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ORIGINAL ARTICLE

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Colon cancer cell vaccine prepared with replication-deficient vaccinia viruses encoding B7.1 and interleukin-2 induce antitumor response in syngeneic mice

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Abstract A replication-deficient recombinant vaccinia virus, NYVAC, was developed by deleting 18 open reading frames in the vaccinia virus genome. Recombinant NYVAC, encoding the murine T cell co-stimulatory gene B7.1 (CD 80) (NYVAC-B7.1) and the murine interleukin-2 gene (NYVAC-IL-2), were prepared and the expression of B7.1 and the secretion of IL-2 were respectively confirmed in vitro. The use of these viruses to prepare a potent tumor cell vaccine was studied in a syngeneic murine CC-36 colon adenocarcinoma model. Mice were immunized on days 1 and 8 with 10^6 irradiated CC-36 cells that were infected with 10^7 plaque-forming units of either NYVAC-B7.1, NYVAC-IL-2 or a control virus, NYVAC-HR, which encodes a vaccinia virus host-range gene. These mice were then challenged with 10^8 viable CC-36 tumor cells on day 15. All mice (10/10) in a group that had received no vaccination and all mice (20/20) in a group that had received a control vaccine of CC-36/NYVAC-HR developed tumor 4-weeks after tumor cell challenge. Interestingly, only 16/20 mice in a group that had received CC-36/NYVAC-B7.1 showed the development of tumor after the same interval. The protection against tumor development and the reduction in tumor burden (as mean tumor diameter, 4 weeks after tumor challenge) were significant in this group when compared to groups that were either unvaccinated or vaccinated with CC-36/NYVAC-HR (mean tumor diameter = 6.51 ± 3.2 mm compared to 26.5 ± 0.9 mm or 26.2 ± 1.8 mm respectively) ($P = < 0.05$). The protection against tumor in a group of mice that received CC-36/NYVAC-IL-2 vaccination was similar to that in the unvaccinated group or the group receiving a CC-36/NYVAC-HR control vaccination. However, in a survival experiment, mice that received either CC36/NYVAC-B7.1 or CC-36/NYVAC-IL-2 vaccination on the day of tumor transplantation survived significantly longer than mice that had not been vaccinated (median survival 60+ days, 60+ days or 23.5 days respectively) ($P = < 0.05$). Interestingly, when a therapeutic tumor vaccination was performed on day 4 after tumor transplantation, mice that had been vaccinated with either CC36/NYVAC-B7.1 or CC-36/NYVAC-IL-2 did not show an improved survival when compared to mice in the control that had not been vaccinated (median survival 28 days compared to 26 days or 25 days respectively). However, mice that had received a therapeutic vaccination with CC-36 cells infected with both NYVAC-B7.1 and NYVAC-IL-2, 4 days after tumor transplantation, survived significantly longer than control mice that had not received any vaccination (median survival 29.5 days compared to 25 days respectively) ($P < 0.05$). These results suggest that a replication-deficient recombinant NYVAC encoding the B7.1 gene and NYVAC encoding the IL-2 gene can be used to produce an effective vaccinia-virus-augmented tumor cell vaccine.

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Introduction

Active specific immunotherapy is one of the experimental therapies for patients with cancer. Our laboratory has been engaged in an active specific immunotherapy approach using vaccinia-virus-augmented melanoma cell vaccine for patients with melanoma. Recently, a phase III, randomized, double-blind, placebo-controlled, multi-institutional active specific immunotherapy trial was performed in our laboratory with this vaccine for patients with stage III melanoma. Although the analysis of data from this trial showed that subsets of patients benefitted with this vaccine, significant improvement in either disease-free interval or overall survival was not observed in all patients [24, 25]. Therefore, improving the potency of this vaccine to make it

more effective has been the objective of this laboratory for the past few years.

The efficacy of a tumor cell vaccine may be enhanced by adding one or more immunomodulating lymphokines to the vaccine. In fact, studies in experimental animal models have shown that a combination therapy of a tumor cell vaccine with lymphokines such as interleukin-2 (IL-2), IL-4, granylocyte-colony-stimulating factor (GCSF), granulocyte/macrophage-colony-stimulating factor (GM-CSF), interferon α (IFNα), and IFNγ enhances the efficacy of the vaccine in inducing immunity [1, 10]. Moreover, tumor cells that secrete lymphokines (IL-2, IL-4, IL-12, IFNγ, IFNα, G-CSF and GM-CSF) by the gene-transduction method have been shown to be better vaccines than vaccines with parental tumor cells that do not secrete these lymphokines [7, 8, 12, 23].

Several methods are used to transduce a cytokine gene into tumor cells [7, 8, 11, 12, 14–17, 21, 23]. In these methods, vectors such as retrovirus vectors [7, 8, 23], adenovirus vectors [12], and vaccinia virus vectors [5, 11, 14–17, 21] have been used to transfer a gene into tumor cells. Since our laboratory has been studying vaccinia-virus-augmented tumor cells as a vaccine in the active specific immunotherapy of cancers, we have used the vaccinia virus vector method to deliver cytokine genes into tumor cells to prepare a potent tumor cell vaccine [21]. Furthermore, the vaccinia virus vector approach has several advantages: it has a double-strand DNA genome, can accommodate more than one gene, contains a special vaccinia virus promotor, and does not integrate into the host genome.

In our preliminary work, a tumor cell vaccine containing recombinant vaccinia virus encoding the IL-2 gene was prepared and studied for its efficacy in a syngeneic CC-36 colon adenocarcinoma model. Results showed that this new vaccine induced more antitumor immunity than did a control vaccine that contained a recombinant vaccinia virus having a non-immunomodulatory gene [21]. Other laboratories have shown that the use of recombinant vaccinia viruses encoding IL-2, IL-12, GM-CSF, or B7.1 in an experimental tumor vaccine increased antitumor immunity in animal models [5, 11, 15–17]. Although this new approach can be translated into the preparation of vaccines for human cancers, the major concern lies in the toxicity of the vaccinia virus, in particular, those with immune deficiencies. The major toxicity includes encephalopathies and eczema. Some toxicities were found to be associated with vaccinia-virus-derived biochemicals, such as thymidine kinase, hemagglutinin, serine protease inhibitors and ribonucleotide reductase [4, 6, 20, 26]. Furthermore, some viral toxicities have been shown to be due to viral replication at the injection site and subsequent dissemination to other locations. In order to prepare a safe and less toxic vaccinia virus, Tartaglia et al. [22] derived a recombinant vaccinia virus, NYVAC, by deleting 18 open reading frames that are associated with the above toxicity. This NYVAC has been shown to produce no detectable ulceration at the site of injection, to be replication-deficient in mice and human cell lines, and to express a greatly reduced pathogenicity in immunocompromized mice when compared with the parental vaccinia vaccine virus [22]. However, this NYVAC still maintains its ability to transcribe and translate the genes that were encoded in it under the control of a vaccinia virus promoter [22]. Therefore, this NYVAC is a safe vaccinia virus vector that can be used to produce vaccinia-virus augmented human tumor cell vaccines [22]. In this report, we have studied the feasibility of using a tumor cell vaccine prepared with the recominant NYVAC-B7.1, containing the gene encoding murine T-cell-co-stimulatory factor B7.1 (CD 80), and the recombinant NYVAC-IL-2, encoding the murine interleukin-2 gene, using the CC-36 murine colon adenocarcinoma model.

Materials and methods

Cell line

The CC-36 colon adenocarcinoma cell line [19] was maintained in Dulbuecco's modified essential medium supplemented with 10% fetal calf serum, 2 mM/ml glutamine, 1 mM/ml pyruvate, 1% nonessential amino acids, 100 units/ml penicillin, and 100 μg/ml streptomycin (all from Gibco, Grand Island, N.Y.). A single-cell suspension of CC-36 tumor cells was prepared by stripping the monolayer of CC-36 cells with trypsin/EDTA (Giboc, Grand Island, N.Y.), washing it once with phosphate-buffered saline, and resuspending these cells in Hank's balanced salt solution. These cells were used in NYVACB7.1-, NYVAC-IL-2 or NYVAC-HR-augmented tumor cell vaccine production and tumor induction.

Mice

Male balb/c mice, 4–6 weeks old, were purchased from Harlan-Sprague-Dawley, Indianopolis, Ind., and used in all the experiments.

Recombinant NYVAC encoding the B7.1 IL-2 or HR gene

The NYVAC encoding the murine B7.1 (NYVAC-B7.1), the NYVAC encoding the murine IL-2 gene and NYVAC encoding the vaccinia virus host-range gene (NYVAC-HR) were produced by Dr. Enzo Paoletti, Virogenetics, Troy, N.Y. The derivation of NYVAC from the Copenhagen strain of vaccinia virus by the deletion of 18 open reading frames is described by Tartaglia et al. [22]. The method for producing foreign-gene-encoding NYVAC is also described elsewhere [22]. Briefly, the cDNA of murine B7.1 was derived from a cDNA library constructed using cells that express murine B7.1. The cDNA sequence of this B7.1 was found to be the same as the published sequence of murine B7.1. This murine B7.1 cDNA gene was cloned into a thymidine-kinase-deletion plasmid, pSD513, under the control of the vaccinia H6 promotor. This plasmid was used as a donor plasmid for the in vivo recombination with NYVAC. The NYVAC encoding the murine B7.1 gene was selected by plaque hybridization using a ^{32}P-labeled DNA probe to murine B7.1 coding sequences. Similarly NYVAC encoding IL-2 was prepared using the cDNA of murine IL-2 that was derived from a murine cDNA library. The NYVAC-HR was also produced by the same methodology using the plasmid pSD513 coding for the vaccinia-virus-host range gene.

The above viruses were multiplied in our laboratory using primary chicken embryo fibroblasts (CEF). The NYVAC-B7.1, NYVAC-IL-2, and NYVAC-HR viruses were quantified as plaque-forming units by a standard method using CEF cells.

Expression of B7.1 on CC-36 cells infected with NYVAC-B7.1

The expression of B7.1 on CC-36 cells infected with NYVAC-B7.1 was analyzed by a direct immunofluorescence method. For this study, 10^6 CC-36 cells were infected with 10^7 pfu NYVAC-B7.1 for 4 h.

These cells were then spun down and treated with 10 µl fluorescein-isothiocyanate-conjugated murine-B7.1-specific antibody (Pharmingen, San Diego, Calif.) for 30 min. The cells were washed twice with phosphate-buffered saline (PBS) and then fixed using PBS containing 0.5% paraformaldehyde and were then analyzed for fluorescence activity using a fluorescence-activated cell sorter (FACScan, Becton Dickinson, Calif.). An isotype-matched antibody and NYVAC-HR-infected CC-36 cells were used as controls.

Expression of IL-2 from NYVAC-IL-2

Production of IL-2 from NYVAC-IL-2 was measured in supernatants derived from a cell culture containing NYVAC-IL-2-infected CC-36 cells. A sample containing 10^6 CC-36 cells in 5 ml complete Dulbecco's modified Eagle medium was infected with either 10^6 or 10^7 pfu NYVAC-IL-2 virus. Supernatants were harvested 2, 4, 6, 8, 10 12, 24, 48, 72 and 96 h after the virus infection and analyzed for mouse IL-2, using a commercial mouse IL-2 enzyme-linked immunosorbant assay kit (Advanced Magnetics Inc., Cambridge, Mass., USA) according to the manufacturer's instructions.

Tumor-protective immunity induced by CC-36 tumor cell vaccine prepared with NYVAC-B7.1 or NYVAC-IL-2

The augmentation of the immunogenicity of tumor cell vaccine prepared with NYVAC-B7.1 or NYVAC-IL-2 was verified in an immunoprophylactic experiment. Four groups of mice were used in this experiment. Mice in the first group (n = 20) were immunized intraperitoneally (i.p.) with 10^6 irradiated CC-36 cells that were infected with 10^7 pfu NYVAC-B7.1 on days 1 and 8. Mice in the second group (n = 20) were immunized intraperitoneally with 10^6 irradiated CC-36 cells that were infected with 10^7 pfu NYVAC-IL-2 on days 1 and 8. Mice in the third group (n = 20) were immunized intraperitoneally with 10^6 irradiated CC-36 cells that were infected with10^7 pfu NYVAC-HR on days 1 and 8. Mice in the fourth group (n = 10) did not receive any vaccination. Mice in all these groups received 10^8 viable CC-36 tumor cells subcutaneously in the right flank region on day 15. Mice were observed for tumor incidence for 60 days. In addition, the mean tumor diameter of each mouse was measured using a vernier caliper every 2–3 days after the first observation of the development of tumor, approximately 1 week after tumor transplantation.

Survival of mice transplanted with live CC-36 tumor cells mixed with irradiated CC-36 cells that were infected with NYVAC-B7.1 or NYVAC-IL-2

Since earlier studies used the method of intratumoral injection of a recombinant vaccinia virus to test the antitumor efficacy of this virus [2, 13], a survival experiment was performed using a mixture of viable CC-36 tumor cells and a vaccine of irradiated CC-36 cells that were infected with NYVAC-B7.1 or NYVAC-IL-2 to determine the antitumor efficacy of these viruses. Mice (n = 10) in the first group received an i.p. injection of 1×10^5 live CC-36 cells. Mice (n = 10) in the second group received an i.p. injection of a mixture of 1×10^5 live CC-36 cells and a vaccine of 1×10^5 irradiated (30 Gy) CC-36 cells that were infected with 10^6 pfu NYVAC-B-7.1 for 4 h. Mice (n = 10) in the third group received an i.p. injection of a mixture of 1×10^5 live CC-36 cells and a vaccine of 1×10^5 irradiated (30 Gy) CC-36 cells that were infected with 10^6 pfu NYVAC-B-7.1 for 4 h. Mice were followed for 60 days to determine survival. Surviving mice on day 60 were sacrificed and evaluated for tumor. Each dead mouse was evaluated for the cause death.

A second survival experiment was performed to determine the therapeutic efficacy of CC-36/NYVAC-B7.1 or CC-36/NYVAC-IL-2 vaccine that was given 4 days after tumor transplantation. In this experiment, on day1, mice were transplanted with 1×10^5 viable CC-36 cells intraperitoneally. On day 4 after tumor transplantation, a group of 10 mice were vaccinated subcutaneously with CC-36/NYVAC-B7.1 (cell:virus ratio 1:10), and a second group of 10 mice were vaccinated subcutaneously with CC-36/NYVAC-IL-2 (cell:virus ratio 1:10) vaccine. Two booster vaccine injections were given on day 10 and day 17 after CC-36 tumor cell transplantation. A group of 10 mice without any vaccination but receiving 1×10^5 viable CC-36 cells intraperitoneally on day 1 served as a control. Mice were followed for 60 days to assess survival.

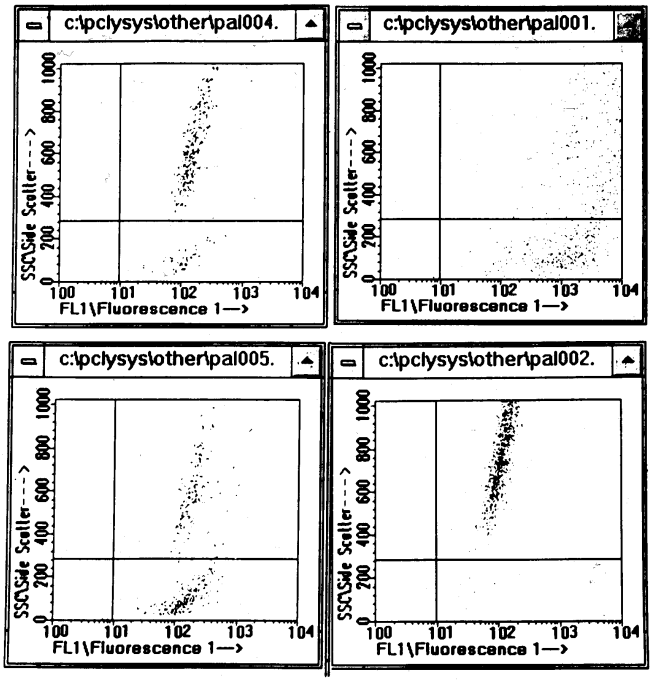

Fig. 1 The expression of murine B7.1 on CC-36 cells infected with NYVAC-B7.1. CC-36 cells (10^6) were infected with 10^7 plaque-forming units (pfu) of NYVAC-B7.1 or NYVAC-HR for 4 h and stained with fluorescein-isothiocyanate-(FITC)-conjugated anti-(murine B7.1) antibody. *Top left* plain CC-36 cells were stained with FITC-conjugated anti-(murine B7.1) antibody. *Top right* NYVAC-B7.1-infected CC-36 cells were stained with FITC-conjugated antibody to murine B7.1. *Bottom left* NYVAC-HR-infected CC-36 cells were stained with FITC-conjugated antibody to murine B7.1. *Bottom right* NYVAC-B7.1-infected CC-36 cells were stained with FITC-conjugated, isotype-matched antibody. Only the CC-36 infected with NYVAC-B7.1 (*top right*) showed the expression of murine B7.1

Subsequent to this survival experiment, a third survival experiment was performed to determine the therapeutic efficacy of a vaccine containing irradiated CC-36 cells that were infected with both NYVAC-B7.1 and NYVAC-IL-2. In this experiment, on day 1, mice (n = 20) were transplanted with 1×10^5 viable CC-36 cells intraperitoneally. On day 4, these mice were vaccinated with CC-36/NYVAC-B7.1/NYVAC-IL-2 vaccine (10^6 irradiated CC-36 cells infected with 5×10^6 pfu NYVAC-B7.1 plus 1×10^6 irradiated CC-36 cells infected with 5×10^6 pfu NYVAC-IL-2) subcutaneously. Two booster vaccine injections were given on day 10 and day 17 after CC-36 tumor cell transplantation. A group of mice (n = 10) that received 1×10^5 viable CC-36 cells intraperitoneally served as a control. Mice were followed for survival for 60 days.

Statistical analysis

The tumor incidence, tumor burden, and the tumor growth kinetics were analyzed by a two-sided Student's t-test. The survival was analyzed by the Wilcoxon test.

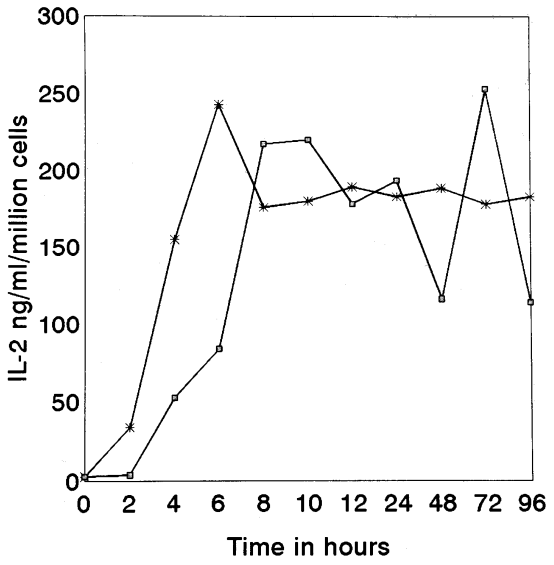

Fig. 2 The production of interleukin-2 (*IL-2*) from CC-36 cells that were infected with NYVAC-IL-2. CC-36 cells (10^6) were infected with NYVAC-IL-2 at a ratio of 1:1 (□) or 1:10 (*) cells:NYVAC-IL-2 (pfu). Supernatants collected at different times after the NYVAC-IL-2 infection were used in an enzyme-linked immunosorbent assay that measured the murine IL-2. The production of IL-2 was seen 2 h after NYVAC-IL-2 infection at a 1:10 ratio and 4 h after infection at a 1:1 ratio

Results

Figure 1 shows the fluorescence-activated cell sorting analysis of cells infected with NYVAC-B7.1 or NYVAC-HR. The expression of murine B-7.1 was observed on CC-36 cells that were infected with NYVAC-B7.1 4-h after infection. The plain CC-36 cells and CC-36 cells that were infected with NYVAC-HR did not show murine B7.1 expression. Moreover, NYVAC-B7.1-infected CC-36 cells treated with a control IgG were negative for B7.1. This study confirms that the NYVAC-B7.1 expresses murine B7.1 co-stimulatory factor on the surface of the CC-36 cells 4 h after NYVAC-B7.1 infection. We have also tested the expression of B7.1 on CC-36 cells 24 h after NYVAC-B7.1 infection. There was no significant increase in the expression of B7.1 aftre 24 h, beyond that recorded 4 h after the NYVAC-B7.1 virus infection (results not shown).

Figure 2 shows the production of IL-2 from the NYVAC IL-2-infected CC-36 cells. The presence of IL-2 could be detected as early as 2 h after NYVAC-IL-2 infection in supernatants derived from CC-36 culture containing virus at a cell-to-virus ratio of 1:10. However, IL-2 could be detected in a supernatant collected 4 h after NYVAC-IL-2 infection at a cell-to-virus ratio of 1:1. The IL-2 production reached the maximum 6 h after the virus infection at both ratios. Approximately 200 ng IL-2 was shown to be produced by 1×10^6 CC-36 cells in 24 h for both the 1:1 and 1:10 cell-to-virus ratios.

In an immunoprophylactic experiment, a group of unvaccinated mice showed 100% (10/10; 0% protection) development of tumor 4 weeks after tumor transplantation. The group of mice vaccinated with CC-36/NYVAC-B7.1 showed 20% (4/20) and 40% (8/20; 4 mice had regression of tumor) protection against the development of tumor 4 and 6 weeks after tumor transplantation. However, 0% protection (0/20) was observed with the group of mice vaccinated with a control vaccine, CC-36/NYVAC-HR, at the same assay times. Moreover, there was no protection (0/20) observed with the group of mice vaccinated with the CC-36/NYVAC-IL-2. Statistical analysis showed that the CC-36/NYVAC-B7.1 vaccination induced a significant protection against the tumor development when compared

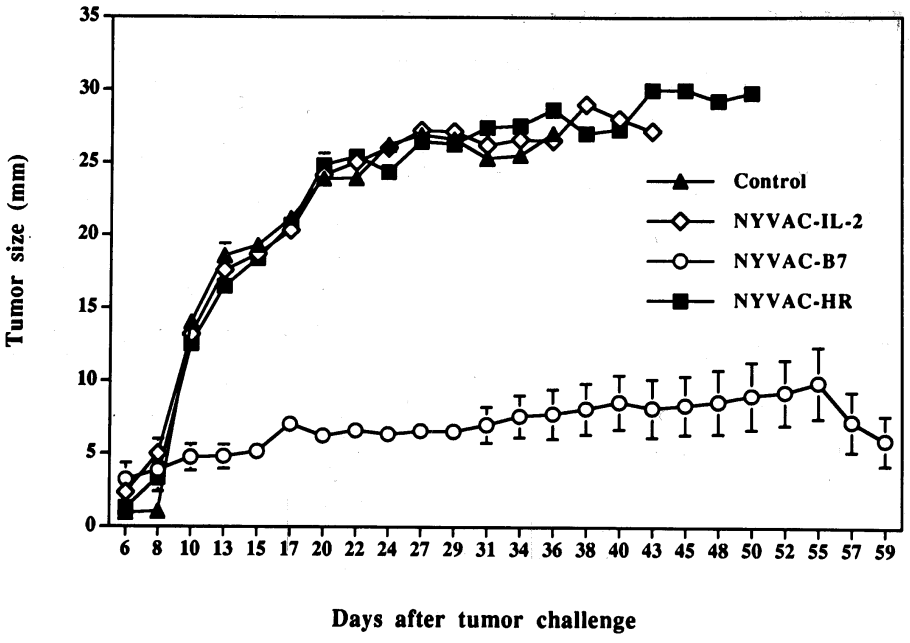

Fig. 3 Mean tumor diameter with standard error (±SE) of mice vaccinated with NYVAC-B7-infected CC-36 cells (O), NYVAC-IL-2-infected CC-36 cells (◇), or NYVAC-HR-infected CC-36 cells (■) and then challenged with 10^8 viable CC-36 tumor cells. The control (▲) did not receive any vaccine. Mice that received NYVAC-B7-infected CC-36 cell vaccine (O) had significantly less tumor growth ($P<0.001$) than other groups

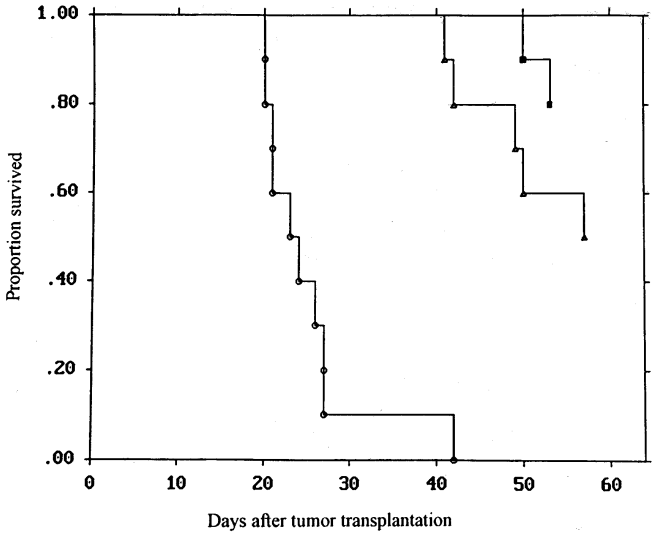

Fig. 4 Survival of mice that received an i.p. injection of viable CC-36 cells (10^5) mixed with a vaccination of irradiated CC-36 cells (10^5) that were infected with 10^6 pfu NYVAC-IL-2 (■) or NYVAC-B7.1 (▲). The control received only 10^5 viable CC-36 tumor cells (O). Significantly more mice survived in the group receiving CC-36/NYVAC-IL-2 or CC-36/NYVAC-B7.1 than in the control group ($P = <0.05$)

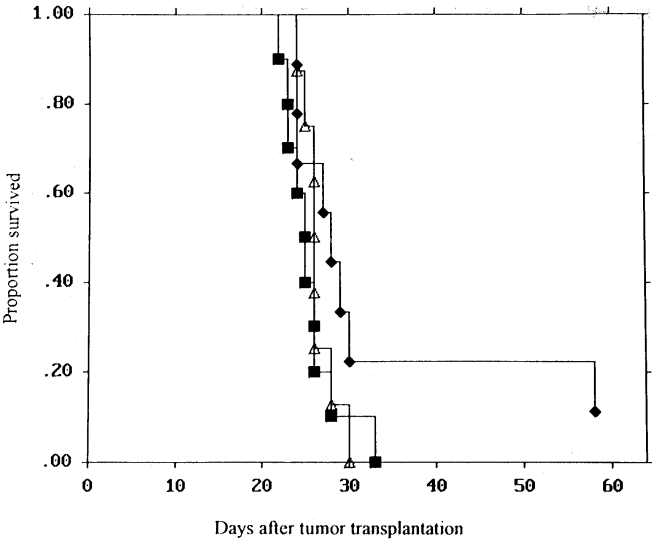

Fig. 5 Survival of mice that had received therapeutic vaccination with 10^6 irradiated CC-36 cells that had been infected with 10^7 pfu either NYVAC-IL-2 (■) or NYVAC-B7.1 (◆). Mice received 10^5 CC-36 cells intraperitoneally on day 1 and s.c. vaccinations on days 4, 10 and 17. The control (Δ) received only 10^5 viable CC-36 tumor cells. No significantly increased survival was observed in the group of mice that received either CC-36/NYVAC-IL-2 or CC-36/NYVAC-B7.1 when compared to the control group

to the unvaccinated control, CC-36/NYVAC-HR vaccination and CC-36/NYVAC-IL-2 vaccination ($P<0.05$). In addition, the group of mice vaccinated with CC-36/NYVAC-B7.1 showed a statistically significant reduction in tumor growth and tumor burden at 2- and 4-week intervals after tumor cell challenge when compared to the groups that received CC-36/NYVAC-HR or CC-36/NYVAC-IL-2 or no vaccination ($P<0.05$) (Fig. 3). The mean tumor diameter of the group vaccinated with CC-36/NYVAC-B7.1 was 6.44 ± 0.6 mm and 6.51 ± 3.2 mm 2 and 4 weeks after the tumor cell challenge respectively. The mean tumor diameters of the unvaccinated group, the CC-36/NYVAC-HR-vaccinated group and the CC-36/NYVAC-IL-2-vaccinated group were 19.3 ± 1.3 mm and 26.6 ± 0.6 mm, 18.4 ± 2.5 mm and 26.3 ± 1.8 mm, and 18.7 ± 2.3 and 27.1 ± 2.4 mm respectively, at the same times after tumor cell challenge.

In the first survival experiment, significantly more of the mice that had received CC-36/NYVAC-B7.1 or CC-36/NYVAC-IL-2 vaccination simultaneously with tumor transplantation survived ($P = <0.05$) than did mice that had received no vaccination (Fig. 4). On day 60, 50% of the mice that had received NYVAC-B7.1-infected CC-36 cells survived compared to 0% of the mice that had received plain CC-36 cells. Similarly, on day 60, 80% of the mice that had received NYVAC-IL-2-infected CC-36 cells survived whereas none of mice that had received plain CC-36 cells survived. In this experiment, no group was treated with CC-36NYVAC-HR since our earlier study suggested that the NYVAC-HR-infected CC-36 cells did not improve survival when compared to plain CC-36 cells (the median survival times were 27 and 26 days respectively). In this study, the median survival times for the group of mice that received CC-36/NYVAC-B7.1 or CC-36/NYVAC-IL-2 were 60+ days or 60+ days respectively, whereas the median survival time for the control group was 23.5 days. On day 60, 2/5 surviving mice in the NYVAC-B7.1 group had tumor, and 3/8 surviving mice in the NYVAC-IL-2 had tumor. Since this experiment used 1 cell to 10 NYVAC-B7.1 or NYVAC-IL-2 in the vaccine, another survival experiment was performed with a vaccine containing 1 cell to 1 NYVAC-B7.1 or NYVAC-IL-2 to determine the effect of virus concentration on the induction of antitumor response. A similar survival advantage was observed with CC-36/NYVAC-B7.1 and CC-36/NYVAC-IL-2 (1:1 cell-to-virus ratio) vaccines when compared to an unvaccinated control (results not shown).

Since the CC-36/NYVAC-B7.1 or CC-36/NYVAC-IL-2 vaccination in the above experiment significantly prolonged survival, a second survival experiment was performed to determine the efficacy of CC-36/NYVAC-B7.1 or CC-36/NYVAC-IL-2 vaccination in mice with 4-day established CC-36 tumor. In this survival study, one primary vaccination was performed on day 4 after tumor transplantation followed by two booster vaccinations that were performed on days 10 and 17 after tumor transplantation. Results from this study show that neither of these vaccines significantly increased survival beyond that of the unvaccinated control group (Fig. 5). Median survival times for the control group, and the groups vaccinated with CC-36/NYVAC-B7.1 or CC-36/NYVAC-IL-2 were 25 days, 28 days, and 26 days respectively. However, when mice were vaccinated on days 4, 10 and 17 with a CC-36 tumor cell vaccine containing both NYVAC-B7.1 and NYVAC-IL-2,

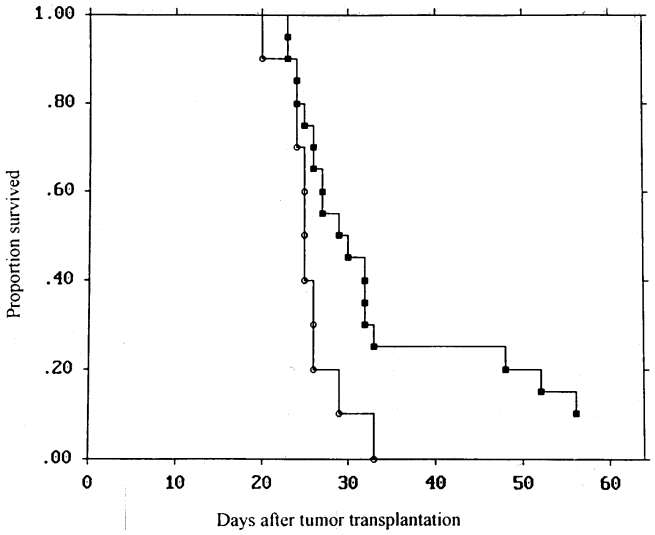

Fig. 6 Survival of mice that had received a therapeutic vaccination with CC-36 cells infected with both NYVAC-IL-2 and NYVAC-B7.1 (■). Mice received 10^5 CC-36 cells intraperitoneally on day 1 and vaccines on days 4, 10 and 17 after tumor transplantation. The control received only 10^5 viable CC-36 tumor cells (O) intraperitoneally. Significantly more mice survived after receiving this vaccine than did mice in the control group ($P < 0.05$)

survival was significantly increased ($P < 0.05$) (Fig. 6). The median survival time following this CC-36/NYVAC-B7.1 plus CC-36/NYVAC-IL-2 vaccine therapy was 29.5 days, compared to 25 days for a control that did not receive a vaccine. Results from this experiment suggest that a CC-36 vaccine containing both NYVAC-B7.1 and the NYVAC-IL-2 induced a better antitumor response than did a vaccine containing either NYVAC-B7.1 or NYVAC-IL-2.

Discussion

Vectors derived from retrovirus [7, 8, 23], adenovirus [12], adeno-associated virus [9] and vaccinia virus [5, 11, 14–17, 21] have been used to transfer a foreign gene into mammalian cells. Recombinant vaccinia viruses encoding the immunomodulating genes such as IL-2, IL-12, GM-CSF, B7.1 and B7.2 have been shown to transduce tumor cells and subsequently express the gene they encode [5, 11, 14–17, 21]. Furthermore, tumor cells augmented with these vaccinia viruses were shown to induce antitumor immunity in syngeneic mice [5, 11, 14–17, 21]. We have shown previously that the use of a recombinant vaccinia virus encoding the IL-2 gene in a colon cancer vaccine induced antitumor immunity in syngeneic mice [21]. Therefore, one can prepare a potent tumor cell vaccine with a recombinant vaccinia virus encoding an immunomodulating gene for patients with cancer. In fact, Eisenlohr et al. have performed a preliminary clinical trial with a recombinant vaccinia virus encoding GM-CSF in patients with melanoma [13].

All the recombinant vaccinia viruses that were used in the above studies were replication-competent, hence there is a risk of infection in individuals who are immunocompromized. Therefore, attempts are being made to prepare a replication-deficient vaccinia virus vector that has negligible toxicity. Miko et al. [15] used UV irradiation to change the replication-competent recombinant vaccinia virus encoding IL-12 to become non-replicating. Furthermore, they showed that a tumor vaccine prepared with this approach induced antitumor activity. On the other hand, Tartaglia et al. produced a replication-deficient vaccinia virus by deleting 18 open reading frames from the genome. The NYVAC showed replication deficiency in rodents and man [22]. Although the NYVAC is replication-deficient in cell lines of human origin, it transcribes and translates the foreign gene encoded in it [3]. We have tested the use of this replication-deficient recombinant vaccinia virus NYVAC in this study to prepare a potent tumor cell vaccine using a murine colon cancer model.

Replication-deficient NYVAC vaccinia viruses containing the genes encoding murine B7.1 and murine IL-2 were prepared and studied for the expression of B7.1 and IL-2 respectively. NYVAC-B7.1 and NYVAC-IL-2 produced B7.1 and IL-2 respectively at 4 h after infecting the CC-36 cells. This is consistent with our earlier report of the production of IL-2 from a proliferating vaccinia virus encoding IL-2 [21]. Interestingly, low-level IL-2 production was evident even 2 h after the NYVAC-IL-2 infection at a 1:10 cell:NYVAC-IL-2 ratio. These results indicate that there may not be any difference between the expression of IL-2 from a proliferating recombinant vaccinia virus encoding IL-2 and that of the replication-deficient NYVAC containing the IL-2 gene.

In the prophylactic experiment, vaccination with CC-36/NYVAC B7.1 produced a significant protection against the development of tumor, and a significant reduction in tumor burden. It is not known why a vaccination with CC-36/NYVAC-IL-2 did not produce protection. Perhaps the IL-2-gene-containing recombinant vaccinia virus alone is less effective at inducing an antitumor response in this model. However, in the first study of survival, mice that received the NYVAC-B7.1-infected CC-36 cell vaccine or NYVAC-IL-2-infected CC-36 cell vaccine lived significantly longer than mice that received no vaccine. The increased survival in these groups may not be due to the elimination of viable CC-36 cells by the infection of NYVAC-B7.1 or NYVAC-IL2 from the vaccine, since these viruses are replication-deficient. Moreover, our earlier experiment showed that mice transplanted with NYVAC- or NYVAC-HR-infected CC-36 cells did not live longer than mice transplanted with plain CC-36 cells, suggesting that NYVAC and NYVAC-HR could not eliminate the CC-36 tumor since these viruses did not contain an immunomodulating gene. Therefore, in the current experiment, the increased survival of mice that received CC-36 cells infected with NYVAC-B7.1 or NYVAC-IL-2 was due to the immunomodulating effect of B7.1 or IL-2 respectively. Surprisingly, in the second study of survival, mice with 4-day-old tumor did not show any antitumor efficacy either following CC-36/NYVAC-B7.1 or CC-36/NYVAC-IL-2 vaccination. However, in the third survival experiment, mice with the same 4-day-old tumor burden survived significantly longer with a CC-36 vaccine

containing both NYVAC-B7.1 and NYVAC-IL-2. This result suggests that a combination of T cell co-stimulatory factor and the IL-2 may play a role in the induction of antitumor response. This is supported by studies from other investigators who have shown that the antitumor efficacy of B7.1-encoding vaccinia virus can be augmented with the addition of soluble IL-12 [18].

The mechanism of induction of an antitumor response by the CC-36 cells infected with NYVAC-B7.1 or NYVAC-IL-2 or both is being studied. An analysis of fresh peripheral blood lymphocytes from mice immunized with these vaccines did not show any CC-36-tumor-specific cytolytic T cell activity (data not shown). We are currently performing a prophylactic experiment using mice depleted in CD4 or CD8 T cells to determine the role of these immune T cells in the induction of antitumor immunity. However, regression of tumor following CC-36/NYVAC-B7.1 vaccination, and long-term survival of mice vaccinated with the CC-36/NYVAC-B7.1 or CC-36/NYVAC-IL-2 or a mixture of these two viruses suggests that NYVAC-B7.1 and NYVAC-IL-2 could be useful in the preparation of an effective tumor vaccine that can induced an antitumor response.

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