefly, cells were pelleted and washed twice with medium, and 2 × 10^4 IxN/2b cells were incubated with serially diluted supernatants of Ad.IL-7/30, Tumor Induction. For transplanted tumors, cells of the indicated lines were washed twice with Dulbecco’s PBS and injected s.c. in a volume of 0.2 ml in the middle of the left flank. Animals without tumors were monitored for at least 60 days. Challenge of mice was done with the same number of viable parental cells as for primary tumor induction s.c. at a distant site.

For induction of tumors by carcinogens, 3-MC (Sigma, Deisenhofen, Germany) was dissolved in sesame oil (Sigma) and injected i.m. in the left hind leg in a concentration of 1 mg/mouse for the induction of sarcomas (2, 4). For skin tumor induction, 0.5 mg/mouse was injected s.c. in the flank, and after 6 weeks, mice were shaved at the injection site. Twice a week, TPA (1.8 nmol) in 0.1 ml of acetone was applied topically to the shaved skin as described previously (38). Tumors were measured with a caliper determining two perpendicular diameters, and mean tumor size is expressed as the mean of the largest diameter and the diameter at a right angle (39, 40). Mice were scored as tumor bearing when the tumor size was ≥ 1.0 cm.

Fig. 3. Failure to induce rejection of nontransplanted tumors by intratumoral Ad.IL-7/B7.1 application. (a) BALB/c and (b) C57BL/6 mice were injected i.m. with 1 mg 3-MC/mouse in the hind leg. Ten weeks later, mice were injected intratumorally with 1 × 10^9 pfu of the adenoviruses Ad.IL-7/B7.1 (●, n = 25) and Ad.βgal (○, n = 25) or PBS (▲, n = 30) once a week for 4 weeks (indicated by arrows), and tumor growth was monitored. c, skin tumors were induced by s.c. injection of 0.5 mg 3-MC/mouse, followed 6 weeks later by topical application of 1.8 nmol of TPA twice weekly for 6 weeks. Adenoviruses Ad.IL-7/B7.1 (■, n = 15) and Ad.βgal (▲, n = 15) or PBS (▲, n = 15) were injected 12 weeks after 3-MC induction intratumorally, and tumor development was monitored. Average tumor size at the time of treatment was 3 mm. Similar results have been obtained with mice injected once intratumorally with adenoviruses and in mice bearing tumors smaller than 3 mm.

Fig. 4. Rejection of transplanted 3-MC-induced fibrosarcomas after intratumoral injection of Ad.IL-7/B7.1. MC51-9 tumor cells (2.5 × 10^5) were injected s.c. into syngeneic 129SvEv mice, grown for 10 days (mean tumor size 3 mm), and injected with 1 × 10^9 pfu of Ad.IL-7/B7.1, Ad.βgal, or PBS. Tumor incidence of mice treated with Ad.IL-7/B7.1 (■), Ad.βgal (○), or PBS (▲) is shown. The number of mice per group was seven. The experiment has been repeated once with a similar result.

Fig. 5. Mice bearing 3-MC-induced fibrosarcomas reject immunogenic transplanted tumors. One mg 3-MC/mouse was injected i.m. into the hind leg of BALB/c mice. Twelve weeks later, when tumors had developed (mean tumor size, 5–8 mm), the mice and the control non-tumor-bearing mice were injected s.c. with 5 × 10^6 TS/A or TS/A-IL-7/B7.1 cells, and tumor growth was monitored. The mean tumor size of 3-MC-treated mice injected with TS/A cells (■) or TS/A-IL-7/B7.1 cells (○), and untreated BALB/c mice injected with TS/A cells (□) or TS/A-IL-7/B7.1 cells (▲) is shown. Bars, SD; the number of mice/group was four.
FAILURE IN REJECTION OF NONTRANSPLANTED TUMORS BY ADENOVIRUSES

B7.1-infected TS/A or MCA1-9 cells for 3–4 days. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was added to the culture, and absorbance at 405 nm was measured 4 h later. The amount of IL-7 was determined by comparison to a standard curve prepared with recombinant murine IL-7 (R&D Systems, Abingdon, United Kingdom). All samples were measured in triplicate.

For T-cell-depleted nude mice, no difference in tumor growth was seen after a slight growth retardation compared to parental TS/A cells, all developed a tumor (five of five mice; data not shown). We then injected Ad.IL-7/B7.1 virus, control virus Ad.βgal, or PBS into TS/A tumors, which were established during a 1-week period of in vivo growth, and monitored the mice for tumor regression (Fig. 2). Intratumoral injection of Ad.IL-7/B7.1 induced rejection in 7 of 10 mice. Ad.βgal-treated tumors grew progressively with a short delay. In T-cell-deficient nude mice, no difference in tumor growth was seen between Ad.IL-7/B7.1-injected mice and Ad.βgal- or PBS-injected mice (Fig. 2). Therefore, T cells are involved in rejection of Ad.IL-7/B7.1-injected TS/A tumors. Tumor-free mice had developed systemic immunity because the injection of 2.5 × 10^5 TS/A cells 60 days after treatment led to their rejection in all of these mice (n = 5), whereas all control mice developed tumors (data not shown). Because it has been shown previously that TS/A tumors resist several ways of vaccinations after 1 day (21) and 4 days of in vivo growth (22), we conclude that adenoviral IL-7/B7.1 gene transfer in vivo is at least as effective as other forms of vaccination.

III-7/B7.1-expressing Adenoviruses Fail to Induce Rejection of 3-MC-induced Nontransplanted Tumors. Next we investigated the therapeutic efficacy of IL-7/B7.1 adenoviruses against tumors in the autochthonous host that were induced by i.m. injection of 3-MC. Ten weeks after 3-MC inoculation, when mice had palpable tumors that were similar in size to that of TS/A tumors at the time of treatment, adenoviruses (Ad.IL-7/B7.1 or Ad.βgal) or PBS was injected into the tumor once a week for 4 weeks, and tumor incidence was monitored (Fig. 3). Whereas Ad.IL-7/B7.1 injection caused a growth delay of up to 4 weeks, not a single mouse rejected the tumor. Comparable results were seen in different inbred mice (BALB/c, C57BL/6, 3b; 129SvEv, data not shown), in mice injected once intratumorally with adenoviruses, and in mice bearing smaller tumors (<3 mm) 10 weeks after 3-MC injection (data not shown). To exclude the possibility that the i.m. site of tumor development that usually results in fibrosarcomas is responsible for the failure of Ad.IL-7/B7.1 adenovirus to cause tumor regression, skin tumors were induced by s.c. suspensions of spleens obtained from four mice/group were prepared and restimulated in vitro at 2 × 10^6 cells/ml with 1 μg/ml β-gal 876–884 peptide known to be presented by MHC I H2-L^d molecules (41) in RPMI 1640 plus 10% FCS, penicillin/streptomycin, MEM, and 2-mercaptoethanol (50 μM). After 5 days of culture, cells were harvested, washed twice, and incubated with 51Cr (1 mCi/ml; DuPont NEN)-labeled CT26, CT26-βgal, or Yac-1 cells at different E:T ratios. After an incubation period of 4.5 h, radioactivity in culture supernatants was determined by a gamma counter (Top Count; Packard). The percentage of specific lysis was calculated as [(sample cpm − spontaneous cpm)/maximal cpm − spontaneous cpm)] × 100%. Spontaneous release did not exceed 17%.

RESULTS

Rejection of Established Transplanted Tumors after Adenoviral IL-7/B7.1 Gene Transfer. We have previously shown that IL-7/B7.1 gene-modified TS/A cells are rejected in a T cell-dependent fashion and induce immunity to parental TS/A cells (24). To analyze the efficacy of in vivo IL-7/B7.1 gene transfer to induce tumor rejection, we constructed an adenovirus harboring IL-7 and B7.1 genes connected by an IRES site (Ad.IL-7/B7.1). The mammary adenocarcinoma cell line TS/A was infected in vitro and analyzed for IL-7 and B7.1 expression (Fig. 1). Two and 5 days after infection, TS/A cells secreted 600 units (150 ng) and 700 units (175 ng) of IL-7, respectively. Similarly, high levels of B7.1 were detected at day 2 and 5. To demonstrate the in vivo activity of adenoviral expressed IL-7 and B7.1, TS/A cells were infected with Ad.IL-7/B7.1 and injected s.c. into mice 8 h later. Eighty percent of the mice (four of five mice) rejected the tumor cells, whereas mice injected with Ad.βgal-infected TS/A cells, after a slight growth retardation compared to parental TS/A cells, all developed a tumor (five of five mice; data not shown).

We then injected Ad.IL-7/B7.1 virus, control virus Ad.βgal, or PBS into TS/A tumors, which were established during a 1-week period of in vivo growth, and monitored the mice for tumor regression (Fig. 2). Intratumoral injection of Ad.IL-7/B7.1 induced rejection in 7 of 10 mice. Ad.βgal-treated tumors grew progressively with a short delay. In T-cell-deficient nude mice, no difference in tumor growth was seen between Ad.IL-7/B7.1-injected mice and Ad.βgal- or PBS-injected mice (Fig. 2). Therefore, T cells are involved in rejection of Ad.IL-7/B7.1-injected TS/A tumors. Tumor-free mice had developed systemic immunity because the injection of 2.5 × 10^5 TS/A cells 60 days after treatment led to their rejection in all of these mice (n = 5), whereas all control mice developed tumors (data not shown). Because it has been shown previously that TS/A tumors resist several ways of vaccinations after 1 day (21) and 4 days of in vivo growth (22), we conclude that adenoviral IL-7/B7.1 gene transfer in vivo is at least as effective as other forms of vaccination.
injection of 3-MC and additional exposure to TPA starting 6 weeks later. Again, tumors (probably papillomas; Ref. 38) were not rejected after Ad.IL-7/B7.1 treatment (Fig. 3c).

**Transplanted 3-MC-induced Tumors Are Rejected after Adenoviral IL-7/B7.1 Gene Transfer.** To test whether 3-MC-induced fibrosarcomas are resistant to adenoviral treatment, one line (fibrosarcoma MC51-9 induced by 3-MC in a 129SvEv mouse) was established in culture. MC51-9 cells could be transduced by adenovirus to express IL-7 and B7.1 comparable to TS/A cells (data not shown). MC51-9 cells were injected s.c. into syngeneic mice, and 10 days later, tumor-bearing mice were treated with Ad.IL-7/B7.1, Ad.βgal, or PBS, and tumor growth was monitored (Fig. 4). Most of the Ad.IL-7/B7.1-treated mice rejected the tumor (seven of eight mice). Ad.βgal- or PBS-treated mice did not reject the tumor.

**Mice Bearing 3-MC-induced Nontransplanted Tumors Reject Immunogenic Transplanted Tumors.** To test whether the failure of Ad.IL-7/B7.1 to induce regression of nontransplanted tumors was caused by an immunosuppressive effect of 3-MC or the tumor-bearing state of the animals, we analyzed whether mice bearing 3-MC-induced tumors could reject transplanted tumors. Therefore, mice were injected with 3-MC, and 12 weeks later, when tumors of approximately 5–8 mm in size had developed, these mice were injected with TS/A or TS/A-IL-7-B7.1 cells. TS/A-IL-7-B7.1 cells were rejected in naive and 3-MC-treated mice (Fig. 5). Parental TS/A cells grew progressively in both groups of mice. This result showed that 3-MC-treated tumor-bearing mice are able to respond to IL-7/B7.1-expressing transplanted tumors. Therefore, immunosuppression should not account for the failure of Ad.IL-7/B7.1 to induce rejection of 3-MC-induced tumors.

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**Fig. 7.** Failure of adenoviral IL-7/B7.1 gene transfer to efficiently attract T cells to nontransplanted tumors. Tumors were established in BALB/c mice by s.c. injection of 2.5 × 10⁵ TS/A cells or i.m. injection of 1 mg 3-MC/mouse. Seven days later (for transplanted TS/A tumors) or 10–12 weeks later (for 3-MC-induced tumors), mice were injected intratumorally with 1 × 10⁹ pfu of Ad.IL-7/B7.1, and tumors were excised five days later. Immunohistochemical analysis of frozen sections with CD8-specific (a–d) and CD4-specific antibodies (e–h) showed increased numbers of T cells in Ad.IL-7/B7.1-injected transplanted TS/A tumors (b and f, respectively) compared to control tumors (a and e, respectively), whereas lymphocytic infiltration in 3-MC-induced tumors (c and g, respectively) was not further increased after administration of Ad.IL-7/B7.1 (d and h, respectively). Magnification, ×200. A representative staining of tumors from three to five mice analyzed is shown.
Comparable in Vivo Transfer Efficiency of Adenoviruses into Transplanted and Nontransplanted Tumors. The different efficacy of IL-7/B7.1 adenoviruses against transplanted and nontransplanted tumors led us to analyze the in vivo transduction efficiency. For example, tumors could differ in interstitial pressure (42), leading to different gene transfer efficacy. Seven-day-old TS/A tumors and 10–12-week-old 3-MC-induced tumors were injected with Ad.β-gal, isolated 5 days later, and analyzed for β-gal expression (Fig. 6, a and b, respectively). Serial cryosections stained with X-gal revealed that TS/A tumors could not be transduced significantly better than 3-MC-induced tumors. Interestingly, repeated injection of Ad.β-gal once a week for 4 weeks, as used for treatment of 3-MC-induced tumors, increased the number of infected tumor cells that seemed to stably express β-gal (Fig. 6c, right side). This is surprising because Ad.β-gal injection into normal tissue results in a strong immune response and clearance of the virus (43, 44).

Failure of Adenoviral IL-7/B7.1 Gene Transfer to Efficiently Attract T Cells to the Nontransplanted Tumor. Rejection of IL-7/B7.1-expressing TS/A tumors is preceded by an increase of tumor-infiltrating T cells (Fig. 7, Ref. 24). To ask whether the failure of Ad.I.L-7/B7.1 to induce tumor rejection of nontransplanted tumors was associated with inefficient T-cell infiltration, we analyzed tumor-infiltrating T cells in transplanted and nontransplanted tumors. Few T cells were detected in TS/A tumors before treatment (Fig. 7). After Ad.I.L-7/B7.1 injection, an increased number of tumor-infiltrating CD4+ and CD8+ T cells was observed. 3-MC-induced tumors were infiltrated by a significant number of T cells before Ad.I.L-7/B7.1 treatment. However, Ad.I.L-7/B7.1 injection did not increase the number of tumor-infiltrating T cells as judged by the immunohistology of multiple sections.

Intratumoral Injection of Ad.β-gal Leads to Induction of Anti-β-gal-specific CTLs. Thus far, we have observed that multiple injections of Ad.β-gal into nontransplanted tumors led to stable β-gal expression (at least 5 weeks), indicating a defective anti-β-gal immune response within the tumor and, additionally, that Ad.I.L-7/B7.1 was unable to efficiently attract T cells into the tumor tissue. This could mean either that intratumoral adenovirus application does not induce T cells or that T cells are induced but do not reach the tumor site. To distinguish between these possibilities, we used β-gal as a surrogate antigen because the usually individual rejection antigens of 3-MC-induced tumors are not known. Ad.β-gal was injected into 3-MC-induced tumors and injected as a control into normal mice, and anti-β-gal-specific CTL responses were measured. Both, normal and tumor-bearing mice had similar β-gal-specific CTL activity (Fig. 8).

DISCUSSION

We have analyzed the efficacy of in vivo IL-7 and B7.1 gene transfer into established tumors to mediate tumor rejection. For this purpose, we used adenoviruses that effectively deliver genes in vivo. Similar adenoviruses containing the genes for IL-2/B7.1 and IL-12/B7.1 have already been shown to induce rejection of established transplanted tumors (19, 20). We transferred IL-7 and B7.1 into tumors because previous studies had shown that tumor cells transduced in vitro to express both genes were effectively rejected upon transplantation. These gene-modified tumor cells had increased immunogenicity and were effective vaccines, as compared to single transfected cells or classical adjuvant (24). With two transplanted tumors, the BALB/c adenocarcinoma TS/A and the 129SvEv 3-MC-induced fibrosarcoma MC51-9, we showed that intratumoral IL-7/B7.1 gene transfer by adenoviruses induces effective T-cell-mediated tumor rejection. For one of the tumors (TS/A) it has been shown that vaccination, e.g., by gene-modified tumor cells (21) or tumor-peptide-loaded dendritic cells (22), fails to induce complete rejection if the tumor had grown for a similar time and to a similar size in mice. Therefore, the in situ gene transfer into the tumor compares favorably with cancer vaccines. Cancer vaccines can be expected from mouse (11, 18, 45, 46) and clinical data (47) to be effective in situations resembling “minimal residual disease.” The approach to convert a tumor in vivo into a vaccine requires a considerably larger amount of tumor cells to successfully transduce the tumor; therefore, this situation reflects a progressed state of tumor development in comparison to “minimal residual disease.” To determine the therapeutic efficacy of the adenoviral gene delivery into nontransplanted tumors is clinically more relevant, and we have shown that the effective approach against transplanted tumors failed against nontransplanted tumors. Several reasons are unlikely to be responsible for the failure: (a) tumor size, because transplanted and nontransplanted tumors were similar in size at the time of treatment. It should be noted that transplanted tumors exceeding 8 mm in average size were not completely rejected (data not shown); (b) site and type of tumors, because both 3-MC fibrosarcomas induced i.m. and 3-MC/TPA-induced skin tumors were resistant to treatment. We cannot exclude that primary tumors induced by other means or grown at other sites are more susceptible to treatment; (c) immune suppression in 3-MC-treated tumor-bearing animals, because the mice rejected immunogenic transplanted tumors (TS/A-IL-7/B7.1); (d) absent or too weak antigens on the 3-MC-induced tumors, because for at least one 3-MC-induced tumor (MC51-9), we showed that the IL-7/B7.1 adenoviruses induced rejection if the tumor was transplanted. It is known that 3-MC-induced tumors vary in inherent immunogenicity (17); however, we could not detect rejection by the treatment in 1 of 200 mice analyzed in the course of the experiments; and (e) we exclude differences in transduction efficacy of transplanted and nontransplanted tumors as demonstrated by comparable β-gal expression after adenoviral gene transfer. A selection of antigen loss variants after treatment seems unlikely, because the delayed tumor growth after Ad.I.L-7/B7.1 treatment was short and similarly observed after Ad.β-gal treatment.

Based on two findings, our results suggest that T cells do not efficiently infiltrate solid nontransplanted tumors in our model. First, adenoviral IL-7/B7.1 expression induced substantial T-cell infiltration and rejection of the transplanted tumor, but both did not occur in the nontransplanted tumor model. Second, adenoviral β-gal expression in
nontransplanted tumors induced undiminished CTLs against β-gal, yet they did not eliminate β-gal-expressing tumor cells. Immune responses against adenoviral and β-gal antigens are well described, and it is known that adenoviral β-gal expression in several tissues is rapidly abolished in an immunological manner (44, 48–50). β-Gal can also act as a surrogate rejection antigen in tumor transplantation experiments (27). We do not know whether the anti-β-gal-specific CTLs are induced directly by adenovirus-infected tumor cells or by adenoviruses that leak out from the tumor tissue and infect antigen-presenting cells (43). In any case, the CTLs appear to be ineffective against β-gal-expressing cells in the nontransplanted tumor, despite the fact that they might be unrelated to any antigens expressed by the tumor and therefore could not be modulated by the growing tumor. It appeared that the nontransplanted tumor contained more T cells as compared to the transplanted tumor before Ad.IL-7/B7.1 treatment. This is not surprising because these tumors had grown considerably longer in mice. The important difference was that after treatment, no substantial increase in infiltrating T cells of the nontransplanted tumor compared to the transplanted tumor was observed, as judged by the immunohistology of multiple tumors.

It has been shown in transplanted (26, 27, 51, 52) and nontransplanted tumor models (16) that B7.1-expressing tumor cells can directly activate CTLs. If mice developing pancreatic carcinomas due to tissue-specific SV40 large T antigen expression additionally expressed B7.1 as transgene on β-islet cells, tumor growth was suppressed until B7.1 expression was down-regulated (16). Reminiscent of our results, the authors further showed that T-cell receptor transgenic large T-specific CD4+ T cells efficiently infiltrated the established tumor in the early stage but not the late stage of tumor development. The problem that T cells do not efficiently infiltrate an established solid tumor has variously been attributed to the tumor stroma (the sum of all nonneoplastic cells of the tumor tissue) and therefore could not be modulated by the growing tumor. It is known that adenoviral and it is known that adenoviral vectors pPBS, pJM17, and pAE1sp1A.

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Interleukin-7/B7.1-encoding Adenoviruses Induce Rejection of Transplanted but not Nontransplanted Tumors

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