Effectiveness of Combined Interleukin 2 and B7.1 Vaccination Strategy Is Dependent on the Sequence and Order: A Liposome-mediated Gene Therapy Treatment for Bladder Cancer

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ABSTRACT

We have developed a novel liposome-mediated immunogene therapy using interleukin 2 (IL-2) and B7.1 in a murine bladder cancer model. A carcinogen-induced murine bladder cancer cell line, MBT-2, was transfected with cationic liposome 1,2-dimyristoyl-sn-glycerol-3-phosphocholine and IL-2 plasmid. The optimized transfection condition generated IL-2 levels of 245–305 ng/10^6 cells/24 h, 100-fold higher than the levels seen with retrovirus transfection. Ninety percent of the peak level of IL-2 production was maintained for up to 11 days after transfection. Animal studies were conducted in C3H/HeJ female mice with 2 × 10^4 MBT-2 cells implanted orthotopically on day 0. Multiple vaccination schedules were performed with i.p. injection of 5 × 10^6 IL-2 and/or B7.1 gene-modified cell preparations. The greatest impact on survival was observed with the day 5, 10, and 15 regimen. Control animals receiving retrovirally gene-modified MBT-2/IL-2 cell preparations had a median survival of 29 days. Animals receiving the IL-2 liposomally gene-modified cell preparation alone had a median survival of 46 days. Seventy-five percent of animals receiving IL-2 followed by B7.1 gene-modified tumor vaccines were the only group to show complete tumor-free survival at day 60. All of these surviving animals rejected the parental MBT-2 tumor rechallenge and survived at day 120 with a high CTL response. In conclusion, liposome-mediated transfection demonstrates a clear advantage as compared with the retroviral system in the MBT-2 model. Multi-agent as opposed to single-agent cytokine gene-modified tumor vaccines were beneficial. These “targeted” sequential vaccinations using IL-2 followed by B7.1 gene-modified tumor cells significantly increased a systemic immune response that translated into increased survival.

INTRODUCTION

The majority of human neoplasms are treated by the traditional modalities of surgery, radiation, or chemotherapy, either independently or in combination. The development of immunotherapeutic models over the past 15 years has led to multiple human protocols for the treatment of cancer (1). These models are predicated on the basic assumption that tumor-specific antigens exist and that the patient’s immune system fails to either recognize or effectively respond to these antigens (2, 3). The goal of immunotherapy is therefore to increase tumor antigen recognition and enhance the antitumor response (4). In studies using the systemic administration of cytokines, such as IL-2, profound inhibitory effects on tumor progression in animal models were seen, but only a limited therapeutic benefit was observed when IL-2 was administered to cancer patients (5–7). This limited efficacy was due in part to the toxicity resulting from the high doses of IL-2 required to stimulate an immune response in humans (8). Other studies using repeated injections of IL-2 directly into the tumor site have attempted to initiate the regression of established tumors or to induce immunological memory (9, 10). These alternative therapeutic options, although generating great interest, have met with sporadic clinical success to date (11).

A new form of immunomodulation using gene transfer techniques is now being actively investigated for a variety of malignancies (12). This treatment requires the insertion of a plasmid DNA encoding a cytokine gene directly into tumor cells. These “gene-modified” tumor cells then produce this cytokine, resulting in enhanced tumor antigen recognition and a documented increase in an antigen-specific immune response (13–15). We have previously investigated this new technique in the MBT-2 murine bladder cancer model (16, 17). The MBT-2 tumor was induced in C3H mice by oral administration of N-[4-(5-nitro-2-furyl)-2-thiazolyl]-formamide, a potent carcinogen that resulted in bladder neoplasms in 80–90% of animals.
This poorly differentiated transitional cell carcinoma, which exhibits metastatic progression, resembles both grossly and histologically, human transitional cell bladder cancer. In addition, treatment regimens that have been successful in this murine model have also shown efficacy in human bladder cancer (19, 20). In our previous research using retrovirus as the transfecting agent, cytokine genes including IL-2 and granulocyte macrophage colony-stimulating factor were successfully incorporated and stably expressed in MBT-2 tumor cells in vitro (16, 17). In vivo experiments showed that i.p. injection of these irradiated cytokine gene-modified cell preparations in tumor-bearing animals resulted in a significant survival advantage.

Although retroviral vectors provide genomic integration and a permanence of transduction, retroviral vector transfection is a time-consuming process and results in low expression of gene product (21). Additional limitations, including the safety issues of replication defects in this viral vector system and the fact that only dividing cells can be transduced, inhibit the potential of retroviral vectors as optimal transfecting agents for human application (22).

This study evaluates an alternative transduction mechanism using cationic liposomes. As reported previously by Vieweg et al. (23), liposome-mediated transfection has several advantages over viral vector systems in the use of promulgating gene-modified tumor cells. Liposome-mediated transfection has no infectious or little immunogenic potential, characteristics that are critical when considering human application (24, 25). Because the transfection is not based on genomic integration, gene product expression is transient (26). As opposed to the retroviral system, multiple copies of the plasmid can be transduced per cell, leading to higher production of the gene for a shorter period of time. We have hypothesized that if gene expression persists long enough for significant immunomodulation, the transient nature of the transfection becomes less important. Using an adeno-associated virus-based plasmid, we evaluated in vitro liposome-mediated transfection in the MBT-2 model. To establish conditions for optimal transfection, we studied four distinct parameters: (a) toxicity [liposomes have inherent lytic effects on mammalian cells when administered in high concentrations (27)]; (b) the critical liposome:DNA ratio producing the least toxicity with the highest gene expression; (c) the time of exposure to the liposome-DNA complex, i.e., the least amount of transfection time to produce the greatest gene expression; and (d) the effect of growth factors, such as FBS, on the potential inhibition of liposome-mediated transfection.

Previous investigations studied the MBT-2 bladder cancer model in vivo by using the injection of MBT-2 cells either intradermally or s.c. to establish the tumor (28–30). Our laboratory devised an alternative method, i.e., orthotopic implantation. This method involves the direct injection of MBT-2 cells into the bladder wall via a small incision in the suprapubic region and allows us to better observe the growth and metastatic spread of bladder tumor cells growing in their normal in situ environment. Additionally, it provides a more physiological approach to evaluate the immune response to bladder cancer cells. Thus, our in vivo studies evaluated the use of liposome-mediated, irradiated, gene-modified cells as "tumor vaccines" in orthotopically implanted MBT-2 tumor-bearing animals. Parameters including vaccination schedule and the amount of transfected cells/vaccine were investigated with a final end point of survival. All animals that survived the initial tumor implantation were rechallenged with parental MBT-2 cells. Necropsy and splenic CTL analysis were performed on those animals that survived the rechallenge.

Initially, we studied a vaccination schema based on multiple injections of single-agent gene-modified MBT-2 cells. These included plasmids containing IL-2 and the adhesion molecule B7-1 genes. B7.1 was well characterized by Freeman et al. (31) and Chen et al. (32) as a T-cell cofactor that is essential for T-cell activation. As the in vivo evaluation progressed, we revised our vaccination schedule to determine the effects of using different gene products at different vaccination times within the same tumor-bearing animals. Thus, one animal would receive IL-2 or B7.1 gene-modified cells as a single agent throughout or in combination in an alternating vaccination regimen. These "targeted" sequential vaccinations evolved as an opportunity to stimulate different aspects of the immune response in a time-dependent manner, based on our knowledge of the cascade of events that dictate successful immunomodulation.

MATERIALS AND METHODS

Plasmids and Cell Lines. The pMP6-IL-2 and pMP6-B7-1 plasmids are adeno-associated virus-based plasmids using a cytomegalovirus promoter and including the murine IL-2 or B7-1 genes, respectively. Their constructs have been described previously (33). The MBT-2 murine bladder carcinoma cell line was obtained from Dr. Timothy Ratliff (The University of Iowa, Iowa City, IA) from a transplantable N-[4-(5-nitro-2-furyl)-2-thiazolyl]-formamide-induced tumor in C3H mice (18). L929 is a well described murine (C3H) fibroblast cell line and was used as a syngeneic control for CTL analysis. Cells were maintained in RPMI 1640 supplemented with 10% FBS and 2 mM l-glutamine at 37°C and 5% CO₂. MBT-2/IL-2 was a stable retrovirally gene-modified cell line (16, 17). All cell lines were tested for Mycoplasma contamination every 6 weeks using a PCR-based assay.

Liposome Preparations and Gene Product Analysis. The liposome used in this study was graciously provided by Vical, Inc. (San Diego, CA). DMRIE/DOPE was composed of positively charged lipid DMRIE in combination with neutral lipid DOPE in a 1:1 molar ratio (27). The lipid reagents were stored at 4°C before use. IL-2 measurement was performed by ELISA (R&D Systems, Minneapolis, MN). B7.1 quantitative analysis was documented by a fluorescence-activated cell sorter using FITC-conjugated rat antimouse B7.1 (PharMingen, San Diego, CA).

Cellular Transfection Procedure. Twenty-four h before transfection, MBT-2 cells were plated on 60-mm dishes at a density of 1 × 10⁶ cells/dish. Various amounts of the liposome (2, 10, 20, 40, 60, 80, 120, and 160 μg) and plasmid DNA (1, 2.5, 5, 7.5, 10, 20, 30, and 40 μg) were diluted separately in serum-free medium to a total volume of 500 μl each and then gently mixed together in polystyrene tubes. This solution was allowed to form complexes at room temperature for 15 min. Adherent MBT-2 cells were rinsed once with serum-free medium. The liposome-DNA complex was then diluted with serum-free medium to a final volume of 4 ml and gently added to the cellular monolayers. After a transfection exposure time
ranging from 15 min to 24 h (0.25, 0.5, 0.75, 1, 2, 3, 4, 8, 12, and 24 h), the transfection solution was rinsed off, and fresh medium with 20% FBS was added. Similar experiments to determine the effect of the growth factor on the transfection process revealed the addition of 10% FBS during the transfection exposure. In separate studies to determine toxicity, cell counts using trypan blue exclusion were performed 12 h after the completion of exposure to the liposome-DNA complex. Twenty-four h after transfection, all cells were exposed to 70 Gy of irradiation. At the next 24 h time point, all cells were rinsed with PBS and then replenished with serum-free medium. Twenty-four h later, supernatants were collected and frozen at −20°C for future IL-2 ELISA determination. Cell counts were performed at this time to be able to report IL-2 concentrations as ng/10^6 cells/24 h. For time course experiments, i.e., evaluation of the gene product secretion over 30 days, all cells were transfected on day 0 and irradiated on day 1 and then had complete medium replaced every 3 days. Cells were rinsed with PBS and replenished with serum-free medium 24 h before supernatant collection. Different sets of cells were transfected in the same study to evaluate gene product secretion at days 3, 6, 9, 12, 15, 18, 21, 24, 27, and 30. Adherent cells at each time point were counted for accurate quantification.

Animal Studies. All animal studies were initiated in 6–8-week-old C3H/HeJ female mice (Jackson Laboratory, Bar Harbor, ME). Freshly prepared MBT-2 cells were implanted orthotopically in the animals. Briefly, animals were anesthetized by an i.p. injection of pentobarbital. Under magnification, a bladder was delivered into the surgical wound, and necropsied with special attention placed on examination of the body weight or mice presenting cachexic status were sacrificed, and CTL analysis was performed on their splenocytes.

RESULTS

Liposome-mediated Gene Transfer in Murine MBT-2 Bladder Carcinoma Cells. At first, the critical ratio of liposome:DNA in the DMRIE/DOPE and pMP6-IL-2 transfection was evaluated and proved to be 4:1. Higher ratios of DNA did not improve IL-2 secretion. Twelve h after a 0.25–1-h exposure to DMRIE/DOPE (2–80 μg), there were 88–93% viable MBT-2 cells as evaluated by trypan blue exclusion. Toxicity was observed at 120 and 160 μg of DMRIE/DOPE with 74% and 68% viable cells, respectively. All studies with FBS present during the transfection revealed a 45–80% reduction of gene product secretion as compared with transfection in serum-free medium. The best gene product secretion with the least cellular toxicity was observed at a transfection time exposure to the liposome-DNA complex of 0.5–1 h. Longer exposure led to slightly less IL-2 production, but with higher cellular toxicity. Transfection times less than 0.5 h resulted in a 34–56% decrease in IL-2 secretion. Therefore, the optimal transfection conditions for 60-mm culture dishes, which produced the least toxicity with the greatest IL-2 secretion at the least amount of exposure time, were 40 μg of DMRIE/DOPE/10 μg of pMP6-IL-2 for 30 min in serum-free medium. Using these optimized parameters, IL-2 secretion ranged from 245–305 ng/10^6 MBT-2 cells/24 h, whereas the previously established MBT-2/IL-2 stable transfectant produced IL-2 levels of 1.7–2.2 ng/10^6 cells/24 h.

To determine the extent of IL-2 secretion in this “transient” liposome-mediated transfection, we studied the gene product expression over a 30-day period. All cells were transfected on day 0 according to the optimized conditions as described above. On day 1, cells received 70 Gy of irradiation, and then IL-2 secretion was measured at 3-day intervals (Fig. 1). In three
separate studies, IL-2 levels reached an average of 280 ng/10⁶ cells/24 h by day 3 and maintained 90% of this peak for up to 9–11 days after transfection. A steady decline in IL-2 production was then observed, until levels of less than 5 ng/10⁶ cells/24 h on day 3 to day 11 after transfection.

Effects of Orthotopic Implantation of MBT-2 Cells in C3H Mice. To determine survival, metastatic potential, and tumor growth in the orthotopic bladder tumor model, a series of confirmatory experiments was performed. Animals were sacrificed on days 3, 7, 10, 14, 17, 21, 24, and 28, and total body weight, tumor volume, and tumor weight were measured. Also, metastatic lesions, if present, were inspected. As shown in Table 1, tumor growth was prominent 14 days after tumor implantation. Animals receiving retrovirally gene-modified MBT-2 cells had a median survival of 29 days. MBT-2 cells (2 × 10⁶) were orthotically implanted in 6–8-week-old C3H mice. Six animals were sacrificed on the days indicated, and total body weight, tumor volume, and tumor weight were measured. Data are a mean of six animals at each time point.

<table>
<thead>
<tr>
<th>Day</th>
<th>Body weight (g)</th>
<th>Tumor volume (mm³)</th>
<th>Tumor weight (g)</th>
<th>Metastases</th>
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<tr>
<td>3</td>
<td>17.03 NT</td>
<td>NT</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>17.59 0.029</td>
<td>0.404</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>17.20 0.119</td>
<td>0.130</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>17.05 0.180</td>
<td>0.220</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>17.36 0.601</td>
<td>0.620</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>14.75 0.637</td>
<td>0.731</td>
<td>Peritoneal (4/6)</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>15.69 0.723</td>
<td>0.863</td>
<td>Peritoneal (6/6)</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>18.28 1.170</td>
<td>1.403</td>
<td>Hydronephrosis (4/6)</td>
<td></td>
</tr>
</tbody>
</table>

a NT, no macroscopically identifiable tumor was observed.

Table 2 Comparison of different MBT-2 tumor vaccination doses

All cells were transfected by the optimized liposome-mediated transfection protocol on day 1, irradiated with 70 Gy on day 2, and harvested for vaccination on day 3. Vaccinations were performed on days 5, 10, and 15 after orthotopic implantation of 2 × 10⁶ MBT-2 cells in C3H mice.

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Dose (cells)</th>
<th>Median survival (days)</th>
<th>Range (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 None</td>
<td></td>
<td>26</td>
<td>22–31</td>
</tr>
<tr>
<td>2 IL-2</td>
<td>1 × 10⁶</td>
<td>28</td>
<td>22–35</td>
</tr>
<tr>
<td>3 IL-2</td>
<td>5 × 10⁶</td>
<td>20</td>
<td>15–25</td>
</tr>
<tr>
<td>4a IL-2</td>
<td>1 × 10⁶</td>
<td>27</td>
<td>20–34</td>
</tr>
<tr>
<td>5a IL-2</td>
<td>2.5 × 10⁶</td>
<td>46</td>
<td>40–52</td>
</tr>
<tr>
<td>6 Retro-IL-2</td>
<td>5 × 10⁶</td>
<td>29</td>
<td>25–34</td>
</tr>
</tbody>
</table>

a Groups 4 and 5 represent a combination of IL-2 gene-modified, radiated cells with nontransfected, radiated cells. Group 5 experienced the longest median survival; however, no cures were obtained.

b IL-2, IL-2-gene-modified MBT-2 cells; NT, non-transfected MBT-2 cells; Retro-IL-2, retrovirally IL-2 gene-modified MBT-2 cells.

animals were found to have relatively small tumor burdens at the time of necropsy. Most of these animals developed several signs of toxicity (i.e., body weight loss, ascites, chattering, and immobility), and this adverse effect was considered to be IL-2 toxicity because these animals were injected with cells producing >1000 ng of total IL-2 secretion per day. An alternative strategy to increase potential tumor antigen presentation while limiting IL-2 toxicity was devised. This required vaccinations of nontransfected MBT-2 cells in addition to IL-2 gene-modified cells. As shown in Table 2, those animals receiving vaccinations of 2.5 × 10⁶ liposomally transfected IL-2-secreting cells in combination with 2.5 × 10⁶ nontransfected cells repeatedly showed a 75% increase in survival as compared with controls. No other combination of gene-modified and nontransfected cells showed any consistent increase in survival. Also, none of these groups exhibited “cure” or survival for 60 days after tumor implantation. Animals receiving retrovirally gene-modified MBT-2/IL-2 cell preparations had a median survival of 29 days.

4 W. A. Larchian, unpublished data.
Targeted Vaccination Studies in the MBT-2 Model. In an attempt to further modulate the immune response with liposome-mediated gene-modified cells in the MBT-2 tumor-bearing animal model, we evaluated the use of a targeted vaccination regimen. The purpose of these studies was to investigate ways of not only increasing antigen recognition but stimulating the cytotoxic arm of the immune response. Therefore, we initiated experiments evaluating various vaccination schedules using combinations of IL-2 and/or B7.1 gene-modified cells (Table 3). MBT-2 cells were transfected using the described optimized liposome-mediated transfection. The vaccination schedule of day 5, 10, and 15 was followed. Tumor-bearing animals received a vaccination with MBT-2 cells containing one transfected plasmid on day 5, followed by MBT-2 cells that were transfected with another plasmid on days 10 and 15. As seen in the previous experiments, control animals and those receiving IL-2 gene-modified MBT-2 cell vaccinations followed the same course (Fig. 2). In those animals receiving B7.1 gene-modified MBT-2 cells alone or B7.1 followed by IL-2, a survival advantage similar to that seen in the group treated with IL-2 alone was demonstrated. In repeated studies, the group of mice given IL-2-secreting cells on day 5 followed by B7.1-expressing cells on days 10 and 15 showed 75% survival at 60 days. At that time, the survivors were orthotopically rechallenged with MBT-2 cells, as were a new set of control animals. None of the survivors received any further vaccinations during the course of the study. The control mice succumbed as before; however, all of the IL-2/B7/B7 survivors remained alive for another 60 days, or 120 days since the original tumor implantation. Interestingly, a set of animals vaccinated simultaneously with IL-2 and B7.1 cotransfected cells revealed no survival advantage.

All survivors were sacrificed at day 120, and necropsy revealed no evidence of primary tumors or metastases in the chest, peritoneum, or pelvis. As shown in Fig. 3, splenocytes harvested from mice vaccinated with IL-2/B7/B7 exhibited very high levels of CTL activity (approximately 50% at an E:T ratio of 100:1). Control target L929 demonstrated insignificant lysis.

DISCUSSION

The role of immunotherapy in the treatment of cancer is currently being defined. The use of gene therapeutic techniques to transfer cytokine genes into tumor cells has resulted in over 50 new human clinical trials encompassing a variety of malignancies in the past several years (35, 36). Our previous studies in the murine MBT-2 bladder cancer model used the retroviral transfection modality. In this report, we have evaluated an alternative transduction modality, cationic liposomes, in an effort to enhance gene transfer and augment the immune response.

Using the MBT-2 model, we have demonstrated a clear advantage of liposome-mediated transfection over retroviral system. With optimized liposome-mediated transfection, we were able to observe a 100–150-fold increase in IL-2 production in a fraction of the transfection time (30 min) as compared with the retroviral system. This magnitude of the enhanced gene product secretion persisted for over 10–14 days. Although “transient” in a nonintegrated form as episomes as compared with the stable genomically integrated retroviral transfection, IL-2 expression by liposome-mediated gene-modified cells proved to be persistent enough to stimulate an immune response, the ultimate goal of this form of gene therapy. Indeed, these advantages could have a profound impact when considering a human model for cytokine gene therapy. First, the liposome-mediated transfection does not require dividing cells, as the retroviral system does. Therefore, all cells available for transfection would be potential candidates for gene modification strategies. Second, the issue of safety concerning replication defects in the retroviral system is obviated. The safety of liposomes has been confirmed by their use in human trials for the treatment of melanoma (37, 38). In addition, the simplicity and
With this in mind, we attempted to understand and modify our approach to liposome-mediated gene-modified cellular vaccines in the MBT-2 tumor-bearing model.

The inclusion of adhesion molecule B7.1, a T-cell co-factor, in the vaccine regimen was an attempt to investigate alternative modes of activating an immune response. Thus, multiple combinations of IL-2 and B7.1 gene-modified tumor vaccines were tested in the in vivo MBT-2 model. The regimen (IL-2/B7/B7) that began with IL-2-secreting cells on day 5, followed by the B7.1-transfected cells on days 10 and 15, had a striking effect on survival. The other groups, which included B7.1 alone (B7/B7/B7) or the alternative schema of B7.1 followed by IL-2 (B7/IL-2/IL-2), did not prove efficacious. So successful was this IL-2/B7/B7 schedule that not only did 75% of the animals experience tumor regression and survive to 60 days after tumor implantation, but all survivors were resistant to orthotopic rechallenge and remained tumor free. The evidence that memory CD8+ response against MBT-2 cells had been achieved in surviving animals is further supported by the CTL analysis (Fig. 3).

Thus far, several previous studies regarding immunogene therapy using B7 and cytokines have shown the controversial results of this strategy in other tumor systems. Gäken et al. (42) have successfully shown that combined expression of murine B7.1 and IL-2 on NC murine adenocarcinoma cells was significantly more effective than either B7.1 or IL-2 alone in inducing the immune-mediated rejection of pre-established NC tumors involving CD4+ lymphocytes. Cayeux et al. (43) have found that immunization with J558-IL2/B7.1 cells followed by challenge with parental murine plasmacytoma J558L cells caused a reduction in systemic protection as compared with J558-B7.1 or J558-IL2 alone. They concluded that “hyperstimulation” of the immune response by genetically modified cancer vaccines could have adverse effects on tumor immunity. It was also true in our current experiment that simultaneously vaccination with IL-2 and B7.1 cotransfected MBT-2 cells resulted in no survival advantage. The differences in immunological mechanisms are not yet completely understood; however, it is suggested that this difference may be due, in part, to the induction of anergy in the potential reactive T cells.

Our results imply that B7.1 is required for successful induction and function of CTLs after IL-2 exposure. B7.1-transfected MBT-2 cells alone were not responsible for tumor regression when used independently; instead, they appeared to be effective only after the initial activation of the immune response by the IL-2 gene-modified MBT-2 vaccinations. One can therefore speculate that the increased attraction of APCs by IL-2 is the initial complementary step in the cascade of immunological events that subsequently permits the function of B7.1 to be realized as a cofactor in the production and stimulation of antigen-specific CTLs. We believe that this concept of timed, programmed, targeted stimulation of the immune response is a logical alternative to continuous single-agent regimens.

Additional studies involving liposome-mediated gene-modified cellular vaccinations in the MBT-2 bladder cancer model are under way. These will focus on the programmed use of multiple cytokines and T-cell factors in targeted vaccination studies. We are currently investigating the different components of the immune response at each activation step to better identify
the mechanisms involved. In addition, the use of liposome transfection with cytokine genes in human urological malignancies will be actively pursued. This alternative form of cancer treatment, which uses gene transfer techniques to enhance tumor antigen recognition, has multiple applications and holds great promise.

In summary, liposome-mediated transfection provides a safe, simple, and highly effective mode of gene transfer as compared with retroviral transfection systems. The use of multiple agents in gene-modified tumor cell vaccinations in a time-dependent fashion has elicited a more significant and substantial immune response than single-agent regimens.

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