

Anti-Tumor Effect of Murine Renal Cell Carcinoma Cells Genetically Modified to Express B7-1 Combined with Cytokine Secreting Fibroblasts

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ABSTRACT

Recently, many experiments have shown that the expression of the costimulatory molecule B7-1 on tumor cells can induce tumor-specific immunity. These results suggest that tumor cells modified to express costimulatory molecules can be used as a potential tumor vaccine. For this purpose, we transduced B7-1 gene into renal adenocarcinoma cells of spontaneous origin (Renca) in BALB/c mouse using the retroviral vector system. Our results indicated that approximately 60% of cells expressed B7-1 gene product using the retroviral vector system, and that B7-1 transduction did not affect the expression of MHC molecules on tumor cells nor the *in vitro* growth rate of tumor cells, but only *in vivo* tumorigenicity. As for the antitumor effect on the remote site, there were no significant differences among parental Renca, Renca lac Z and Renca B7-1 sublines, although tumors grew a little more slowly in the mice injected with Renca B7-1 cells as a vaccine. Even if the growth of tumors was significantly delayed in the mice treated by Renca B7-1 as a vaccine combined with the injection of BALB/c3T3 IL-12 near to the tumor on the same or following day, no significant antitumor effects were observed when the Renca B7-1 cells were injected as a vaccine compared with cytokines near the vaccine site. These results indicated that B7-1 gene transduction can decrease the tumorigenicity of murine renal cell carcinoma cells, but fails to induce sufficient antitumor response when it is used as a tumor vaccine. It is necessary to develop immunogenicity, by such means as irradiation or a combination of appropriate cytokines, to stimulate effective tumor immunity in a therapeutic setting.

Key words: B7-1, IL-12, Gene therapy, Murine renal cell carcinoma

Whereas 30% of patients diagnosed with renal cell carcinoma (RCC) have metastatic disease at presentation, patients with metastatic RCC have a median survival of 6-10 months and a 2-year survival of 10-20%. Neither surgery, radiation therapy, hormonal therapy nor chemotherapy has altered the natural history of this disease^{5,7,15)}. In the past, cytokines such as interferons and interleukin-2 (IL-2) have been tested on a large scale in the treatment of advanced RCC. However, the rates of objective response (partial and complete) have been disappointing, only rarely exceeding 20%¹⁸⁾.

Tumor vaccines are based on weakly immunogenic specific tumor antigens admixed with adjuvants in order to elicit, restore, or augment antitumor immune responses against residual or metastatic tumor cells. Cellular cytotoxicity is con-

sidered to play a major role in eliminating tumor cells. Activation of cellular toxicity requires at least three synergistic signals: the presentation of specific tumor antigen, the costimulatory signal and the propagation signal. Among the known costimulatory molecules, the B7 family of membrane proteins appears to be the most potent. B7-1 is a member of the Ig superfamily, and it is expressed on the majority of antigen presenting cells, such as dendritic cells, activated macrophages, and activated B cells. B7-1 can also interact with its counter receptor, CD28, on T cells. Binding of the T cell receptor (TCR) with peptide-MHC complexes in the absence of costimulation results in T cell inactivation or "anergy"¹⁹⁾. Several reports have suggested that dendritic cells infiltrating into the tumor failed to express B7-1. That B7-1 failed to be expressed in the tumor is perhaps one of the

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reasons why the tumor escapes immune surveillance²). Hence, one strategy to enhance the immune recognition of tumor cells is to express costimulatory molecules on their surface, thus enabling them to present specific tumor antigens as well as provide the necessary costimulatory signals.

Several experiments have shown the ability of B7-1 transduction to alter the capacity of neoplastic cells to elicit protective immunity against tumors. In some experiments, animals were inoculated with B7-1 transduced tumor cells^{1,3,4,16}. This inoculation led to primary rejection of the modified tumor cells. We may infer from these experiments that genetically engineered B7-1 tumor cells might possibly be useful as a therapeutic vaccine for RCC.

The retroviral vector system can afford high titer and high gene expression. This method would obviate the need for selection of transduced cells among a bulk tumor cell population and thereby both minimize the time required for culturing primary tumor cells prior to vaccination and maximize the antigenic heterogeneity represented in the vaccinating inoculum.

Therefore, to address the possibility of gene therapy using B7-1 expressed RCC cells as a vaccine, we prepared genetically engineered murine RCC cells to express the B7-1 molecule, then characterized these cells and evaluated their antitumor effect in the murine model. We also determined the synergic effect of Renca B7-1 cells combined with fibroblasts genetically engineered to secrete IL-2 and IL-12.

MATERIALS AND METHODS

Tumors

Renca, a renal cell carcinoma of spontaneous origin in BLAB/c mice¹³, was generously provided by Dr. Fujioka (Iwate Medical School, Iwate, Japan) and maintained in Dulbecco's modified Eagle medium (DMEM) (GIBCO BRL, Life Technologies Inc., Rockville, MD, USA) supplemented with 20% fetal bovine serum (FBS) (GIBCO BRL, Life Technologies Inc., Rockville, MD, USA). BALB/c 3T3 cells, a non-transformed embryonic murine mesenchymal line, were also used.

Recombinant retroviral vectors and transduction to tumor cells

DNA sequences encoding the B7-1, IL-12 p35, IL-12 p40, and lac Z were inserted into the retroviral vector MFG and the resulting constructs were introduced into ψ CRIP cells to generate a recombinant virus with the amphotropic host range⁶. Renca cells were exposed to viral supernatants including B7-1 or lac Z.

Briefly, 5×10^5 of Renca cells were inoculated into a 100 mm Petri dish and were incubated until the

logarithmic phase. Then these cells were transduced with 2 ml of retroviral supernatants (MFG-B7-1 or MFG-lac Z) in the presence of polybrene (4 μ g/ml) for 24 hr, and were further incubated in DMEM supplemented with 10% FBS for 2 days.

BALB/c 3T3 cells were transduced by IL-2, IL-12 p35, IL-12 p40 and lac Z gene according to the methods described previously¹⁴. BALB/c 3T3 IL-12 cells can secrete 248.5 ng of biologically active IL-12/10⁶ cells/48 hr estimated by the proliferative response of 4 days human PHA-activated lymphocytes¹⁴. The secretion of IL-2 by BALB/c 3T3 IL-2 cells was confirmed by ELISA methods (Mouse ELISA IL-2, Endogen, Inc., MA, USA). BALB/c 3T3 IL-2 cells can secrete 657.3 ng/10⁶ cells/48hr. Genetically engineered murine fibroblasts can serve as the vehicle for cytokine delivery.

Immunohistochemistry for Renca B7-1

For detection of B7-1 expression, 5×10^3 cells were seeded on a Chamber Slide in each well and incubated at 37°C for 24 hr. After fixing in a mixture of 50% acetone/50% methanol for 1 min, sections were incubated with rat anti-mouse B7-1 monoclonal antibody (Sumitomo Denko, Yokohama, Japan) for 30 min at room temperature, then stained using Vectastain ABC kit (VECTOR Laboratories, Inc. Burlingame, CA) according to the manufacturer's instructions. DAB was used as the peroxidase substrate and methyl green as a counter stain. Diluted normal rat serum was used as a negative control for rat anti-mouse B7-1 monoclonal antibody.

Immunofluorescent flow-cytometry analysis

The alteration of the surface antigens, MHC class I antigen, class II antigen and B7-1 on Renca cells after gene transduction was analyzed. FITC-conjugated anti-MHC class I (H-2K^d) and class II (I-A^d) monoclonal antibody were purchased from Meiji Nyugyo Co. Ltd. (Tokyo, Japan), and FITC-conjugated anti-B7-1 monoclonal antibody was from Sumitomo Denko (Yokohama, Japan). Each kind of cells was incubated with the respective antibody in phosphate-buffered saline (PBS) supplemented with 5% FBS for 45 min at 4°C. After washing the cells 3 times with PBS, the respective cells were analyzed by flow cytometry (EPICS Elite Flow Cytometer, Coulter, Hialeah, FL).

Cell proliferation assay

To characterize the *in vitro* growth rate, we enumerated the number of each kind of cells sequentially. Ten thousand of each cell (parental cell, Renca lac Z and B7-1) were inoculated into a 35 mm Petri dish containing DMEM supplemented with 10% FBS. The total number of cells in each dish was examined on days 1, 2, 3 and 5.

Animal experiments

BALB/c mice (female, 6–8 weeks old) were purchased from Charles River Japan Inc. (Kanagawa, Japan) and were housed in micro isolator cages, fed *ad libitum*, given acid water, and maintained in a specific pathogen-free environment. All experiments were conducted in accordance with the University of Hiroshima Animal Care guidelines.

For the tumorigenicity experiment, we divided the mice into 3 groups, one for parental Renca cells, one for Renca lac Z cells and one for Renca B7-1 cells. Each group contained 5 mice. All cell lines that were prepared for injection were collected by trypsin from a culture flask in 5 ml Hanks' balanced salt solution (HBSS). The cells were then washed twice with HBSS containing 10% FBS and resuspended at a concentration of 1×10^7 cells/ml in HBSS. Parental and modified tumor cells (1×10^6) were inoculated into BALB/c mice subcutaneously (s.c.) at the right posterior flank in a volume of 0.1 ml. The mice were examined for tumor development and the tumors were measured in two dimensions with calipers twice weekly.

As a treatment model, we injected parental Renca cells into mice s.c. at the right posterior

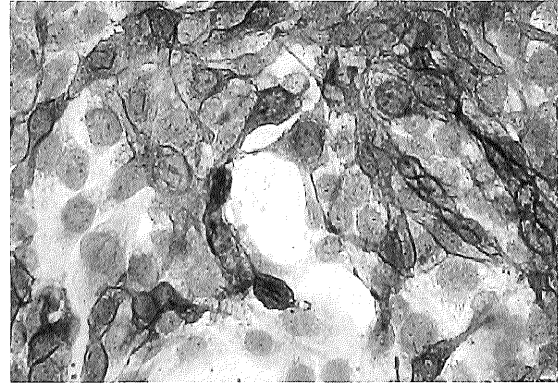


Fig. 1. Immunohistochemistry for Renca B7-1. For detection of B7-1 expression, 5×10^3 cells were seeded on a Chamber Slide and incubated for 24 hr. After fixing in a mixture of 50% acetone/50% methanol, the sections were incubated with rat anti-mouse B7-1 monoclonal antibody, then incubated with diluted biotinylated anti-rat antibody from Vectastain ABC kit. DAB was used as the peroxidase substrate and methyl green as a counter stain. Approximately 60% cells were successfully transfected by B7-1 gene.

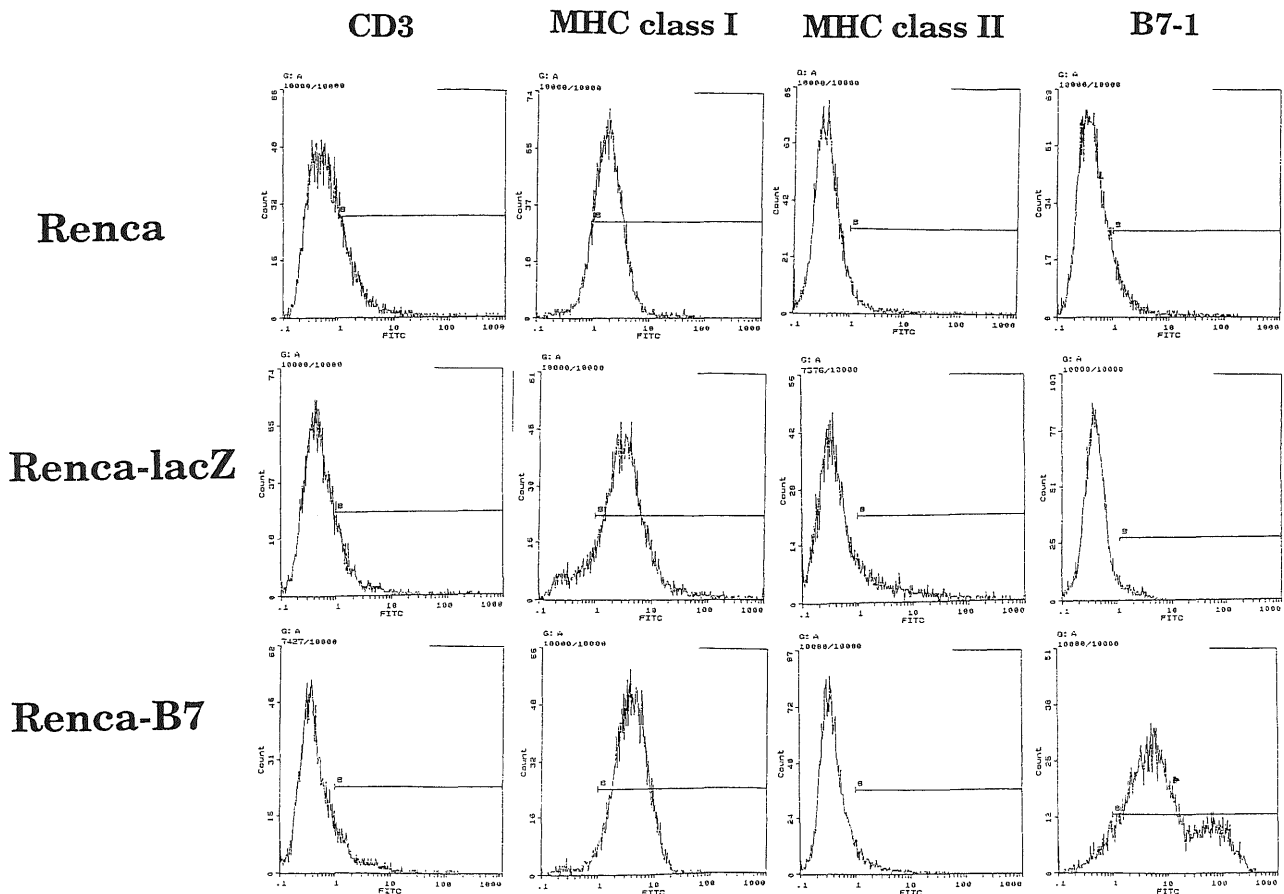


Fig. 2. Immunofluorescent by flow cytometry analysis.

Parental Renca cells, Renca lac Z cells and Renca B7-1 cells were incubated with FITC-conjugated anti-MHC class I (H-2K^d) and class II (I-A^d) monoclonal antibody in PBS supplemented with 5% FBS for 45 min at 4°C. After washing the cells 3 times with PBS, the respective cells were analyzed by flow cytometry. There were no significant changes in the level of MHC molecule expression on the parental Renca cells, Renca-lac Z cells and Renca B7-1 cells. The B7-1 transfectant, Renca-B7-1, expressed a high density of B7-1 molecules on the cell surface.

flank at a dose of 1×10^6 and 1×10^6 of Renca-B7-1 cells at the opposite site as a vaccine, on the same or following day. As a control, we also injected 1×10^6 of parental Renca cells or Renca-lac Z cells. Then, the mice were examined for tumor development of parental cells on the right flank and these tumors were measured in two dimensions with calipers twice weekly.

To examine the synergic effect of B7-1 combined with cytokines, we injected 1×10^6 of parental Renca cells into mice s.c. at the right posterior flank. On the same or following day, we injected 1×10^6 of Renca-B7-1 cells at the opposite site as a vaccine combined with an injection of BALB/c 3T3 IL-12, BALB/c IL-2 or BALB/c lac Z either near the tumor site or near the vaccine site.

RESULTS

Expression of Costimulatory Molecule and MHC Antigens on Tumor Cells

The results from the immunohistochemistry by monoclonal anti-B7-1 antibody showed that Renca cells failed to express the B7-1 molecule, but that Renca B7-1 cells did express the B7-1 molecule. Approximately 60% cells were successfully transduced by the B7-1 gene (Fig. 1). Because MHC antigens are important in the induction of an immune response, we analyzed the expression of these surface molecules by flow cytometry using immunofluorescence. As shown in Fig. 2, all Renca subline cells can express MHC class I (H-2K^d), but

not MHC class II (I-A^d). There were no significant changes in the level of MHC class I expression by B7-1 transduction. The parental Renca cells and Renca lac Z cells, which were transduced by the lac Z marker gene, did not express detectable levels of B7-1 antigen. The B7-1 transfectant, Renca B7-1, expressed a high density of B7-1 molecules on the cell surface.

In Vitro Growth Rate.

There was no apparent difference in the *in vitro*

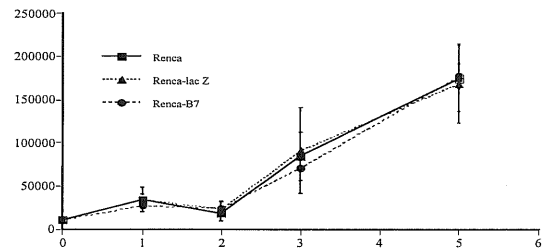


Fig. 3. Cell proliferation assay.

To characterize *in vitro* growth rate, 1×10^4 of each cells (parental Renca cells, Renca lac Z cells and Renca B7-1 cells) were inoculated into a 35 mm Petri dish containing DMEM supplemented with 10% FBS, and the total number of cells in each dish was enumerated on days 1, 2, 3 and 5. There were no apparent differences among the parental Renca cells, Renca-lac Z and Renca-B7-1 cells. The mean value and standard deviation of triplicate dishes from representative experiments are shown.

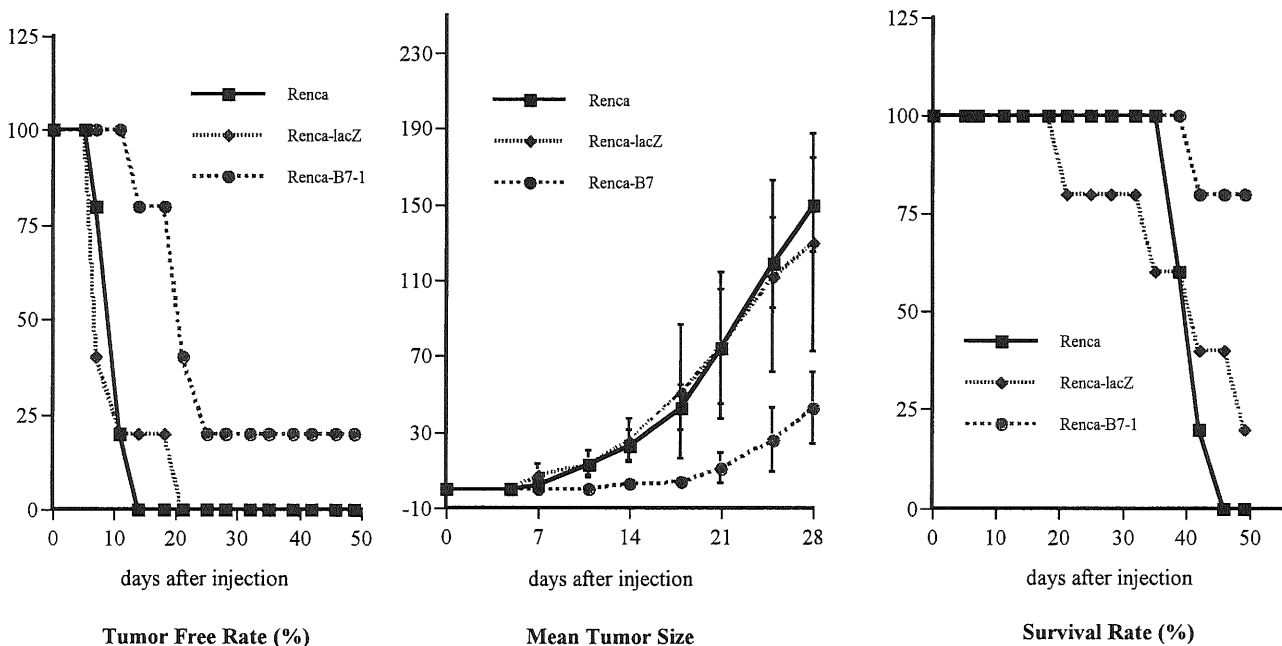


Fig. 4. Tumorigenicity experiment.

To examine the effect of B7-1 transduction on tumorigenicity *in vivo*, 1×10^6 cells of parental Renca cells, Renca-lac Z cells or Renca-B7-1 cells were injected into the right flank of syngenic BALB/c mice. The mice were examined for tumor development and the tumors were measured in two dimensions with calipers twice weekly. Renca-B7-1 showed a marked delay in the appearance of tumors after s.c. injection compared to the parental Renca cells and Renca-lac Z cells. Each group contained 5 mice. The mean value and standard deviation from representative experiments are shown.

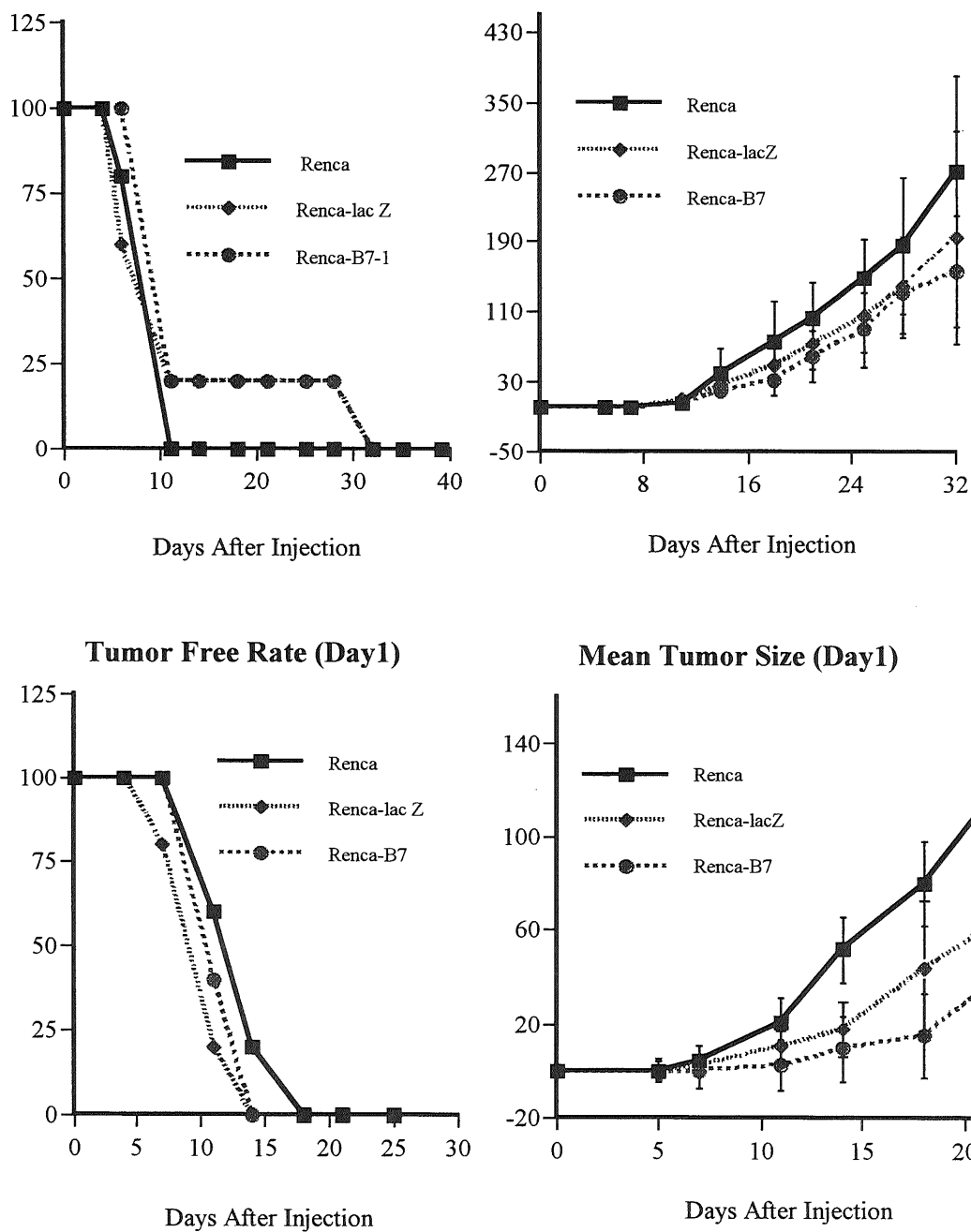


Fig. 5. Renca B7-1 for treatment purpose.

To examine the antitumor effects of Renca-B7-1 cells, we injected parental Renca cells into mice s. c. at the right posterior flank at a dose of 1×10^6 cells and 1×10^6 of Renca B7-1 cells at the opposite site as a tumor vaccine, on the same or following day. As a control, we also injected 1×10^6 of parental cells and Renca lac Z cells. There were no significant differences among the parental Renca, Renca lac Z and Renca B7-1 sublines, although tumors grew a little more slowly in the mice injected with parental Renca B7-1 cells than those injected with Renca cells. Each group contained 5 mice. The mean value and standard deviation from representative experiments are shown.

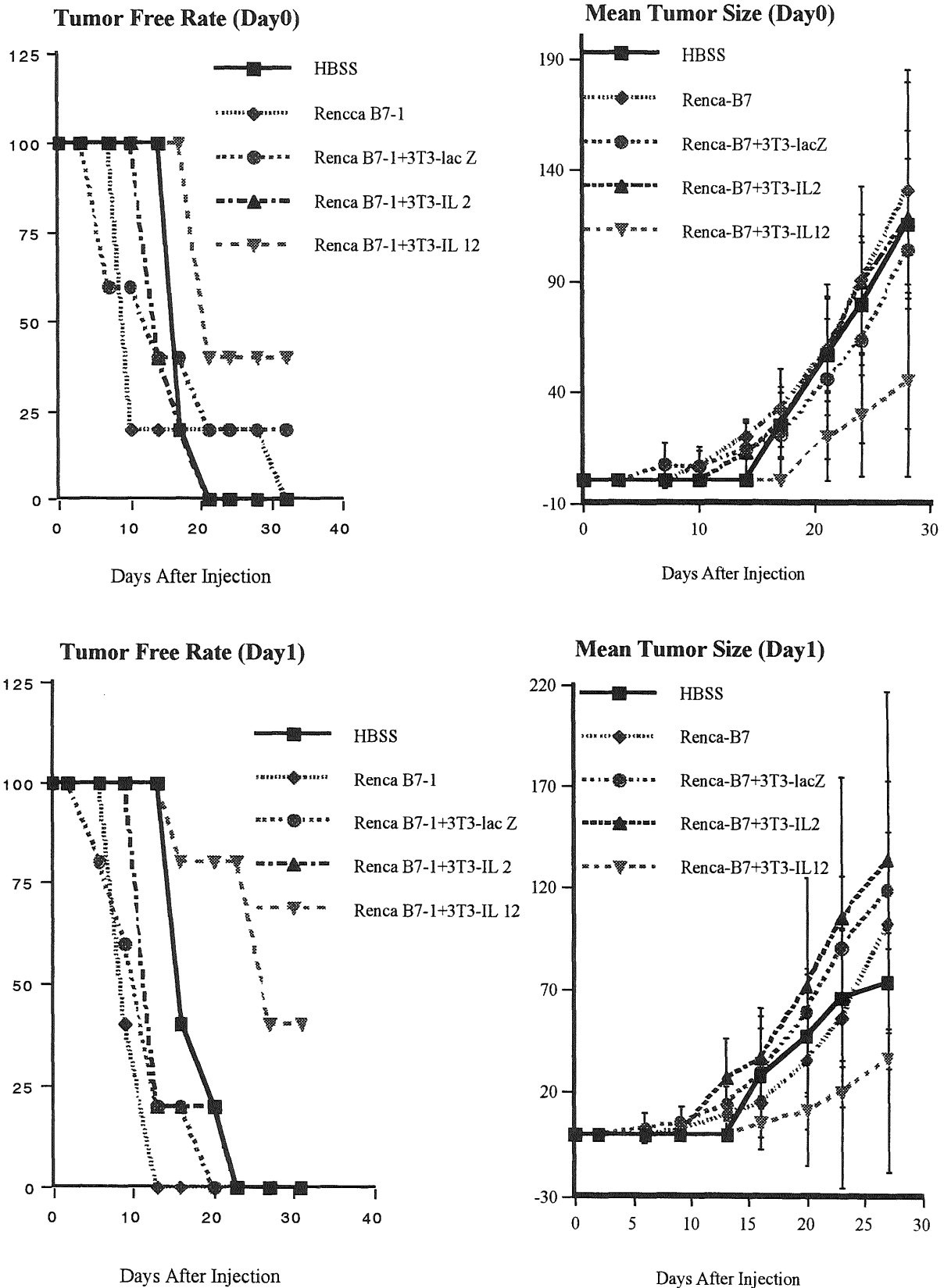


Fig. 6. The synergistic effects of Renca B7-1 combined with cytokines near the tumor site.

To examine the synergistic effects of B7-1 combined with cytokines, we injected 1×10^6 of parental Renca cells at the right posterior flank. on the same or following day, we injected 1×10^6 of Renca B7-1 cells into mice s. c. at the opposite side as a tumor vaccine combined with injection of BALB/c 3T3 IL-2 cells, BALB/c IL-12 cells, or BALB/c lac Z cells near the tumor site. The appearance and the growth of tumors were delayed apparently in the mice treated with Renca B7-1 combined with BALB/c 3T3 IL-12 on the same or following day, compared with those treated with HBSS, Renca B7-1, Renca B7-1 combined with BALB/c IL-2 and BALB/c 3T3 lac Z cells. Each group contained 5 mice. The mean value and standard deviation from representative experiments are shown.

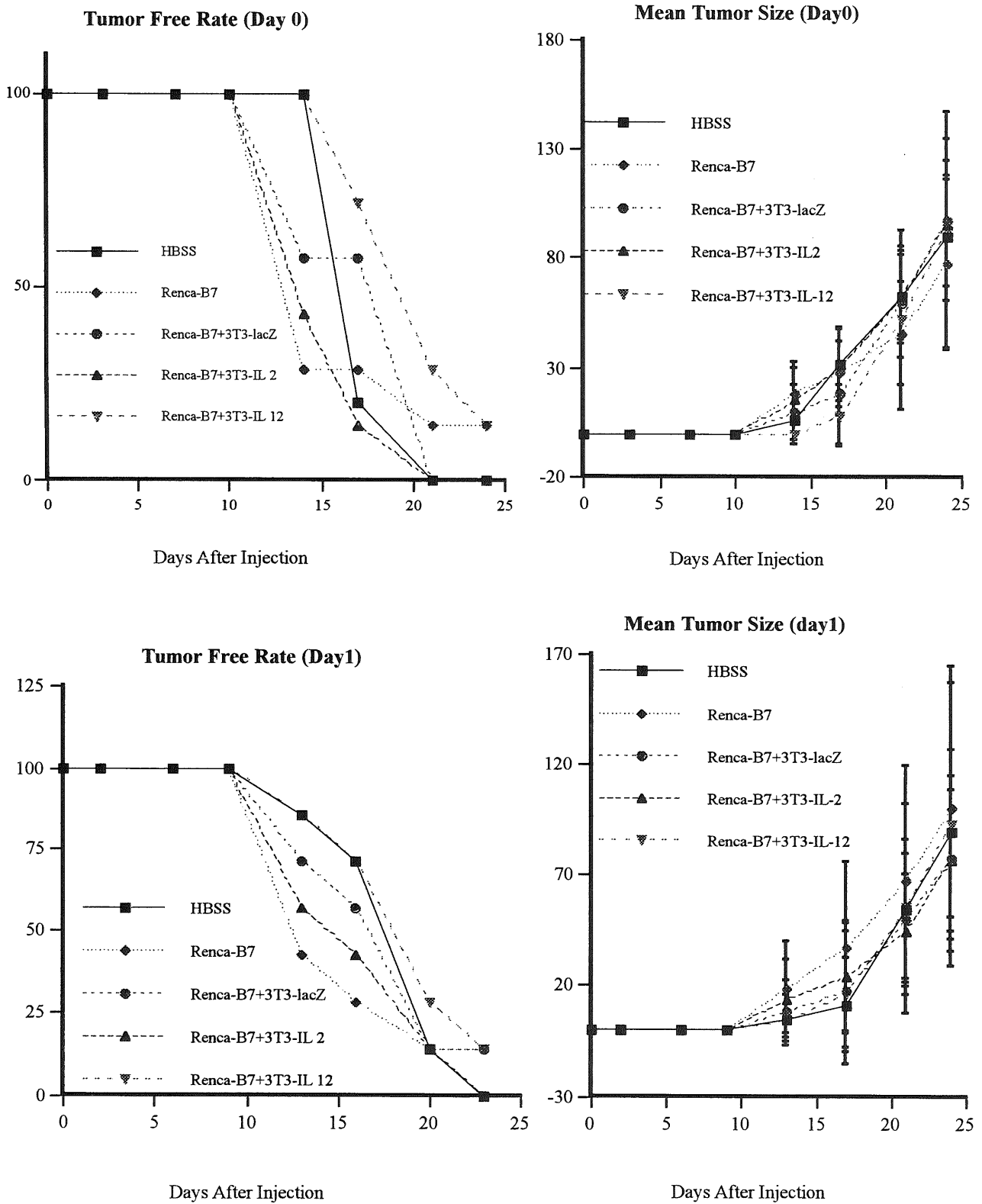


Fig. 7. The synergic effects of Renca B7-1 combined with cytokines near the vaccine site. In another experiment, we injected 1×10^6 of parental Renca cells at the right posterior flank and injected 1×10^6 of Renca B7-1 cell at the opposite site as a vaccine combined with BALB/c IL-2, BALB/c 3T3 IL-12 or BALB/c 3T3 lac Z cells near the vaccine site. There were no significant differences among HBSS, Renca B7-1, Renca B7-1 combined with BALB/c IL-2, BALB/c 3T3 IL-12 and BALB/c 3T3 lac Z cells. Each group contained 7 mice. The mean value and standard deviation from representative experiments are shown.

growth among parental Renca cells, Renca lac Z cells and Renca B7-1 cells (Fig. 3). This result indicated that B7-1 gene transduction did not alter the *in vitro* growth of Renca subline cells.

In Vivo Studies of Renca Sublines.

To examine the effect of B7-1 transduction on tumor growth *in vivo*, 1×10^6 cells of parental Renca cells, Renca lac Z cells or Renca B7-1 cells were injected into the right flank of syngenic BALB/c mice. Renca B7-1 showed a marked delay in the appearance of tumors after s.c. injection compared to parental Renca cells and Renca lac Z cells. All mice injected with parental Renca cells and Renca lac Z cells developed tumors within 3 weeks, but 40% of mice injected with Renca-B7-1 cells were free from tumors at the same time, and about 20% of mice were free from tumors at the end. Renca lac Z and parental Renca exhibited almost the same degree of tumor growth and survival rate, but Renca B7-1 gave a significantly delayed tumor growth and high survival rate as compared with the above two groups (Fig. 4).

To examine the antitumor effects of Renca B7-1 cells, we injected parental Renca cells into mice s. c. at the right posterior flank and Renca B7-1 cells at the opposite site as a tumor vaccine on the same or following day. We also injected parental cells and Renca lac Z cells as a control. There was no significant difference among parental Renca, Renca lac Z and Renca B7-1 sublines, although tumors grew a little more slowly in the mice injected with Renca B7-1 cells than in those injected with parental cells or Renca lac Z cells (Fig. 5).

To examine the synergic effects of B7-1 combined with cytokines, we injected parental Renca cells at the right posterior flank and Renca B7-1 cells into mice s. c. at the opposite side as a tumor vaccine combined with BALB/c 3T3 IL-2 or BALB/c 3T3 IL-12 cells near the tumor on the same or following day. BALB/c 3T3 lac Z cells were injected as a control. There was no significant difference between Renca B7-1 combined with BALB/c 3T3 lac Z and BALB/c 3T3 IL-2, but the appearance and the growth of the tumor were delayed apparently in the mice treated with Renca B7-1 combined with BALB/c 3T3 IL-12 on the same or following day (Fig. 6). Almost all mice treated with Renca B7-1 combined with BALB/c 3T3 lac Z or BALB/c IL-2 developed a tumor finally, but 40% of mice treated with Renca B7-1 combined with BALB/c 3T3 IL-12 cells were free from tumors. In another experiment, we injected Renca B7-1 cells at the opposite site as a vaccine combined with BALB/c 3T3 IL-2, BALB/c 3T3 IL-12 or BALB/c 3T3 lac Z cells near the vaccine site. There was no significant difference among HBSS, Renca B7-1, Renca B7-1 combined with BALB/c 3T3 IL-2, BALB/c 3T3 IL-12 and BALB/c 3T3 lac Z cells (Fig. 7).

DISCUSSION

Early researches suggested that the transduction of B7-1 gene into murine tumors could induce the rejection of the tumors and protect against subsequent challenge from B7 negative parental tumors. These results indicated that B7-1 transfectants can be used as a potential vaccine. For this reason, we transduced B7-1 gene into murine renal cell carcinoma (Renca) cells by means of the MFG retroviral vector system. Our results indicated that the MFG retroviral vector system has a high efficacy for B7-1 gene transduction. Approximately 60% cells expressed B7-1 molecules successfully.

Renca cell can express MHC class I (H-2K^d), but failed to express MHC class II (I-A^d) and B7-1. This is similar to the RCC-1 cell line which constitutively expresses MHC class I, LFA-3 and ICAM-1 molecules¹⁷. There were no significant changes in the level of MHC molecule expression on parental Renca cells, Renca-lac Z cells and Renca-B7-1 cells suggesting that B7-1 gene transduction does not affect the expression of MHC molecules.

In vitro and *in vivo* experiments indicate that B7-1 gene transduction does not affect *in vitro* growth but does affect *in vivo* tumorigenicity. B7-1 gene transduction decreased the tumorigenicity of Renca cells and this response might be mediated by immune system. This result is similar to that of another researcher, Wu et al¹⁹, who mixed B7-1^{hi}-expressing B-16 tumor cells with wild-type B-16 cells in different proportions (0, 25, 50, 75, and 100%) and injected them subcutaneously into C57BL/6 mice. Tumors grew within 3 weeks in all of the mice injected with 0, 25, 50, and 75% B7-1^{hi}-expressing B-16 cells, but only 20% of the mice injected with 100% B7-1^{hi}-expressing B-16 cells got tumors. This result, together with ours, suggests that the heterogeneity of B7-1 tumor cells within the whole tumor population injected may influence the outcome in tumor growth.

Although tumors grew a little more slowly in the mice injected with Renca B7-1 cells as a vaccine than those injected with parental Renca cells or Renca lac Z cells, all mice eventually developed tumors suggesting that the protection effect of Renca B7-1 as a tumor vaccine at a remote site is not sufficient. Recently, more and more experiments have shown that B7-1 expression in some tumors resulted in little or no local antitumor response^{2,3,20,21} and may reduce systemic antitumor immunity. A possible reason is the immunogenicity of tumor cells. Chen et al³ studied the effect of B7 costimulation on the antitumor immunity of murine tumor lines with varying immunogenicity. Immunogenic tumors regressed completely when transduced with B7 gene, while nonimmunogenic tumors remained tumorigenic after transduction of the B7 gene. Immunization with B7 transduced immunogenic tumors enhanced protective immu-

nity and increased specific CTL activity against the respective wild-type tumors as compared with immunization with non-transduced or mock-transduced tumors. In contrast, immunization with nonimmunogenic tumors transduced with B7 did not provide protective immunity and did not increase specific CTL activity. These results suggested that tumor immunogenicity is critical to the outcome of costimulation of T cell-mediated tumor immunity by B7. Therefore, the immunogenicity of Renca cells may be responsible for the difficulty in providing systemic protective immunity and may increase specific CTL activity toward the parent cells by Renca cells after transduction of B7-1 gene. Our result, together with those of others, indicated that B7-1 signals influence the activation and differentiation of T cells in a manner that is far more complex than was originally thought.

For this reason, we used BALB/c 3T3 transfectants BALB/c 3T3 IL-2 and BALB/c 3T3-IL12 to provide cytokine to see whether this combination therapy is better than B7-1 alone. IL-2, produced by CD4+T cells, is the principal cytokine responsible for progression of T lymphocytes from the G₁ to S phase of the cell cycle to induce T cell growth. IL-12, secreted by APCs, stimulates the differentiation of naive CD4+ T cells to the Th1 subset and CD8+ T cells into mature, functionally active CTLs through inducing transcription of IFN- γ by NK cells. IL-12 can induce powerful and prolonged proliferation in activated T cells in the presence of an appropriate costimulate signaling through the CD28 receptor induced by B7-transfected cells or certain anti-CD28 antibodies²⁰.

There were no significant differences between Renca B7-1 cells combined with BALB/c 3T3 lac Z and IL-2, but both the appearance and growth of tumors were apparently delayed in the mice treated with Renca B7-1 cells as a tumor vaccine combined with BALB/c 3T3 IL-12 cells near the tumor site. However, the antitumor activity of Renca B7-1 cells as a vaccine was not observed when BALB/c 3T3 IL-12 cells were injected near the vaccine. Although we could not rule out the possibility of direct antitumor effect of IL-12 when BALB/c 3T3 IL-12 cells were injected near the tumor site combined with Renca B7-1 at opposite site, the synergic effect of Renca B7-1 combined with BALB/c 3T3 IL-12 seems a possibility. Renca B7-1 combined with BALB/c 3T3 IL-2 did not show any antitumor activity in this study. These results indicate that we should carefully choose appropriate cytokines and arrange the employ pathway when we use this transfectant combined with cytokines.

It is known that irradiation of tumor cells often increases their inherent immunogenicity, but the mechanisms responsible have not been ascertained. Some authors have reported that MHC

class I or class II molecules show enhanced expression upon the irradiation of tumor cells^{8,9}, and others have suggested that B7 costimulatory molecules were upregulated by irradiation^{11,12}. Our preliminary experiment showed that Renca B7-1 cells combined with cytokines provided a fairly effective antitumor activity after X-ray irradiation (data not shown). We can speculate that the immunogenicity of Renca B7-1 cells could be increased by irradiation and that the treatment result might be enhanced.

In conclusion, our results suggested that B7-1 gene transduction was able to decrease the tumorigenicity of murine renal cell carcinoma cells, but failed to induce sufficient systemic antitumor response when it was used as a tumor vaccine even combined with immunostimulatory cytokines. It is necessary to determine the intrinsic immunogenicity of the parental tumor when we use this gene in a tumor. Although further examination is necessary, irradiation of this transfectant may be a possible way to cause these B7 transfectants to stimulate effective tumor immunity in a therapeutic setting.

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