Enhancing Efficacy of Recombinant Anticancer Vaccines With Prime/Boost Regimens That Use Two Different Vectors

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**Background:** The identification of tumor-associated antigens and the cloning of DNA sequences encoding them have enabled the development of anticancer vaccines. Such vaccines target tumors by stimulating an immune response against the antigens. One method of vaccination involves the delivery of antigen-encoding DNA sequences, and a number of recombinant vectors have been used for this purpose. To optimize the efficacy of recombinant vaccines, we compared primary and booster treatment regimens that used a single vector (i.e., homologous boosting) with regimens that used two different vectors (i.e., heterologous boosting). **Methods:** Pulmonary tumors (experimental metastases) were induced in BALB/c mice inoculated with CT26.CL25 murine colon carcinoma cells, which express recombinant bacterial β-galactosidase (the model antigen). Protocols for subsequent vaccination used three vectors that encoded β-galactosidase—vaccinia (cowpox) virus, fowlpox virus, naked bacterial plasmid DNA. Mouse survival was evaluated in conjunction with antibody and cytotoxic T-lymphocyte responses to β-galactosidase. **Results:** Heterologous boosting resulted in significantly longer mouse survival than homologous boosting (all $P<.00001$, two-sided). Potent antigen-specific cytotoxic T lymphocytes were generated following heterologous boosting with poxvirus vectors. This response was not observed with any of the homologous boosting regimens. Mice primed with recombinant poxvirus vectors generated highly specific antibodies against viral proteins. **Conclusions:** The poor efficacy of homologous boosting regimens with viral vectors was probably a consequence of the induction of a strong antiviral antibody response. Heterologous boosting augmented antitumor immunity by generating a strong antigen-specific cytotoxic T-lymphocyte response. These data suggest that heterologous boosting strategies may be useful in increasing the efficacy of recombinant DNA anticancer vaccines that have now entered clinical trials. [J Natl Cancer Inst 1997;89:1595–1601]

The identification of tumor-associated antigens has enabled the development of recombinant and synthetic anticancer vaccines for use in clinical trials (1,2). A number of different recombinant and synthetic vectors, including recombinant vaccinia virus (rVV), recombinant fowlpox virus (rFPV), and recombinant DNA plasmids (pDNA), developed to express large quantities of the tumor-associated antigens have been demonstrated to effectively elicit potent humoral (antibody) and cell-mediated immune (e.g., cytotoxic T lymphocytes [CTLs]) responses in experimental murine model antigen systems (3–5).

Most significantly, these approaches have been shown to have therapeutic efficacy in the treatment of tumor-bearing mice (3–5). Several of these vaccination strategies are now being tested in clinical trials on patients with metastatic cancer at the National Cancer Institute and elsewhere.

The induction of an effective immune response against cancer antigens may require repetitive immunizations, since the aim of these experimental vaccines is the treatment of established tumors. Few studies have examined the therapeutic effect on tumor growth of repetitive boosting with recombinant vectors. It has been reported, however, that humoral and cellular immune responses are considerably reduced against the heterologous protein products generated by the rVV in individuals who have had previous exposure to the virus (6,7). Preimmunization of mice with a wild-type vaccinia virus followed by a recombinant version of the same virus has been shown to preclude the induction of immunity to the recombinant antigen for the natural lifespan of the animal (8). While these studies were limited to recombinant vaccinia vaccines, many recombinant vectors including fowlpox, influenza, adenovirus, and bacteria are also likely to induce anamnestic responses to the vector following repeated boosting.

In this study, we compared different vaccination strategies of priming and boosting either with a single vector (homologous boosting) or with two different vectors (heterologous boosting). We analyzed the generation of antigen-specific secondary CTLs, antibody responses, and most importantly, the elicitation of therapeutic antitumor immunity to develop optimal strategies for immunization against cancer.

**Materials and Methods**

**Tumor cell lines.** CT26.WT is a clone of the N-nitroso-N-methylurethane-induced (H-2d), undifferentiated colon carcinoma of BALB/c murine origin. After transduction with a retrovirus encoding the LacZ gene, CT26.WT was subcloned to generate the β-galactosidase (β-gal)-expressing cell line CT26.CL25 (4). Cell lines were maintained in complete medium consisting of RPMI-1640 medium, 10% heat-inactivated fetal calf serum (Biofluids, Rockville, MD), 0.03% L-glutamine, 100 μg/mL streptomycin, 100 U/mL penicillin, and 50 μg/mL gentamicin sulfate (National Institutes of Health Media Center). CT26.CL25 was maintained in medium containing 400 or 800 μg/mL G418 (Life Technologies, Inc. [GIBCO BRL], Gaithersburg, MD).

**Mice.** Female BALB/c mice, 6–10 weeks old, were obtained from the Animal Production Colonies, National Cancer Institute-Frederick Cancer Research and Production Colonies, National Cancer Institute-Frederick Cancer Research and...
Development Center, National Institutes of Health (Frederick, MD). The mice received care in accordance with the guidelines set forth by the animal research advisory committee of the National Institutes of Health.

**Peptides.** The synthetic peptide TPFHARIGL, representing the naturally processed H-2 Ld-restricted epitope spanning 876–884 of the gene, was provided by J. Haynes (Agracetus, Middleton, WI). Closed, circular plasmid DNA was isolated by the use of Wizard Minipreps DNA purification kits (Promega Corp., Madison, WI). Plasmid DNA and gold were coprecipitated by the addition of 200 μL of 2.5 M CaCl₂, during vortex mixing as previously described (9). DNA-coated gold particles were delivered into the abdominal epidermis by use of the hand-held helium-driven Accell gene delivery system (provided by Geniva, Middleton, WI). Each animal received 10 nonoverlapping deliveries per immunization at a pressure of 400 psi of helium.

**Recombinant viruses.** The rVV vaccine VJS6 was engineered such that the E. coli lacZ gene encoding for β-gal was under the control of the early/late vaccinia virus 7.5K promoter from plasmid pSC65 (5). The recombinant stocks were initially propagated in the BSC-1 monkey kidney cell line to create a crude lysate, which was then further purified over a sucrose cushion (10). The rFPV vaccine used in these studies (rFPV.bg40k) contains the E. coli lacZ gene under control of the vaccinia virus 40K promoter inserted into the BamHI region of the fowlpox virus genome as previously described (Therion Biologics Corp., Cambridge, MA) (4).

**In vivo treatment studies.** BALB/c mice were challenged intravenously with 10⁷ CT26.CL25 tumor cells to establish pulmonary metastases (11). Three days later, four groups of mice (37–40 mice per group) were primed with either no immunogen, 10 μg of pCMV/β-gal administered intradermally with the gene gun, 10⁷ plaque-forming units (PFU) of rVV-β-gal VJS6 administered intravenously, or 10⁸ PFU of rFPV.bg40k administered intravenously. Fourteen days after tumor inoculation, each group was divided into four groups of nine to 10 mice, each of which was subsequently boosted with the same amount of either no immunogen, pCMV/β-gal DNA, rVV-β-gal, or rFPV.bg40k to compare the 16 possible heterologous and homologous immunization strategies. These mice were followed for long-term survival.

**Statistical analysis.** Kaplan–Meier plots were generated, and the Mantel–Haenszel test was used to compare the survival of mice belonging to the different treatment groups; in each case, an adjustment was made for the number of tests accomplished. This experiment was performed two times with similar results. The P values stated are derived from two-sided tests.

**Generation of CTL and antibody responses.** BALB/c mice (eight mice per group) were vaccinated with either no immunogen, 10 μg of DNA encoding β-gal intradermally with the gene gun, 10⁷ PFU of rVV (VJS6) intravenously, or 10⁸ PFU of rFPV.bg40k intravenously. Twenty-one days later, each group of primed mice was divided into four groups of two mice each and were boosted with each immunogen to compare all heterologous and homologous possibilities. For the determination of the optimal kinetics of an in vivo secondary CTL response, mice were killed on days 2, 4, 6, or 8 after the second vaccination, at which time their spleens were removed and CTL lytic activity was assessed against β-gal-expressing tumor cells without an in vitro stimulation step in a standard 6-hour ⁵¹Cr release assay described below (data not shown). For all other experiments, mice were killed at the optimal time point, i.e., 4 days following the second vaccination, and in vivo CTL lytic activity was assessed. Pooled serum (from two mice per group) was also taken 8 days after the boost to evaluate antibody reactivity to β-gal protein via an enzyme-linked immunosorbent assay (ELISA).

**⁵¹Cr release assay.** Six-hour ⁵¹Cr release assays were performed as previously described (12). Briefly, for the radioactive labeling of the target cells, 2 × 10⁶ of either CT26.WT cells, CT26.CL25 cells, or CT26.WT cells pulsed with β-gal peptide were incubated with 200 μCi of the γ emitter Na⁺⁵¹CrO₄ in 0.2 mL of complete medium for 90 minutes. Peptide-pulsed CT26.WT cells were incubated with 1 μg/mL (approximately 1 μM) antigenic β-gal peptide (TPFHARIGL) during ⁵¹Cr labeling as previously described (3–5). Target cells were then mixed with effector cells for 6 hours at 37 °C at the effector-to-target cell ratios indicated (100:1, 50:1, 25:1, etc.). The amount of ⁵¹Cr released was determined after 6 hours of incubation, and the percentage of specific lysis was calculated as follows: percent specific lysis = ([(experimental cpm – spontaneous cpm)/(maximal cpm – spontaneous cpm)])(100).

**ELISA.** Serum from immunized mice was collected 21 days after the primary immunization and 8 days after the final boost to be analyzed for the presence of antibodies against β-gal protein, wild-type vaccinia virus, or wild-type fowlpox virus by ELISA, as previously described (3). Specifically, microtiter plates were either dried down overnight at 37 °C in a nonhumidified incubator with 200 ng/50 μL per well of purified β-gal protein (Sigma Chemical Co., St. Louis, MO) or coated with either wild-type vaccinia virus (5 × 10⁹ PFU/50 μL per well) or wild-type fowlpox virus (5 × 10⁹ PFU/50 μL per well) at 4 °C overnight. Incubation of 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) on each well for 1 hour to prevent nonspecific antibody binding was followed by a second 1-hour incubation with 50 μL of fivefold dilutions (starting at 1:100) of test sera. After being washed with 1% BSA in PBS, horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin G (IgG) F(ab)₂, fragments (1:5000 dilution in 1% BSA in PBS) (Amersham International, Amersham, U.K.) were added for 1 hour at 37 °C to detect antibodies immobilized on the wells. The resulting complex was detected by the chromogen o-phenylenediamine (Sigma Chemical Co.). Absorbance was read on a Titrtek Multiskan Plus Reader (Flow Laboratories, McLean, VA) with the use of a 490-nm filter.

**Western blot analysis.** Mouse antisera obtained 21 days after the primary immunization was tested in a western blot for reactivity against β-gal protein, wild-type vaccinia virus, and wild-type fowlpox virus. To this end, 5 μg of β-gal protein, 6.6 × 10⁶ PFU of wild-type vaccinia virus, and 2 × 10⁷ PFU of wild-type fowlpox virus were dissolved in sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis sample buffer, boiled for 5 minutes, and subjected to electrophoresis by use of a 6%–18% linear gradient SDS–polyacrylamide gel. After electrophoresis, proteins were transferred for 2 hours onto nitrocellulose paper (0.45-μm pore size) by use of transfer buffer at room temperature at 25 V. The blots were then incubated in PBS containing 5% nonfat dry milk for 1 hour at room temperature. Ten milliliters of a 1:200 dilution of antisera in PBS with 2% nonfat dry milk was added to each nitrocellulose strip, and the strips were incubated for 2 hours at room temperature with gentle agitation. After the blots were washed with PBS containing 0.05% Tween-20, they were incubated with horseradish peroxidase-conjugated sheep anti-mouse IgG F(ab)₂ fragments (1:1000 dilution in 1% BSA in PBS) to visualize antibody binding. Bound immunoglobulin was then detected by the incubation of the blots for approximately 3 minutes in 3,3′-diaminobenzidine tetrahydrochloride (Sigma Chemical Co.) dissolved in distilled water. The reaction was stopped by washing the nitrocellulose blots for 5 minutes with distilled water.

**Results**

**Prolongation of Survival of Tumor-Bearing Mice by Boosting With Heterologous Vectors.** To compare the effect of repetitive immunization of the recombinant vaccine vectors on tumor growth, long-term survival studies were performed (Fig. 1). BALB/c mice challenged intravenously with 10⁷ CT26.CL25 (β-gal)+ tumor cells were immunized 3 days later with either no immunogen, pDNA (pCMV/β-gal), rVV expressing β-gal (VJS6), or rFPV expressing β-gal (rFPV.bg40k). Seventeen days after tumor inoculation, each group of primed mice was divided into four groups and boosted with each immunogen to compare all possible heterologous and homologous immunization strategies.

Mice immunized 17 days after tumor administration with either pDNA (pCMV/β-gal) or rFPV (rFPV.bg40k) had a slight increase in survival compared with that of nonimmunized mice (Fig. 1, A). Two immunizations with rVV (VJS6) significantly prolonged survival compared with that of unvaccinated mice, but this result was not statistically different from the result obtained with one immunization 3 days after tumor challenge (Fig. 1, B). Mice that received a boost with a heterologous recombinant viral vector, rFPV, after an initial immunization with rVV had a longer survival time than mice that received the homologous prime and boost with rVV (P < 0.0001; Fig. 1, B). Indeed, 50% of the heterologously boosted mice survived longer than 60
days. A similar pattern was observed for rFPV immunization (Fig. 1, C). Whereas one immunization with rFPV did not significantly enhance survival compared with that of control mice, a prolongation of survival was seen with two sequential immunizations of rFPV ($P<.0001$). However, mice administered rFPV and boosted with the heterologous vector rVV had a significant increase in survival compared with that of the mice that received two doses of rFPV ($P<.00001$); 80% of the mice that received the heterologous combination survived for greater than 60 days (Fig. 1, C).

For pDNA immunization, a small but significant increase in survival was observed in the group of mice that received a prime and a boost with pDNA ($P = .0026$) (Fig. 1, D). Boosting pDNA immunization with either heterologous viral vector, rVV or rFPV, significantly extended longevity compared with the lifespan of the untreated group ($P<.0001$) or with the results obtained with a prime and boost of pDNA ($P = .0002$ and $P = .0028$, for boosting pDNA immunization with rVV and rFPV, respectively) (Fig. 1, D). Conversely, boosting with pDNA increased the lifespan of mice primed with either rVV or rFPV compared with the lifespan of mice immunized two times with pDNA ($P = .0026$ and $P = .0046$ for rVV and rFPV priming, respectively) (Fig. 1, E). No statistically significant difference in survival was observed between mice primed with either rVV or rFPV and boosted with pDNA and the groups of mice that received a homologous prime and boost of either rFPV or rVV (Fig. 1, D and E). Considered together, these data suggested that immunizing and boosting with two different vectors expressing the same tumor-associated antigen prolonged survival of tumor-bearing mice more efficiently than multiple immunizations with the same vector.

**In Vivo Secondary CTL Responses Induced in Mice Immunized and Boosted With Different Vectors Expressing the Same Tumor-Associated Antigen**

To determine the effect of the different immunization schema on the induction of an antigen-specific CTL response, we im-
mumized the mice with the different heterologous and homolo-
gous combinations of the pDNA, rVV, and rFPV vaccines as
described in the “Materials and Methods” section (Fig. 2).

Unprimed mice administered rVV or rFPV and tested for
CTL reactivity 4 days later failed to induce a lytic response
against either CT26.CL25 (β-gal+) or CT26.WT (β-gal–). Mice
primed with either rVV or rFPV and tested 21 days later did
not elicit β-gal-specific CTLs (data not shown). No CTL activity
was observed when mice were immunized and boosted with
the same vector, either rVV or rFPV (Fig. 2). However, boosting
the rVV-primed mice with a different vector, rFPV, induced anti-
gen-specific CTLs (Fig. 2). Mice primed with rFPV also
did not induce anti-β-gal CTLs. However, rFPV-primed
mice boosted with the heterologous vector rVV elicited antigen-
specific CTLs (Fig. 2). Mice primed with pDNA induced β-gal-
specific CTLs only when they were boosted with either rVV
or rFPV (Fig. 2). The order of this immunization appeared to be
important because, when either an rVV or an rFPV immuniza-
tion was followed by a booster with pDNA, no lytic activity was
observed (data not shown). These studies suggest that repetitive
vaccination with the same vector does not promote the expan-
sion of antigen-specific CTLs. However, the immunization stra-
 egy using two different recombinant vectors expressing the same
antigen does induce enhanced lytic activity.

Augmented Anti-β-gal Antibody Responses Elicited After
a Boost With Any Combination of pDNA, rVV, or rFPV

To study antigen-specific humoral immunity by use of the
different combinations of the rDNA, rVV, and rFPV vaccines,
we tested serum samples, harvested 8 days after the boost, by
ELISA for antibody reactivity against β-gal protein. β-gal-
specific antibody titers were increased following a primary
immunization with rVV with boosts of either pDNA, rVV, or
rFPV. (Titers increased from 1:50 with no boost to 1:250 for
each group [Fig. 3, A]).) After rFPV immunization, β-gal
titers were also dramatically boosted by a second immunization with
either pDNA (titer = 1:6250), rVV (titer = 1:3000), or rFPV
(titer = 1:1500). The β-gal-specific antibody titers were also
boosted when either pDNA, rVV, or rFPV was administered as
a boost following pDNA priming; these titers ranged from 1:200
to 1:2500 for each (Fig. 3, A). Mice that were primed with rVV
demonstrated lower titers against β-gal after the boost than those
that were primed with either pDNA or the rFPV. An enhance-
ment of the anti-β-gal antibody response was observed regard-
less of boosting with either a homologous vector or a heterolo-
gous vector expressing the same tumor-associated antigen.

Induction of Vector-Specific, High-Titered Antibodies
After a Single Immunization With Either rVV or rFPV

To characterize vector-specific humoral immunity induced by
immunization with either of the pDNA, rVV, or rFPV vaccines,
we tested serum samples harvested 21 days after the primary
immunization by ELISA and western blot for antibody reactivity
against wild-type vaccinia virus or wild-type fowlpox virus
(Figs. 3, B, and 4).

High titers of anti-vaccinia virus antibody were seen in the
serum from mice primed with rVV (titer = 1:31 250), but not in
the serum from mice immunized with pDNA or rFPV (Fig. 3,
B). Western blot analysis demonstrated that immunization with
rVV induced antibodies against both a single band of β-gal
protein (Fig. 4, lane 4) and multiple bands of wild-type vaccinia
virus (Fig. 4, lane 5), but no reactivity was observed against
wild-type fowlpox virus (Fig. 4, lane 6). Similarly, titers of
anti-fowlpox virus antibodies were found only in the sera of
mice primed with rFPV (titer = 1:1250; Fig. 3, B). Western blot
analysis showed that vaccination with rFPV induced antibodies
that recognized β-gal protein (Fig. 4, lane 7), 14–20 bands of
wild-type fowlpox virus (Fig. 4, lane 9), and no bands of wild-
type vaccinia virus (Fig. 4, lane 8). The antibodies induced by
vaccinia virus did not react with either wild-type
vaccinia virus or wild-type fowlpox virus, but they did recognize
β-gal protein both by ELISA and by western blot analysis (Figs.
3, B, and 4). These data show that high titers of vector-specific
antibodies were induced by immunization with either vaccinia
virus or fowlpox viruses.

Altogether, vaccination strategies using different recombi-
nant vectors expressing the same tumor-associated antigen re-
sulted in no cross-reactive antibodies among vectors, enhanced

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**Fig. 2.** In vivo secondary cytotoxic T-lymphocyte (CTL) responses in mice immunized with different homologous and heterologous vaccina-
tion regimens. Previously untreated BALB/c mice (eight per group)
were primed with either no immunogen (None), 107 plaque-forming
units (PFU) of VJS6 (rVV) expressing β-galactosidase (β-gal) intra-
venously, 107 PFU of rFPV.bg40k (rFPV) intravenously, or 10 μg of
pCMV/β-gal (pDNA) intradermally with the gene gun. Twenty-one
days later, each group of mice was divided into four groups and boosted
with the same amount of either no immunogen, rVV, rFPV, or pDNA to
to compare heterologous and homologous vaccination regimens. Mice
were killed 4 days after the boost, and a standard 6-hour 3HCT release
assay was performed immediately to assess secondary in vivo CTL
responses against β-gal as described in the “Materials and Methods”
section at effector-to-target cell (E:T) ratio of 100:1 and after twofold
dilutions of the effectors to give E:T ratios of 50:1, 25:1, etc. CT26.WT
(β-gal–, ○) and CT26.CL25 (β-gal+, ●) served as targets. This
experiment was repeated seven times with similar results.
CTL responses, and prolonged survival of tumor-bearing mice. Thus, this strategy of immunizing and boosting with alternating recombinant vectors may represent a more potent means of enhancing an immune response against a desired antigen than repetitive immunizations with the same vector.

Discussion

Exposure of the immune system to an antigen results in "immunological memory," which in turn enhances the body’s ability to respond to a repeat exposure to the same antigen. The rationale to employ prime/boost regimens is based on observations of secondary immune responses, which are faster and larger than the first (primary) immune response (13,14). Specifically, a priming dose of an antigen results in the activation and expansion of clonotypes capable of recognizing a particular peptide antigen presented in the context of its restricting major histocompatibility complex (MHC) molecule. It has been hypothesized that secondary immune responses are more vigorous than primary immune responses not only because of an increased number of antigen-specific precursor T cells but also because of a "memory" state of T-lymphocyte activation. Although the mechanisms of T-cell memory are still the subject of some debate (i.e., whether there is a persistence of antigen or a persistence of particular T cells), this memory can last for the lifetime of the vaccinated animal and results in a rapid expansion of memory T lymphocytes upon re-exposure to relatively lower concentrations of the relevant antigen (15,16).

To our knowledge, this study represents the first report to suggest that immunizing and boosting with two different recombinant vectors expressing the same tumor-associated antigen may be better than repeated vaccinations with the same recombinant vector for the active treatment of malignant disease. We selected rVV, rFPV, and pDNA as vectors for this study because they deliver antigen intracellularly to mediate powerful CTL responses and antitumor immunity (3–5,17–19).

Whereas boosting with the identical recombinant vectors augmented antibody reactivity against β-gal, immunizing and boosting with the same immunogen failed to elicit secondary CD8⁺ T-lymphocyte responses, as shown by lysis of antigen-expressing tumor cells (Fig. 2). These data may be a result of an increase in precursor CTL frequency following the boosting immunization that is below the level of detection of our in vivo primary CTL assay. However, Murata et al. (20) also have recently demonstrated that priming and boosting with the same

| Antibody Reactivity | Serum from Mice harvested the day of the boost (21 days after the initial priming immunization) were tested by ELISA for antibody reactivity against wild-type vaccinia virus (left panel) or wild-type fowlpox virus (right panel) as described in the "Materials and Methods" section. Serum titers to either β-gal protein, wild-type vaccinia virus, or wild-type fowlpox virus were calculated with the use of the dilution observed at an optical density of 0.3. |

**Fig. 3.** Antibody reactivity of sera from mice immunized with different homologous and heterologous vaccination regimens. Previously untreated BALB/c mice were primed with either no immunogen (None), 10 μg of β-galactosidase (β-gal) DNA (pDNA) intradermally with the gene gun, 10⁷ plaque-forming units (PFU) of VJS6 (rVV) expressing β-gal intravenously, or 10⁷ PFU of rFPV.bg40k (rFPV) intravenously. Twenty-one days later, each group of mice (two mice per group) was boosted with the same amount of each immunogen to compare all heterologous and homologous immunization regimens. A) Sera harvested 8 days after the boost were assayed by enzyme-linked immunosorbent assay (ELISA) for antibody reactivity against β-gal protein as described in the "Materials and Methods" section. B) Sera from mice harvested the day of the boost (21 days after the initial priming immunization) were tested by ELISA for antibody reactivity against wild-type vaccinia virus (left panel) or wild-type fowlpox virus (right panel) as described in the "Materials and Methods" section. Serum titers to either β-gal protein, wild-type vaccinia virus, or wild-type fowlpox virus were calculated with the use of the dilution observed at an optical density of 0.3.

**Fig. 4.** Western blot of purified β-galactosidase (β-gal) protein, wild-type vaccinia virus, and wild-type fowlpox virus with the use of serum samples from mice immunized with recombinant vaccinia virus (rVV), recombinant fowlpox virus (rFPV), and recombinant DNA plasmids (pDNA). Mice were immunized one time with either 10 μg of pCMV/β-gal (pDNA) intradermally with the gene gun (left panel), 10⁷ plaque-forming units (PFU) of VJS6 (rVV) intravenously (middle panel), or 10⁷ PFU of rFPV.bg40k (rFPV) intravenously (right panel). Twenty-one days later, serum was harvested and tested at a 1:200 dilution by western blot against nitrocellulose blots of 5 μg of β-gal protein (lanes 1, 4, and 7), 6.6 × 10⁶ PFU of wild-type vaccinia virus (lanes 2, 5, and 8), or 2 × 10⁷ PFU of wild-type fowlpox virus (lanes 3, 6, and 9) as described in the "Materials and Methods" section. The blots were then washed and incubated with horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin G (Fab')₂ fragments (1:1000 diluted in 1x phosphate-buffered saline with 1% bovine serum albumin) to visualize antibody binding. Bound immunoglobulin was then detected by incubating the blots for approximately 3 minutes in 3,3’-diaminobenzidine tetrahydrochloride dissolved in distilled water. The reaction was stopped by washing the nitrocellulose blots for 5 minutes with distilled water. The molecular weight markers Mn(K) are shown on the right of the figure.
recombinant virus did not result in the expansion of the secondary CD8+ T-cell population specific for the heterologous antigen. In the present study, a strong secondary β-gal-specific lytic response was observed when mice were immunized and boosted in any order with the two different recombinant poxvirus vectors expressing the same antigen. In agreement with these findings, Murata et al. (20) demonstrated a 20- to 30-fold expansion in the number of antigen-specific CD8+ precursor T cells in mice primed with a recombinant influenza virus and boosted with a recombinant vaccinia virus expressing the same MHC class I-restricted epitope. Similarly, Hodge et al. (21) showed that splenocytes derived from mice primed with rVV expressing the tumor-associated antigen, carcinoembryonic antigen (CEA), and boosted with the recombinant avian poxvirus ALVAC-CEA demonstrated a fourfold increase in CEA-specific T-cell proliferation as compared with mice primed and boosted with rVV expressing CEA alone.

Pre-existing neutralizing antibodies against the viral structural proteins may reduce the CTL responses against tumor-associated antigens and antitumor immunity by rapidly neutralizing the boosting recombinant viral infection and thus reducing the expansion of precursor T cells against the tumor-associated antigen (6–8). In accordance with these studies, we observed high titers of specific anti-vector antibodies after one immunization with the recombinant viruses. Antiviral CTLs may also play a role in the elimination of recombinant virus and thus the reduced tumor-associated antigen-specific secondary CTL and antitumor responses (22). The limited immunogenicity to the tumor-associated antigen observed after a boost with the same viral vector may also be due to a specific suppression of responses against weaker determinants by stronger or immunodominant determinants (23–25). Vaccinia virus and fowlpox viruses both produce more than 200 proteins responsible for their structure, transcription, and replication that could function as potential immunodominant epitopes. The abundant expression of vaccinia virus structural proteins by the replicating rVV could also account for the weak induction of anti-β-gal antibodies (Fig. 3, A).

Although some of the proteins expressed by fowlpox virus and vaccinia virus share homology, it is not known whether the subset of proteins that shares homology also shares immunodominant determinants (26,27). Our studies demonstrated that little or no antibody cross-reactivity exists between rVV and rFPV (Figs. 3, B, and 4). This lack of cross-reactivity between vaccinia virus and fowlpox virus may contribute to the enhancement of CTL expansion and antitumor immunity following a boost with a heterologous vector.

Vaccination with recombinant plasmid DNA is accomplished by the expression of plasmid DNA encoding a single antigen of interest preceded by a eukaryotic promoter and therefore shares no epitopes in common with either viral vector (3,28–30). In the case of immunization with pCMV/β-gal, we did not observe antibodies cross-reactive with either rVV or rFPV. The use of plasmid DNA may eliminate problems of memory responses to vector-related proteins, although humoral responses to DNA in this setting have not been adequately studied. Despite this characteristic, DNA boosted by DNA was not as effective as a DNA prime boosted by either of the recombinant viral vectors. The poorer efficacy of DNA vaccination may be due to a number of reasons. DNA vaccination may not produce the quantity of the antigenic protein required to elicit therapeutic immune responses in an active treatment regimen. By contrast, viral vectors produce large quantities of antigen that persist for long periods. In addition, viruses express many immunogenic proteins that may serve as adjuvants that boost immunity against the recombinant protein. Our results demonstrated, however, that DNA could be utilized both as a priming or as a boosting agent to prolong the survival of tumor-bearing mice.

These data help to establish a strategy of immunizing and boosting with alternating recombinant vectors that could be useful in the clinical use of recombinant anticancer vaccines. However, unlike many of the recently identified tumor-associated antigens cloned from melanoma that are nonmutated differentiation antigens, β-gal is a foreign molecule to a mouse, and the responses induced by these recombinant constructs may not be the same as those responses induced by self antigens. Future studies will be aimed at generating immunity against self molecules with the use of the strategy of immunization with heterologous recombinant vectors encoding self antigens, such as the murine homologues of the human melanoma antigens. Clinical trials applying the use of alternating recombinant vectors are also currently ongoing in the National Cancer Institute in patients with metastatic melanoma. This strategy represents a novel approach to enhance the potency of therapeutic recombinant vectors for the treatment of established disease in vivo.

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Notes

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