Effect of Canarypox Virus (ALVAC)-Mediated Cytokine Expression on Murine Prostate Tumor Growth

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Background: **Canarypox virus, ALVAC, does not replicate in infected mammalian cells and has potential as a vector for gene therapy in the treatment of cancer.** ***Purpose:*** **Recombinant viruses carrying DNA sequences encoding interleukin 2 (ALVAC–IL-2), interferon gamma (ALVAC–IFN γ), tumor necrosis factor-α (ALVAC–TNF-α), or the co-stimulatory molecule B7-1 (ALVAC–B7-1) were investigated as agents for the treatment of a newly defined mouse prostate tumor model.** ***Methods:*** **RM-1 mouse prostate cancer cells, which are syngeneic (i.e., same genetic background) to C57BL/6 mice, were used. The expression of foreign gene products in vitro in infected RM-1 cells was measured by immunoprecipitation, bioassay, or flow cytometry. The effects of foreign gene product expression on RM-1 tumor cell growth in C57BL/6 mice were measured after subcutaneous injection (in the back) of 5×10^5 uninfected or infected cells; measurements included determinations of time to a measurable tumor size, tumor size as a function of time, and survival. The induction of protective immunity by uninfected and infected RM-1 cells was tested by injection of lethally irradiated (70 Gy) cells and subsequent challenge with uninfected cells. The generation of cytotoxic T cells was monitored by use of a ^{51}Cr release assay. Severe combined immunodeficient (SCID) mice were used to determine whether T or B lymphocytes were involved in ALVAC vector-mediated antitumor responses. Data were analyzed by use of Pearson's modification of the chi-squared test and Kaplan–Meier survival methods. Reported *P* values are two-sided.** ***Results:*** **The level of foreign gene product expression in ALVAC-infected RM-1 cells was dependent on the multiplicity of virus infection used; a multiplicity of five viruses per infected cell was chosen for subsequent experiments. RM-1 tumor growth in C57BL/6 mice was not affected by tumor cell expression of IL-2 alone, IFN γ alone, or B7-1 alone; however, expression of TNF-α alone significantly delayed tumor growth at early time points (compared with parental ALVAC-infected tumors, *P* = .0001 at day 21 and *P* = .037 at day 28). Tumor cell expression of both TNF-α and IL-2 completely inhibited tumor growth in 60%-100% of treated mice. No protection against subsequent tumor challenge was detected in mice previously exposed to RM-1 cells expressing both TNF-α and IL-2. Cytotoxic T-lymphocyte activity toward RM-1 cells was not observed in C57BL/6 mice that rejected tumors. Tumor cell expression of TNF-α and IL-2 also resulted in tumor growth inhibition in SCID mice.** ***Conclusions:*** **RM-1 mouse prostate cancer cells are readily infected by ALVAC vectors, and foreign gene products are efficiently expressed. Inhibition of RM-1 tumor growth by tumor cell expression of TNF-α and IL-2 appears to involve nonspecific antitumor activity. [J Natl Cancer Inst 1997;89:428-36].**

A promising approach to cancer immunotherapy is immunization with modified tumor cells carrying cytokine or immunomodulatory genes. The strategy of using tumor cell vaccines as a source of tumor antigens eliminates the need for knowledge of the specific molecular identity of immunodominant antigens. One of the most important concepts underlying the use of cytokine gene-transduced tumor cell vaccines is that the cytokine is produced at high concentrations at the tumor site and systemic concentrations are generally quite low. This paracrine physiology more closely mimics the natural biology of cytokine action than does the systemic administration of recombinant cytokines (*1*).

The most efficient approach to gene transfer is through the use of viral vectors, such as retroviruses (*2,3*). Retroviral vectors have the property of mediating stable and durable gene transfer into the chromosomal DNA, but a limiting factor for clinical applications is their low efficiency of infection. Current retrovirus-based systems for gene transfer require ex vivo manipulation; therefore, they represent a less than optimal approach for routine clinical use.

Other studies have utilized vaccinia virus for gene transfer. Vaccinia virus is a large DNA virus that replicates entirely in the cytoplasm of infected cells. It has the properties of a wide host range, rapid infection, and high expression of inserted gene sequences (*4*). Numerous endogenous viral genes are dispensable for replication, and at least 25 000 base pairs of foreign DNA can be added to the vaccinia virus genome without any effect on viral replication (*4*). Antitumor activity has been demonstrated by in vitro and in vivo infection with recombinant vaccinia viruses that carry sequences encoding interleukin (IL)-1β (*5*), IL-2 (*6*), IL-12 (*7*), and the co-stimulatory molecules B7-1

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See ''Notes'' following ''References.''

(CD80) or B7-2 (CD86) (*8*). The presentation of foreign antigens by recombinant vaccinia viruses has also elicited antitumor immune responses (*9,10*).

The concept of recombinant vaccinia virus as a live vaccine stems from its successful use against smallpox (*4*). However, severe adverse reactions, such as progressive vaccinia, eczema vaccinatum, and encephalitis, have been reported. In the United States in 1968, the rates of complications and of death following primary vaccination were 74 and one per million individuals, respectively (*11*). Vaccination was contraindicated for candidates with eczema or for persons with immune dysfunction.

The significant adverse reaction rate associated with smallpox vaccination has prompted the development of highly attenuated poxviruses for recombinant vaccines. Three approaches have been investigated: 1) the adaptation of existing vaccinia viruses that were attenuated by serial passage in tissue culture for smallpox vaccination, 2) the selective deletion of specific vaccinia virus genes (*12*), and 3) the use of poxviruses that have a narrower host range than vaccinia virus (*12-19*).

The use of a canarypox vector (ALVAC) offers significant potential advantages over the use of conventional vaccinia virus vectors. ALVAC is restricted to avian species for productive replication; however, it has the ability to infect mammalian cells and to express inserted gene sequences (*12-14,16,17*). The highly attenuated character of ALVAC has been demonstrated as follows: 1) no progeny virus is detected in any of the human cell lines tested, 2) it is considerably less virulent in normal and newborn mice when given by intracranial inoculation than the other vaccinia strains (WR, Wyeth, and VC-2), and 3) no disseminated lesions are found in genetically or chemically immunocompromised mice after intraperitoneal inoculation (*12,13*). A series of studies with ALVAC vectors that carry sequences encoding the rabies virus glycoprotein (*14,15*), the measles virus fusion and hemagglutinin glycoproteins (*16*), the feline leukemia virus env and gag proteins (*17*), and the human immunodeficiency virus-1 (HIV-1) envelope glycoprotein (*18,19*) in animals and humans have shown that ALVAC vectors are well tolerated and that immune responses or protective immunity against challenge with the cognate pathogens can be induced. No significant local or systemic reactions were reported.

In this study, ALVAC vectors carrying sequences that encode either IL-2 (i.e., ALVAC–IL-2), interferon gamma (IFN γ) (i.e., ALVAC–IFN γ), tumor necrosis factor-α (TNF-α) (i.e., ALVAC–TNF-α), or B7-1 (i.e., ALVAC–B7-1) were investigated as potential agents for the treatment of a newly defined mouse prostate tumor model (*20*). This study demonstrates the utility of the prostate tumor model, the effective delivery of cytokines by recombinant ALVAC vectors, and the potential utility and flexibility of these vectors in cancer therapy.

Materials and Methods

Animals and Tumor Cells

The mouse prostate cancer model RM-1, syngeneic to C57BL/6 mice, was obtained from Dr. Timothy C. Thompson of Baylor College of Medicine, Houston, TX (*20*). This model was generated by transduction of cells with the ras and myc oncogenes and yields a poorly differentiated carcinoma (*21*). Cultured cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (BioWhittaker, Inc., Walkersville, MD) containing 10% fetal calf serum (FCS) and 10 m*M* HEPES buffer (pH 7.0). Male C57BL/6 mice and male severe combined immunodeficient (SCID) mice were purchased at 6-8 weeks of age from Charles River Laboratories (Wilmington, MA). Upon arrival, the mice were allowed to acclimate for a minimum of 2 days before use. They were provided with food and water ad libitum. SCID mice were maintained under sterile conditions in microisolator cages and given autoclaved food and water ad libitum. All animal studies were approved by the Washington University Animal Review Board and were performed in accordance with institutional guidelines.

Viral Vectors and Infection

ALVAC is a canarypox virus-based vector that is restricted to avian species for productive replication (*12,13*). The canarypox strain from which ALVAC was derived was isolated from a pox lesion on an infected canary. The isolated virus was attenuated by 200 serial passages in chick embryo fibroblasts and was subjected to four successive rounds of plaque purification under agarose. One plaque isolate, designated ALVAC, was selected for amplification. All amplifications and plaque titrations were performed on primary chick embryo fibroblasts. Parental ALVAC (CPpp) and ALVAC vectors encoding murine IL-2 (vCP275), murine IFN γ (vCP271), human TNF-α (vCP245), and murine B7-1 (vCP268) were developed at Virogenetics Corporation (Troy, NY) as previously described (*14,16-19*). The foreign gene expression of each vector was confirmed by immunoprecipitation (*22*) or bioassay (Genzyme, Cambridge, MA; data not shown) of the products or by flow cytometry (*see below*).

RM-1 cells were harvested and plated with DMEM containing 10% FCS and 10 m*M* HEPES buffer (pH 7.0) on the day before infection. The medium was changed to DMEM with 2% FCS, and the viral vectors were added to the cells at the multiplicity of infection (MOI)—plaque-forming units per cell—shown in each experiment. The cells were then incubated at 37 °C in an atmosphere containing 5% CO_2 for 5 hours. The infected cells were removed from the tissue culture plates by treatment with 10 m*M* EDTA (pH 7.2), and they were washed with 0.1 *M* phosphate-buffered saline (PBS) (pH 7.2). The cells were then resuspended to the desired concentration in DMEM without FCS and injected subcutaneously into the backs of mice. When cells were tested for gene expression in vitro, the medium was replaced (after the 5-hour incubation) with DMEM containing 10% FCS and 10 m*M* HEPES buffer (pH 7.0). The cells were incubated for another 24 hours.

IL-2 Assay

The helper T-cell clone HT-2 was used for the biologic assay of IL-2, as previously described (*23*). Briefly, aliquots of 5×10^4 IL-2-starved HT-2 cells (obtained from Dr. John Russell, Washington University School of Medicine) were incubated with the serially diluted culture fluids from RM-1 cells infected with ALVAC–IL-2 as described above. IL-2-dependent HT-2 cell proliferation was monitored by staining with 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide (XTT) in the presence of phenazine methosulfate as described previously (*23,24*), and the staining intensity was measured at OD_{495}. The concentration of IL-2 was calculated and compared with a serially diluted standard (human recombinant IL-2; Chiron Therapeutics, Emeryville, CA).

Flow Cytometry

RM-1 cells infected with ALVAC–B7-1 were harvested after treatment with 10 m*M* EDTA (pH 7.2) and washed with 0.1 *M* PBS (pH 7.2) that was supplemented with 10% horse serum as described previously (*25*). One microgram of hamster anti-B7-1 monoclonal antibody (MAb) (Pharmingen, San Diego, CA) was added to 10^6 RM-1 cells in a volume of 1.0 mL, and the mixture was incubated on ice for 15 minutes. The cells were washed twice with 0.1 *M* PBS (pH 7.2) supplemented with 1% FCS. One microgram of secondary antibody, a fluorescein isothiocyanate (FITC)-conjugated goat anti-hamster immunoglobulin G MAb (Pharmingen), was then added, and the mixture was incubated for 15 minutes on ice. The cells were washed twice with 0.1 *M* PBS (pH 7.2) supplemented with 1% FCS. One microgram of 7-aminoactinomycin D (Sigma Chemical Co., St. Louis, MO) was added to assess viability, and the cells were washed with 0.1 *M* PBS (pH 7.2) supplemented with 1% FCS. Samples were analyzed for fluorescence with a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

Tumor Inhibition Studies

RM-1 cells were infected with virus and injected into the backs of mice as described above. Preliminary experiments had shown that an MOI of less than 10

had no toxic effect on RM-1 cells over a 3-day period (data not shown). Expression of recombinant gene products was determined to be dependent on MOI (*see below*). In all experiments described in this article, a final MOI of 5 was used. If two vectors were combined, an MOI of 2.5 for each was used, producing a final MOI of 5 for the experimental group. RM-1 tumor outgrowth, determined by time to a measurable tumor (approximately 2 mm in diameter) and by tumor size as a function of time, was monitored twice a week. Survival was measured in some experiments. When survival was measured, mice appearing ill from tumor burden were killed for humane reasons. The results of each experiment were reproduced in a minimum of three independent experiments, using five animals per group, and the reported results are summary results of combined experiments.

Immunization Study

Irradiated RM-1 cells infected with ALVAC vectors were inoculated subcutaneously into the backs of mice (day −10). Subsequent rechallenge with untreated RM-1 cells was performed by subcutaneous injection in the contralateral back 10 days after immunization (day 0). Infected and irradiated RM-1 cells were used to reimmunize the mice 4 days after rechallenge (day 4), and the tumor growth of untreated RM-1 cells was monitored. For both immunization and rechallenge, 5×10^5 cells were used.

^{51}Cr Release Assays

Splenocytes (effector cells) were harvested from mice inoculated with tumor cells. Red blood cells were lysed with ACK lysing buffer (0.15 *M* NH_4Cl, 1 m*M* $KHCO_3$, and 0.1 m*M* Na_2EDTA [pH 7.2]), and effector cells were resuspended and serially diluted in complete RPMI-1640 medium supplemented with 10% FCS. Experimental target cells (10^7) in 100 mL complete RPMI-1640 medium supplemented with 10% FCS were incubated with 100 μCi $Na^{51}CrO_4$ for 90 minutes. The EL-4 thymoma cell line, derived from C57BL/6 mice, was used as a control. Serially diluted effector cells at effector-to-target ratios ranging from 6:1 to 100:1 were incubated with target cells (10^4) at 37 °C in an atmosphere containing 5% CO_2 for 6 hours. The amount of ^{51}Cr released into the culture fluids was ascertained by use of a gamma counter. The percentage of specific lysis was calculated as follows:

$$\frac{\text{Experimental } ^{51}\text{Cr release (cpm)} - \text{spontaneous } ^{51}\text{Cr release (cpm)}}{\text{Maximum } ^{51}\text{Cr release (cpm)} - \text{spontaneous } ^{51}\text{Cr release (cpm)}} \times 100.$$

Maximum release was determined by the addition of cetrimonium bromide to target cells, while spontaneous release was determined by incubation of target cells in the absence of effector cells. In none of the experiments did spontaneous release of ^{51}Cr from target cells exceed 20% of maximum ^{51}Cr release.

TNF-α Assay

RM-1 cells and αL929 cells (a TNF-α-sensitive cell line provided by Dr. Charles Evans of the National Cancer Institute, Bethesda, MD) were tested for TNF-α sensitivity as described (*25*). Briefly, 1×10^4 cells/well were plated in 96-well flat-bottom tissue culture plates and incubated at 37 °C in an atmosphere containing 5% CO_2 for 48 hours. Subsequently, the cells were incubated with serially diluted TNF-α at 37 °C in 5% CO_2 for 24 hours. The cells were then washed once with 0.1 *M* PBS (pH 7.2), stained with crystal violet for 15 minutes, washed twice with 0.1 *M* PBS (pH 7.2), and dried overnight. The stain in each well was eluted from the cells with 100% methanol; its absorbance was measured by means of a microplate reader (model 450; Bio-Rad Laboratories, Richmond, CA) equipped with a 595-nm interference filter.

In experiments testing the effects of ALVAC–TNF-α on RM-1 proliferation in vitro, RM-1 cells were plated at a concentration of 5×10^3 cells/well in a 96-well microtiter plate. One group (replicates of eight wells) was transfected with ALVAC–TNF-α at an MOI of 5 as described above. The growth of ALVAC–TNF-α-treated cells was compared with the growth of untreated RM-1 cells as follows: Plates were incubated at 37 °C and harvested at daily intervals over a 3-day period. At each harvest, the plates were washed three times with prewarmed (37 °C) 0.1 *M* PBS (pH 7.2) and stained with XTT plus phenazine methosulfate as described previously (*24,26*). The plates were air dried, the dye was extracted with 100% methanol, and the level of dye retention, which is proportional to cell number, was determined at OD_{495}. The data are reported as OD units.

Statistical Analyses

Differences were analyzed by use of Pearson's modification of the chi-squared test. Survival was estimated by the Kaplan–Meier method. Reported *P* values are two-sided.

Results

IL-2 Production and B7-1 Expression by RM-1 Cells Infected With ALVAC–IL-2 or ALVAC–B7-1 In Vitro

The day before infection, RM-1 cells were plated in six-well tissue culture plates at a density of 10^6 cells/well. The cells were incubated with varying MOIs of ALVAC–IL-2 or ALVAC–B7-1 for 5 hours. After being washed, the infected cells were incubated for another 24 hours, and the culture fluids of cells infected with ALVAC–IL-2 were tested for IL-2 production. The cells infected with ALVAC–B7-1 were tested for B7-1 expression by flow cytometry after immunofluorescent staining with an FITC-conjugated antibody. IL-2 production levels in infected cell cultures reflected the MOIs used (Fig. 1, A). In fact,

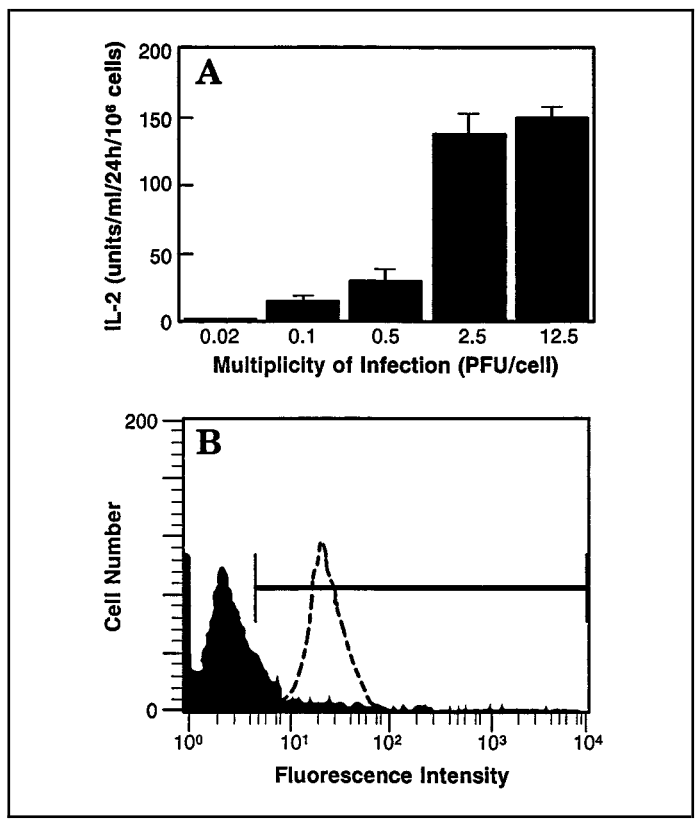

Fig. 1. Exogenous protein expression by RM-1 mouse prostate cancer cells infected with canarypox virus (ALVAC) vectors in vitro. **A)** Interleukin 2 (IL-2) production by RM-1 cells infected with ALVAC–IL-2 at varying multiplicities of infection (MOIs). Each data point was determined from triplicate cultures, and data are reported as the means ± standard error. IL-2 concentration was calculated by measuring growth of IL-2-starved HT-2 cells that were incubated with serially diluted culture fluids from infected cells (*see* ''Materials and Methods'' section for details). **B)** Expression of the co-stimulatory molecule B7-1 on the surface of RM-1 cells 24 hours after infection with parental ALVAC (shaded area) or the ALVAC–B7-1 (unshaded area) at an MOI of 5 plaque-forming units (PFU)/cell. Cells were incubated with a hamster anti-B7-1 monoclonal antibody (MAb), followed by incubation with a fluorescein isothiocyanate-conjugated goat anti-hamster immunoglobulin G MAb. Samples were analyzed by flow cytometry. The X axis shows fluorescence intensity, and the Y axis shows cell number.

concentrations of 1.9, 15, 30, 140, and 150 U/mL per 24 hours per 10^6 cells were measured at MOIs of 0.02, 0.1, 0.5, 2.5, and 12.5, respectively. RM-1 cells infected with ALVAC–B7-1 at an MOI of 2, 5, 10, or 20 expressed B7-1 on the cell surface of 81%, 86%, 97%, or 95% of infected cells, respectively; B7-1 expression at an MOI of 5 is shown in Fig. 1, B. All subsequent experiments were performed at MOIs of 5. ALVAC–IFN γ and ALVAC–TNF-α-infected RM-1 cells produced cytokines in patterns similar to the pattern described for IL-2 (data not shown).

Effect of Infection With ALVAC Recombinant Vectors on RM-1 Tumor Growth

To test the effects of cytokines or B7-1 expression on local tumor growth, we injected 5×10^5 RM-1 cells infected with the respective ALVAC vectors into the backs of male C57BL/6 mice. Tumor sizes and the survival of mice receiving RM-1 cells infected with ALVAC vectors expressing cytokines or the B7-1 co-stimulatory molecule were compared with those of mice receiving untreated RM-1 cells or the parental ALVAC vector. As shown in Fig. 2, A, only RM-1 tumor cells producing TNF-α exhibited a significant delay in tumor growth. The delay occurred at early time points (compared with parental ALVAC-infected tumors: day 21, $P = .0001$; day 28, $P = .037$). Furthermore, the ALVAC–TNF-α-induced inhibition resulted in significantly prolonged survival (versus mice with parental ALVAC-infected tumors: $P = .048$, Fig. 2, B); however, all mice ultimately succumbed to tumor cell challenge.

Effect of Combining ALVAC Recombinant Vectors on RM-1 Tumor Growth

ALVAC–TNF-α was combined with other ALVAC recombinant vectors because TNF-α provided the best effect on RM-1 tumor growth as a single agent. RM-1 cells infected with ALVAC–TNF-α plus ALVAC–IL-2 induced small nodules (2-3 mm in diameter) at the injection sites of some mice. The histology of the nodules showed the absence of tumor cells, neutrophil infiltration, and necrosis (Fig. 3). Within 2 weeks, the nodules regressed, and the antitumor effects appeared to result in complete inhibition of tumor growth, since the mice remained tumor free for more than 100 days (Fig. 4, A; data not shown). The results were reproducible in multiple experiments involving more than 40 mice and showed a 60%-100% survival rate (Fig. 4, B) depending on the experiment, with most providing 100% tumor inhibition as indicated below. Tumor growth was not significantly inhibited in mice when the other ALVAC combinations were used (Fig. 4, A).

Mechanism of Tumor Regression by ALVAC–TNF-α and ALVAC–IL-2 Combinations

A series of studies was initiated to determine the mechanism by which ALVAC–TNF-α and ALVAC–IL-2 mediated antitumor activity. First, the ability of RM-1 cells to induce protection against a second tumor challenge was established. Mice were inoculated subcutaneously with irradiated RM-1 cells (5×10^5 cells; irradiated with 70 Gy) and rechallenged with varying numbers of RM-1 cells 10 days later (Fig. 5, B). Partial protection was observed at the 10^5 rechallenge dose when tumor growth was compared with that observed in naive (i.e., previously untreated) mice inoculated with an equivalent cell dose (Fig. 5, A), suggesting that RM-1 is weakly immunogenic. Thus, RM-1 cells appear to be a suitable model for immunotherapy studies. Subsequently, mice were immunized with irradiated RM-1 cells or irradiated RM-1 cells that had been infected with ALVAC recombinant vectors (determined to produce cytokine levels equivalent to those in nonirradiated cells; data not shown) and rechallenged with 5×10^5 RM-1 cells as described in the ''Materials and Methods'' section (Fig. 5, C). The 5×10^5 rechallenge dose was chosen as a rigorous test of the presence of protection (*see* data in Fig. 5, B). Tumor outgrowth was slightly delayed, but no significant protection was seen in mice immunized with the ALVAC–TNF-α plus ALVAC–IL-2 combination. Likewise, no RM-1-specific cytotoxic T lymphocytes (CTLs) were observed in mice immunized with irradiated RM-1 only, in mice immunized with irradiated RM-1 infected with

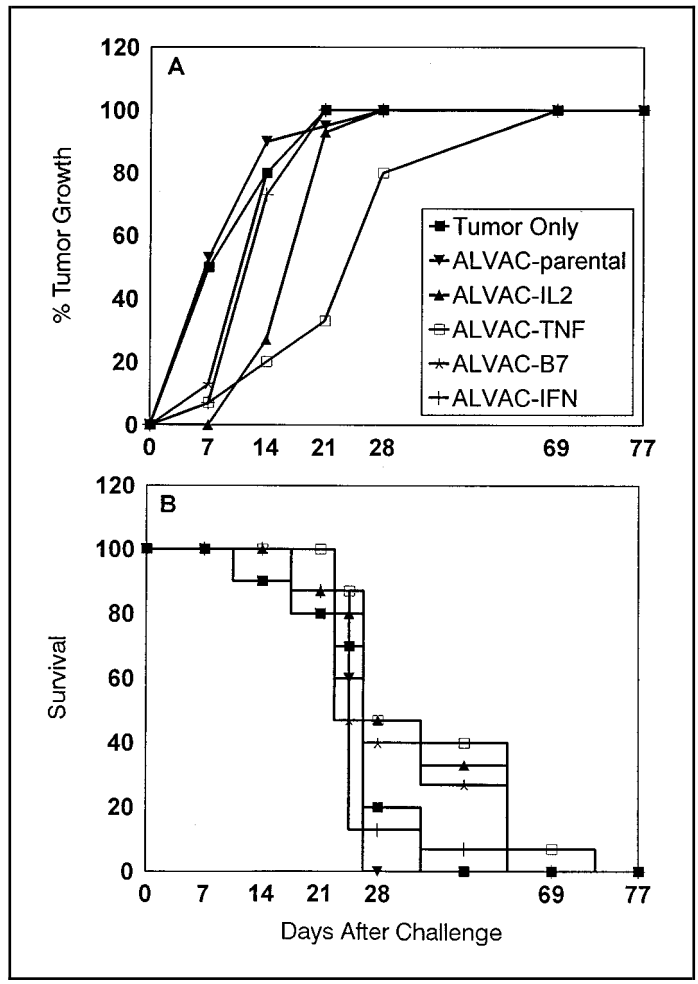

Fig. 2. In vivo tumor growth and survival analyses. RM-1 mouse prostate cancer cells were either not infected (tumor only) or infected in vitro with parental canarypox virus (ALVAC-parental) or ALVAC vectors encoding interleukin 2 (ALVAC-IL2), tumor necrosis factor-α (TNF-α) (ALVAC-TNF), B7-1 (ALVAC-B7), or interferon gamma (ALVAC-IFN) at a multiplicity of infection of 5 plaque-forming units/cell. C57BL/6 mice were inoculated subcutaneously in their backs with 5×10^5 RM-1 cells. The reported data are combined from three separate experiments, with five animals per treatment group in each experiment. **A)** Tumor growth delay, determined as described in the ''Materials and Methods'' section. **B)** Survival of mice. ALVAC–TNF-α significantly prolonged the survival of mice (two-sided $P = .048$ versus mice with tumors infected by parental ALVAC). *See* ''Materials and Methods'' section for more details.

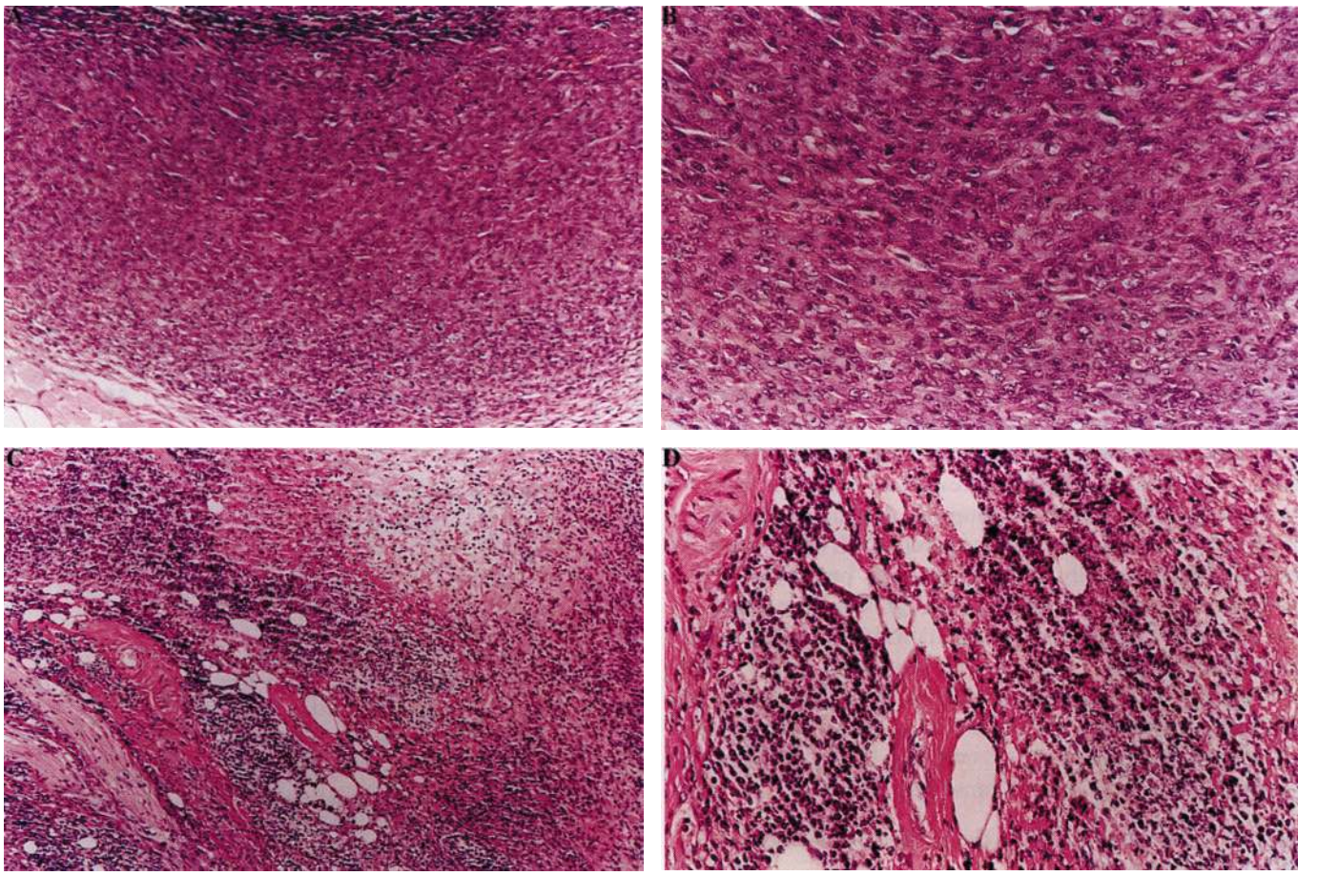

Fig. 3. Histology of tumors formed by RM-1 mouse prostate cancer cells. **A** and **B**: Tumors formed by uninfected RM-1 cells are poorly differentiated carcinomas. **C** and **D:** Regressing tumors containing RM-1 cells infected with recombinant canarypox virus (ALVAC) vectors encoding tumor necrosis factor-α and interleukin 2. Note neutrophil infiltration and tumor necrosis (hematoxylin–eosin; original magnification ×100 [A and C] and ×400 [B and D]).

ALVAC recombinant vectors, or in mice that had experienced regression of RM-1 tumors as shown in Fig. 4, A (data not shown). The expression of major histocompatibility complex class I molecules and susceptibility to allogeneic CTL lysis were verified (data not shown). Taken together, these data suggest that the effects of ALVAC–TNF-α plus ALVAC–IL-2 combination therapy reflect a localized, nonspecific phenomenon.

We performed studies to determine whether the antitumor activity resulted from TNF-α-induced cytotoxicity (Fig. 6). RM-1 cells were observed to be resistant to the effects of exogenous TNF-α, whereas αL929 cells exhibited susceptibility (Fig. 6, A). Another TNF-α assay was performed with TNF-α and/or IL-2. It showed that the growth of RM-1 cells was not sensitive to TNF-α when they were also exposed to IL-2 (data not shown). In addition, RM-1 cells transfected with ALVAC–TNF-α proliferated at a rate identical to that of control, nontransfected cells (Fig. 6, B). Similarly, ALVAC–IL-2 had no effect on RM-1 cell proliferation in vitro (data not shown). These data suggest that the inhibitory effects of the ALVAC recombinant vectors are independent of direct antiproliferative or cytotoxic effects caused by the vectors.

To determine whether T cells or B cells were involved in the ALVAC-mediated antitumor response, we inoculated SCID mice with infected RM-1 cells in the same manner. Inhibition of RM-1 growth was observed in all SCID mice of the ALVAC–TNF-α plus ALVAC–IL-2 group (Fig. 7). These data suggest that nonspecific, host-associated antitumor mechanisms are involved in ALVAC–cytokine-mediated antitumor activity.

Discussion

The availability of prostate cancer models for immunologic studies was limited to the Dunning Copenhagen rat model for many years. This model has a number of limitations, including the aggressive nature of Copenhagen rats and the expense of housing large numbers of rats for immunotherapy studies. In 1989, Thompson et al. (*21*) introduced a mouse prostate cancer model, which mimics the multistep carcinogenesis of human prostate cancer. Prostate cancer cell lines derived from primary mouse prostate cancers have been developed and provide relevant models for prostate immunotherapy studies. In our study, the mouse prostate tumor cell line, RM-1, of Thompson et al. was used to demonstrate the utility of nonreplicative ALVAC recombinant vectors for gene therapy studies. Our data demonstrate the utility of the model and provide a basis for future immunotherapy studies.

ALVAC is a canarypox virus that is replication deficient in mammalian cells (*13*). Nevertheless, when it is engineered to express extrinsic gene products, authentic expression and pro-

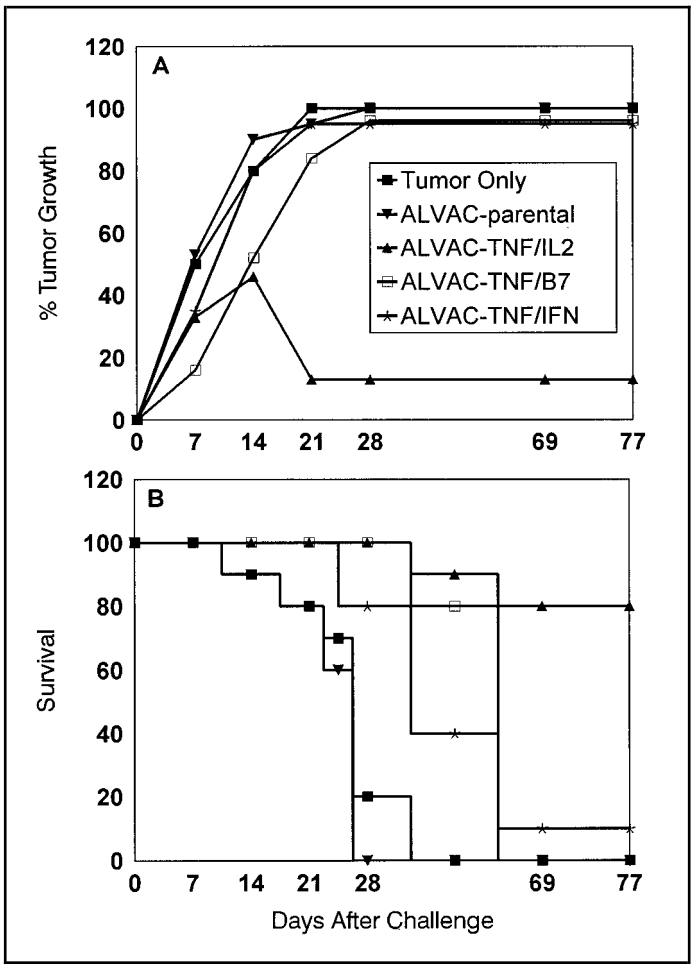

Fig. 4. Effects of combining various canarypox virus (ALVAC) vectors with an ALVAC vector that encodes tumor necrosis factor-α (TNF-α) on growth of tumors formed by RM-1 mouse prostate cancer cells and on mouse survival. Mice were inoculated subcutaneously with 5×10^5 RM-1 cells that were uninfected (tumor only) or infected with parental ALVAC at a multiplicity of infection (MOI) of 5 plaque-forming units per cell (ALVAC-parental), ALVAC–TNF-α plus ALVAC–IL-2 (interleukin 2) at an MOI of 2.5 for each (ALVAC-TNF/IL2), ALVAC–TNF-α plus ALVAC–B7-1 at an MOI of 2.5 for each (ALVAC-TNF/B7), or ALVAC–TNF-α plus ALVAC–IFN γ at an MOI of 2.5 for each (ALVAC-TNF/IFN). **A)** Tumor growth delay, determined as described in the ''Materials and Methods'' section. **B)** Survival. Survival increases were significant for the ALVAC–TNF-α plus ALVAC–IFN γ combination (two-sided $P = .048$) and the ALVAC–TNF-α plus ALVAC–IL-2 combination (two-sided $P = .00002$).

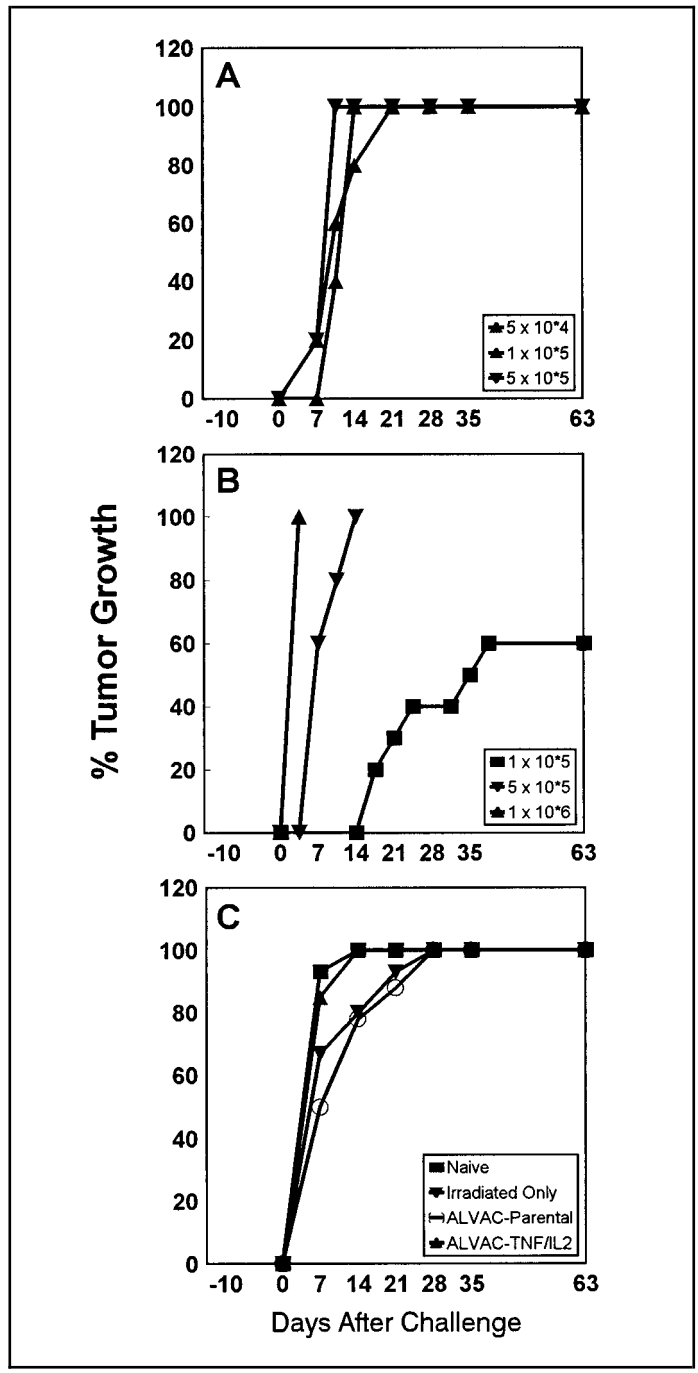

Fig. 5. Protection against tumor challenge after exposure to canarypox virus (ALVAC)-infected cells. **A)** Growth of tumors formed by RM-1 mouse prostate cancer cells in naive (i.e., previously untreated) mice receiving various doses of RM-1 cells (injected subcutaneously). **B)** Effect of previous exposure to irradiated RM-1 cells on the growth of tumors induced by various challenging doses of RM-1 cells. **C)** Effects of previous exposure to irradiated RM-1 cells alone or irradiated RM-1 cells that were infected with ALVAC vectors on the growth of tumors induced by a challenging dose of 5×10^5 RM-1 cells. In panels A and B, 10*4, 10*5, and 10*6 = 10^4, 10^5, and 10^6, respectively. TNF = tumor necrosis factor-α; IL2 = interleukin 2.

cessing are observed in vitro in mammalian cells [Fig. 1; (*14-16*)]. The inability of ALVAC to replicate productively in nonavian species provides an exquisite safety barrier against the occurrence of vaccine-associated or vaccine-induced complications that are seen with replicating vaccinia virus in the vaccination for smallpox (*11*). The Recombinant DNA Advisory Committee has reduced the biologic containment level for ALVAC to biohazard safety level 1 (*27*). Recent phase I clinical trials of rabies glycoprotein recombinant and HIV-1 glycoprotein 160 recombinant ALVAC vectors demonstrated that the experimental vaccines were well tolerated and induced humoral and cellular immune responses in humans (*15,19*).

In our study, we demonstrated that ALVAC vectors can effectively deliver sequences encoding immunomodulatory gene products to RM-1 prostate cancer cells. Moreover, the growth of RM-1 cells expressing ALVAC-delivered TNF-α or the combination of TNF-α and IL-2 was not significantly inhibited in vitro. RM-1 cells infected with ALVAC recombinant vectors were observed to produce high levels of cytokines and to express the B7-1 co-stimulatory molecule effectively (Fig. 1). ALVAC-induced expression of single cytokines or co-stimulatory mol-

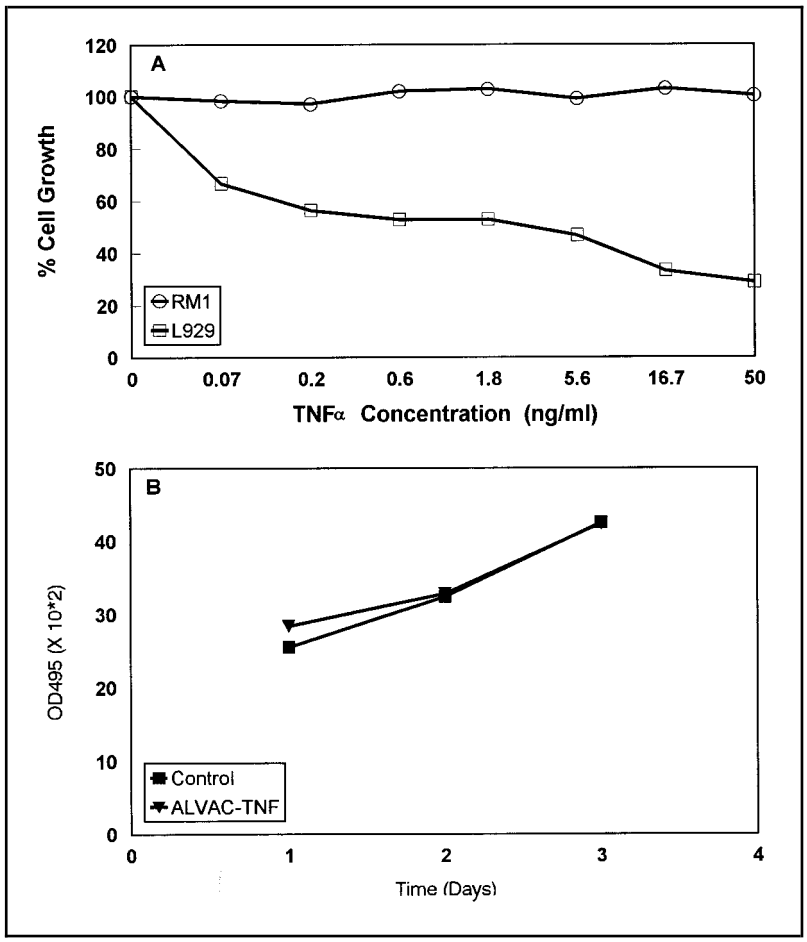

Fig. 6. Effects of tumor necrosis factor-α (TNF-α) on tumor cell growth in vitro. **A)** RM-1 mouse prostate cancer cells (○) and αL929 cells (a TNF-α-sensitive cell line) (□) were incubated with various concentrations of recombinant TNF-α for 24 hours. Cell growth was measured by crystal violet uptake (*see* ''Materials and Methods'' section for details). **B)** RM-1 cells were plated at a concentration of 5×10^3 cells/well in a 96-well microtiter plate. One set was transfected with a canarypox virus (ALVAC) vector encoding TNF-α (▼) at a multiplicity of infection of 5 plaque-forming units/cell as described in the ''Materials and Methods'' section, while the others served as uninfected controls (■). Data are reported as OD_{495} units from staining with 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-5[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide in the presence of phenazine methosulfate. *See* ''Materials and Methods'' section for details. $10*2 = 10^2$.

ecules (ALVAC–IL-2, ALVAC–IFN γ, and ALVAC–B7-1) did not effectively prevent tumor growth in vivo (Fig. 2). Infection with ALVAC–TNF-α significantly inhibited tumor growth and was thus used in combination studies to assess the effects of multiple cytokine delivery on tumor growth. These data demonstrate that ALVAC–TNF-α in combination with ALVAC–IL-2 completely inhibited RM-1 tumor growth in 60%-100% of treated mice. In some experiments, ALVAC–TNF-α combined with ALVAC–IFN γ inhibited tumor growth, but the inhibition was variable, occurring in only one of three experiments similar to the one shown in Fig. 4. While consistent and significant inhibition of tumor growth was observed with the combination of ALVAC–TNF-α and ALVAC–IL-2, protection against a subsequent tumor challenge was not observed (Fig. 5, B).

In previous studies (*5-8*) with replicative recombinant vaccinia viruses that encode IL-1β, IL-2, IL-12, B7-1, or B7-2, tumor growth inhibition has also been observed. Hodge et al. (*8*), using vaccinia vectors expressing B7-1 or B7-2 and MC38 colonic adenocarcinoma cells, observed complete inhibition of tumor growth and partial protection against rechallenge by the

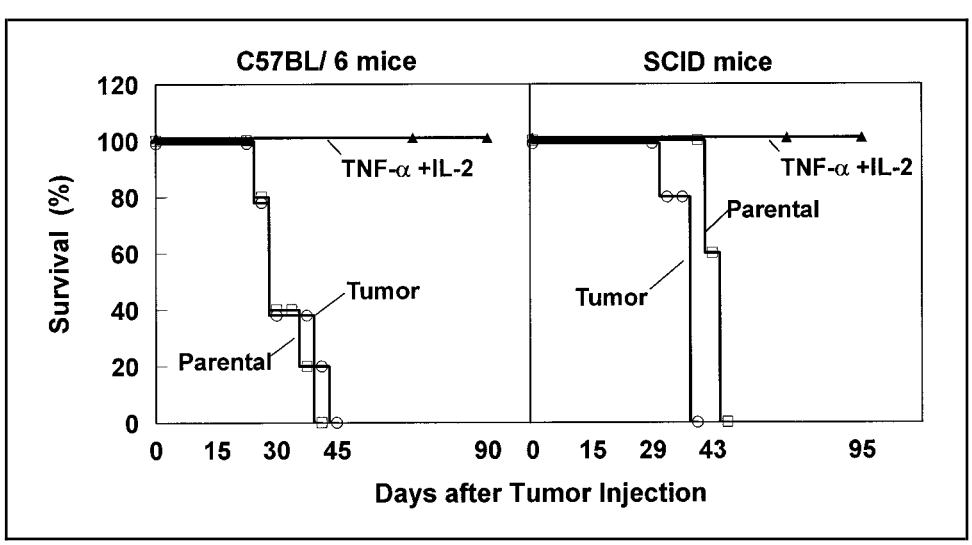

Fig. 7. Survival of C57BL/6 mice and of severe combined immunodeficient (SCID) mice. Mice were inoculated subcutaneously with 5×10^5 RM-1 mouse prostate cancer cells that were previously infected with the following canarypox virus (ALVAC) recombinant vectors at the multiplicity of infection (MOI) indicated for each one (total MOI of 5 plaque-forming units [PFU]/cell for all): parental ALVAC (MOI = 5) (□); ALVAC–tumor necrosis factor-α (TNF-α) (MOI = 2.5) plus ALVAC–IL-2 (interleukin 2) (MOI = 2.5) (▲); or no virus (○). In this experiment, ALVAC–TNF-α plus ALVAC–IL-2 provided 100% survival in both C57BL/6 mice and SCID mice.

parental tumor. Their recombinant vaccinia vectors induced the expression of B7-1 and B7-2 by tumor cells, as was observed in our studies with ALVAC–B7-1. However, in our studies, B7-1 did not inhibit RM-1 tumor growth. The reason for this difference is unclear. B7-1 is known to be necessary for the complete activation of helper T cells (*28*). The rationale for using B7 expression in immunotherapy studies is to provide the stimulus necessary for the activation of helper T cells and, thus, to provide the cytokine support required for CTL activation (*29*), although B7 expression has been shown to enhance CTL development independent of cytokine production (*30*). Chen et al. (*31*) demonstrated that the effectiveness of B7 expression was associated with the immunogenicity of a tumor. Immunity to tumors that were considered to be immunogenic was enhanced by B7 expression, but no effect was observed for nonimmunogenic tumors. Also, Wu et al. (*32*) suggested that uniform expression of B7 molecules by all tumor cells is required for functional B7-induced tumor immunity. Perhaps either the immunogenicity of RM-1 cells varies from that of MC38 cells or the level of B7-1 expression in our study was different from that reported by Hodge et al. (*8*).

In this study, experiments with single agents resulted in some inhibition of tumor growth but no effect on animal survival. The only single-agent regimen that induced significant inhibition of tumor growth was infection with ALVAC–TNF-α (Fig. 2, A). Previous studies (*33-35*), using tumor cell vaccines expressing TNF-α, produced variable results, with antitumor activity apparently dependent on the level of TNF-α produced and the tumor cell line tested. Asher et al. (*33*), using WP-4 sarcoma cells, showed a dose-dependent inhibition of tumor growth by TNF-α. The tumor inhibition was mediated by $CD4^+$ and $CD8^+$ T cells, and protective immunity was observed. In contrast, Allione et al. (*34*) did not observe tumor-specific immunity, although they did observe inhibition of tumor growth at the site of inoculation. Our study demonstrated that RM-1 tumor cells were resistant to direct TNF-α-induced cytolysis or growth inhibition, suggesting that the effect of TNF-α on tumor outgrowth was mediated by the immune system.

Enhanced antitumor activity was observed when ALVAC–TNF-α and ALVAC–IL-2 were combined. RM-1 tumor growth was completely inhibited in most experiments, but protective immunity could not be demonstrated. Several investigators have used cytokine combinations as antitumor agents with varying success. Allione et al. (*34*) combined cytokine-transfected tumor cells with *Corynebacterium parvum* but observed no enhancement of the antitumor effects. In contrast, Rosenthal et al. (*36*) used combined IL-2- and IFN γ-transfected tumor cells and observed enhanced antitumor activity; in their model, protective immunity was observed only when both cytokines were used simultaneously. Similarly, Dranoff et al. (*37*) observed improved antitumor activity with IL-2 in combination with TNF-α or other cytokines by using retrovirally transfected B16 melanoma cells as a model. In our model, ALVAC-delivered cytokine combinations also enhanced antitumor activity. Although RM-1 tumor growth was effectively inhibited with the combination of ALVAC–TNF-α and ALVAC–IL-2 in syngeneic C57BL/6 mice, the combination was equally effective in SCID mice. Similar results have been observed for IL-2- or IFN γ-transduced tumor cells injected into athymic nude mice (*2,3,35,38*). TNF-α and IL-2 are produced by the Th1 subset of $CD4^+$ lymphocytes and are key regulators of the cell-mediated immune response against neoplastic cells. IL-2, which is one of the most commonly used cytokines for gene therapy, stimulates the proliferation and lytic capacity of natural killer cells and T lymphocytes and induces local tumor rejection and tumor-specific immunity (*1,2,9,34,35*). Taken together, these data suggest that the antitumor activity induced by ALVAC–TNF-α and ALVAC–IL-2 results from the activation of non-B-cell, non-T-cell, host-mediated mechanisms.

One of the problems in testing a combination therapy is the need to perform multiple transfections. In the clinical setting, this requirement is problematic. Our study demonstrates the utility of nonreplicative ALVAC vectors in combination therapy. Moreover, use of these vectors provides a simple means of performing sequential immunizations with alternate vectors, which may provide further enhancement of immune activation. Efficient infection was obtained with several kinds of tumor cell lines (mouse bladder tumor and prostate tumor cell lines) and normal mouse prostate cells (our unpublished observations). Poxvirus is also useful for in vivo infection, as was found by Lee et al. (*39*) for recombinant vaccinia virus and by us (unpublished observations).

We conclude that ALVAC is useful for gene sequence delivery in immunotherapy studies because of its infection efficiency, its capability of high gene product expression, its safety, and its ease of handling. ALVAC-mediated expression of IL-2 and TNF-α induced nonspecific antitumor activity that resulted in a survival advantage. Further studies characterizing ALVAC recombinant vectors are in progress.

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Notes

Supported by the CaPCURE Foundation.

Manuscript received August 26, 1996; revised December 13, 1996; accepted January 8, 1997.