
ABSTRACT

Adenocarcinoma of the prostate is the most common cancer in men. The majority of cancers are discovered once they have already metastasized, and there is no effective therapy for prostatic cancer at this stage. The use of cytokine-secreting tumor cell preparations as therapeutic vaccines for the treatment of advanced prostate cancer was investigated in the Dunning rat R3327-MatLyLu prostatic tumor model. IL-2 secreting, irradiated, tumor cell preparations were capable of curing animals with s.c. established tumors, and induced immunological memory that protected animals from subsequent tumor challenge. Immunotherapy was less effective when tumors were induced orthotopically, but nevertheless led to improved outcome, significantly delaying, and occasionally preventing, recurrence of tumors after resection of the cancerous prostate. Granulocyte-macrophage colony stimulating factor secreting tumor cell preparations were less effective, and interferon-γ secreting cells had only a marginal effect. Induction of a potent immune response in tumor bearing animals against the nonimmunogenic MatLyLu tumor supports the view that active immunotherapy warrants further investigation as a potential therapeutic approach to prostate cancer.

INTRODUCTION

Cancer of the prostate is the most commonly diagnosed cancer in men and is the second most common cause of cancer death in Western civilization (1). Progression of prostatic cancer in humans is not accompanied by a vigorous antitumor immune response, although weak immune responses have been occasionally documented in some patients (2). Several attempts to actively immunize patients with adenocarcinoma of the prostate have shown little or no therapeutic benefit (3, 4). Thus, like most other human neoplasms, prostate cancer is largely a nonimmunogenic cancer. This, together with the demonstration that spontaneously occurring tumors in animals do not induce protective immunity (5, 6), and that no increase in tumor incidence is seen in T-cell-deficient mice (7), have led to the belief that naturally occurring tumors do not encode tumor-specific antigens capable of inducing an effective immune response, and that active immunotherapy of cancer may be futile. In a landmark study, van Pel and Boon (8) have shown that a nonimmunogenic murine tumor could be converted to an immunogenic form which was capable of inducing systemic immunity against the parental tumor. This finding demonstrated that the parental tumor did in fact encode tumor antigens, but that in its original form the tumor was incapable of presenting the tumor antigen(s) to the immune system. If this conclusion applies to human cancer as well, active immunization of patients against nonimmunogenic forms of cancer may be possible.

Rat models have been used to study prostate biology because the rat prostate has many features in common with the human prostate. In addition, spontaneous prostatic tumors have been observed in several strains of rats. The most thoroughly studied and readily available tumor is the Dunning tumor and its derivatives (9–12). Several sublines with varying degrees of hormone dependency and metastatic behavior have been isolated from the original tumor, providing a spectrum of prostatic cancers with distinctive individual properties which correspond to various stages of prostate cancer in humans. One such line, R3327-MatLyLu, is an anaplastic androgen independent tumor which spontaneously metastasizes to the lymph nodes and the lung (10). Furthermore, since the MatLyLu tumor does not exhibit any signs of intrinsic immunogenicity (see below), it constitutes an excellent model to explore immunotherapeutic protocols for the treatment of metastatic human prostatic cancer. A growing number of studies have shown that genetically engineered tumor cells expressing cytokines such as IL-2, IFN-γ, IL-4, IL-6, IL-7, or GM-CSF could immunize mice against a subsequent challenge with parental tumor cells (13–25). It was recently demonstrated that cytokine secreting, growth inactivated, tumor cell preparations were also active in tumor bearing animals, reducing tumor progression and even curing some animals of a preestablished tumor (20–24). We therefore tested whether irradiated MatLyLu tumor cell preparations genetically engineered to express either IL-2 (MAT/IL-2), GM-CSF (MAT/GM), or IFN-γ (MAT/IFN) were capable of inducing an immune response against the nonimmunogenic parental tumor, and altering the course of disease in tumor bearing animals.

MATERIALS AND METHODS

Generation of Cytokine Secreting Prostatic Tumor Cells. R3327-MatLyLu cell lines stably expressing the human IL-2, the mouse IFN-γ, or the murine GM-CSF were constructed by Rosenthal et al.4 Retroviral vectors carrying the corresponding cDNAs and the bacterial neomycin resistance gene were used to introduce and express each cytokine in MatLyLu cells. MatLyLu clones transduced with the retroviral vector DC/AD/R (25) were found to express the highest levels of cytokine, and were chosen for further study (12.8 ng/ml/106 cells/24 h of IL-2, 14.2 ng/ml/106 cells/24 h of IFN-γ, and 90 ng/ml/106 cells/24 h of GM-CSF, as determined by standard bioassays and ELISA). Secretion of cytokines had no discernible effects on cell morphology or in vitro growth rate when compared to parental MatLyLu cells or MatLyLu cells transduced with another retroviral vector encoding the human ADA minigene (26). Wild type- or adenosine deaminase-transduced MatLyLu cells did not secrete detectable levels of either IL-2, IFN-γ, or GM-CSF.

Tumor Cell Lines and Animal Studies. R3327-MatLyLu is an androgen independent, highly metastatic, and anaplastic tumor of rat origin, which spreads to the lymph node (Ly) and invariably to the lung (L). This prostate cancer cell line has been described in detail (11). CopMeElα, a syngeneic mammary adenocarcinoma cell line, was provided by Dr. J. T. Isaacs, The Johns Hopkins University, Baltimore, MD. Cells were cultivated in RPMI supplemented with 10% heat inactivated fetal calf serum and 1% glutamine (GIBCO Laboratories, Grand Island, NY). Copenhagen rats were obtained by the Mammalian Genetics and Animal Production Section, National Cancer Institute. Only mature animals 2 to 3 months of age (150–200 g) were used in the experiments. Tumor cell injections were carried out using freshly prepared

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3 The abbreviations used are: IL, interleukin; IFN, interferon; GM-CSF, granulocyte macrophage-colony stimulating factor; CTL, cytotoxic T-lymphocyte.

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MatLyLu cells either once (A), 3 times 5 days apart (•), or left untreated (•). Fourteen days after the third injection, animals were inoculated s.c. with 1.0 \times 10^4 live tumor cells. Fourteen days later, pooled splenocytes were isolated and cultured for 6 days in the presence of irradiated MatLyLu cells used as stimulators. At the end of the culture period, effector cells were cocultured with several targets at the indicated effector to target ratios, and cytotoxicity was determined in a 5-h europium-diethylenetriaminepentaacetate release assay (28). Europium-diethylenetriaminepentaacetate release was measured by time resolved fluorescence (Delfia fluorometer; Wallac, Inc., Gaithersburg, MD).

RESULTS

Immunogenic animal tumors are not thought to be good models for human cancer (5, 6). To determine whether parental MatLyLu cells are intrinsically immunogenic, one group of rats was given i.p. injections of 5.0 \times 10^6 irradiated (5000 rads) MatLyLu cells either once (A), 3 times 5 days apart (•), or left untreated (•). Fourteen days after the third injection, animals were inoculated s.c. with 1.0 \times 10^4 live MatLyLu cells. Tumor volume was determined with the aid of microcalipers.

cell suspensions. Cell viability was determined by trypan blue dye exclusion. After trypsinization, the cells were washed 3 times in phosphate buffered saline to remove any traces of serum or media.

Prostatic tumors were induced orthotopically by injection of 5 \times 10^3 tumor cells into the ventral prostatic lobe of Copenhagen rats and s.c. by implantation of 1 \times 10^4 MatLyLu cells into the right hind leg of syngeneic Copenhagen rats. It was previously determined that these cell numbers lead to tumor take in 100% of the animals (27). Tumor growth was assessed biweekly by abdominal palpation and microcalipers. Immunizations were carried out 3, 6, and 9 days post-tumor implantation with 1.0 \times 10^6 irradiated (5000 rads), cytokine secreting MatLyLu cell preparations injected intradermally into the left hind leg.

Tumor Recurrence Model. Tumors were induced intraprostatically with 1 \times 10^4 cells and 4 days later the cancerous prostate was surgically removed. It was previously determined that when prostatectomy was performed 4 or more days after implantation of MatLyLu cells, tumors recurred locally in 100% of the animals, despite meticulous surgical removal of the cancerous prostatic tissue. One day following prostatectomy, treatment with 1 \times 10^6 irradiated MatLyLu cells was initiated and repeated twice, 3 days apart. Animal care was in accordance with institutional guidelines. When animals showed signs of morbidity, they were euthanized by pentobarbital overdose.

Europium Release Assay. Three groups of rats (3 animals/group) were immunized 3 times with 5.0 \times 10^6 irradiated (5000 rads) MatLyLu cells. Fourteen days later, pooled splenocytes were isolated and cultured for 6 days in the presence of irradiated MatLyLu cells used as stimulators. At the end of the culture period, effector cells were cocultured with several targets at the indicated effector to target ratios, and cytotoxicity was determined in a 5-h europium-diethylenetriaminepentaacetate release assay (28). Europium-diethylenetriaminepentaacetate release was measured by time resolved fluorescence (Delfia fluorometer; Wallac, Inc., Gaithersburg, MD).

RESULTS

Immunogenic animal tumors are not thought to be good models for human cancer (5, 6). To determine whether parental MatLyLu cells are intrinsically immunogenic, one group of rats was given i.p. injections of 5 \times 10^6 irradiated MatLyLu cells once, and another group received injections 3 times at 5-day intervals. Two weeks following the third injection, rats were either challenged s.c. with 1.0 \times 10^4 live MatLyLu cells (the minimal dose of cells leading to tumor formation in 100% of injected animals), or spleen cells were isolated to determine whether tumor specific CTLs were induced in the vaccinated rats. Upon challenge with unmodified MatLyLu cells, all rats developed tumors and no difference was seen in the rate of tumor growth (Fig. 1) or survival (data not shown) between vaccinated and age-matched control rats. As shown in Fig. 2, tumor specific CTLs were not detected in rats immunized with parental MatLyLu cells. Thus, the MatLyLu tumor did not exhibit any evidence of intrinsic immunogenicity as judged by the stringent criteria set forth by Hewitt et al. (5), or standard cytotoxicity assays (Fig. 2). In stark contrast to unmodified MatLyLu cells, IL-2 secreting cells induced a strong CTL response which specifically recognized parental MatLyLu cells, as well as the related R3327-G subline (9), but not the unrelated adenocarcinoma CopMeElia cell line of the same genetic background. Thus, experimental manipulation of the parental MatLyLu tumor to express IL-2 converted this nonimmunogenic tumor to an immunogenic form capable of inducing an in vivo tumor specific immune response.

To assess the therapeutic value of cytokine secreting MatLyLu cells as cellular vaccines in rats carrying an established tumor, 1.0 \times 10^6 parental MatLyLu cells were injected s.c. and treatment with various tumor cell preparations was initiated 3 days later. Unless otherwise specified, treatment in this and subsequent experiments consisted of 1.0 \times 10^6 irradiated cells injected intradermally, followed by 2
Since it was noted that the site of tumor implantation can greatly influence its biological properties (29), orthotopic tumor induction may represent a more accurate approach to assess the effectiveness of a particular treatment protocol. Consequently, MatLyLu tumors were induced by inoculation of $5 \times 10^3$ MatLyLu cells into the ventral lobe of the rat prostate. In dose-finding experiments, tumors formed in 100% of animals that received $5 \times 10^3$ or more parental MatLyLu cells. Tumor growth could be monitored by abdominal palpation, though reliable quantitation of tumor size was rendered more difficult due to its internal location and uneven shape. Similar to human prostate cancer, orthotopic implantation of the MatLyLu tumor resulted in metastatic spread to pelvic and retroperitoneal lymph nodes, while s.c. established MatLyLu tumors metastasized preferably to inguinal lymph nodes and lungs (11, 27). However, unlike prostate cancer in humans, metastasis to the bones was not evident, most likely due to the significantly reduced life span of the animal. As seen in Fig. 3B, when the tumor was established in the prostate and treatment with tumor cell preparations was initiated 3 days later, only treatment with IL-2 secreting MatLyLu cells was capable of producing a clear survival benefit. However, unlike treatment of rats bearing a s.c. implanted tumor, rats could not be cured of their preestablished tumors.

Since treatment of tumor bearing rats was initiated only 3 days post implantation of the tumor cells, it was important to determine the state of the tumor implant at the start of the treatment protocol. Fig. 4A shows a cross-section through the ventral prostatic lobe and adjacent bladder of the rat, which was inoculated 3 days earlier with $5 \times 10^3$ parental MatLyLu cells. The presence of a tumor mass at the center of the prostatic lobe is evident. As shown in Fig. 4B, the tumor consisted of an infiltrating, high-grade carcinoma with signs of neovascularization (27). Similar histological pictures were obtained from 2 additional tumor bearing rats. It is therefore evident that, at the time of initiation of treatment with the cytokine secreting tumor cell preparations, a well established neovascularized tumor was present in the prostate consisting of rapidly dividing tumor cells. (It may be argued that since it takes 3 to 5 days for the development of an immune response, the first wave of tumor specific T-cells will encounter a 6–8-day-old, highly developed, tumor.)

In a comparative study, it was recently shown that GM-CSF secreting tumor cells were the most active preparation capable of inducing antitumor immunity against the nonimmunogenic, highly metastatic murine B16-F10 melanoma (24). We therefore compared the therapeutic effect of IL-2 and GM-CSF-secreting tumor cell preparations in rats carrying a 3-day-old MatLyLu tumor. As shown in Fig. 5A, treatment of s.c. implanted tumor bearing rats with IL-2 secreting cells led to complete tumor regression in all animals (5 of 5), while only 2 of 5 animals treated with GM-CSF secreting cells were cured of their preestablished tumor. No difference in the effect of IL-2 versus GM-CSF secreting tumor cell preparations was observed in rats bearing an orthotopically induced tumor (Fig. 5B). Provided that murine-derived GM-CSF is interchangeable with rat-derived GM-CSF in vivo, as is the case in vitro, our results show that in this experimental model, IL-2 secreting tumor cell preparations are equal or superior to GM-CSF secreting cells.

In order to determine whether rats exhibiting long term tumor regression (Fig. 5A) would be resistant to a second challenge with tumor cells, the “cured” rats were given s.c. injections of $5 \times 10^4$ MatLyLu cells (5 times the minimal tumorigenic dose). No tumors formed in rats “cured” of their original tumor by treatment with IL-2 secreting cells (5 of 5), while tumors grew at a similar rate in 2 of 2 animals “cured” by treatment with GM-CSF secreting cells, and in a control group consisting of age-matched animals (5 of 5). This result indicates that immunological memory was established in rats cured of
Fig. 4. Histological analysis of a 3-day-old tumor induced by intraprostatic inoculation of $1.0 \times 10^5$ MatLyLu cells. A, hematoxylin and eosin stain showing the anatomical structures of the ventral prostate (P), adjacent bladder (B), and centrally located solid tumor mass infiltrating the prostatic stroma (arrow). No secondary tumor deposits within the gland. (Magnification, $\times 4$). B, higher magnification ($\times 200$) revealed high-grade tumor with various mitoses and signs of neovascularization (arrows).

DISCUSSION

Using an increasingly relevant animal model to explore the utility of cytokine secreting tumor vaccines for the treatment of prostate cancer, we have shown that inactivated, IL-2-secreting, tumor cells were most effective in reducing tumor growth and prolonging the survival of tumor bearing animals. We also observed that MatLyLu tumors orthotopically implanted in the prostate were less susceptible to treatment with cytokine secreting tumor cells than s.c. implanted tumors (Figs. 3 and 5). The ectopically and orthotopically implanted tumors appear to grow at a similar rate, and rats bearing a tumor at either site succumb to disease within 3.5 weeks (Figs. 3 and 5). It is, macroscopic tumors were evident in rats treated with IL-2 secreting MatLyLu cells (Fig. 6A). However, in the majority of these animals (4 of 5), the residual tumor was not completely eliminated since tumors grew, albeit with a significant delay, and the rats eventually succumbed to disease (Fig. 6B).
therefore, difficult to attribute the superior effect seen in rats bearing s.c. implanted tumors to slower tumor growth at this site (although in some experiments we observed reduced tumor growth in s.c. implanted animals). As previously suggested (30, 31), it is more likely that the prostate constitutes a less permissive environment than the skin for the development of an effective antitumor immune response, highlighting the importance of using orthotopically implanted tumors to assess the effectiveness of therapeutic protocols (29). The modest though reproducible therapeutic benefit seen in animals bearing an orthotopically implanted tumor is encouraging (Figs. 3B, 5B, and 6), and suggests that additional studies may lead to improved outcome.

Growth inactivated (irradiated) MatLyLu cells were used to vaccinate animals not only to simulate a clinical setting but also to demonstrate that the parental tumor cells were devoid of intrinsic immunogenicity. The potential utility of cytokine gene modified tumor vaccines was initially demonstrated using live tumor cell preparations, which was possible because the cytokine-secreting cells lost their ability to grow in vivo, i.e., to form tumors in the animal (13–19). However, in such studies the intrinsic immunogenicity of the tumor cells could not be determined since it was unclear whether the cytokine contributed to the induction of antitumor immunity or whether its only function was to inhibit tumor growth in the animal. Indeed, it was recently shown that several tumors used in previous studies were immunogenic when administered in an unmodified, though irradiated, form, suggesting that the cytokine secreted from the live tumor cells was not responsible for the observed antitumor effect (24).

The MatLyLu prostatic carcinoma line is analogous to hormone-refractory, metastatic human prostate cancer in many ways (9–11). Using stringent testing criteria set forth by Hewitt et al. (5), we have shown that MatLyLu cells are nonimmunogenic in the rat (Figs. 1 and 2), and therefore represent a useful model for immunological studies addressing a similar situation in human cancer. The finding that genetically modified, IL-2 secreting, MatLyLu cells were capable of inducing a significant immune response against the parental tumor demonstrates that MatLyLu cells encode potent tumor rejection antigens which can be made accessible to the immune system upon proper manipulation of the tumor cells. That nonimmunogenic tumors can be
experimentally manipulated to induce an immune response against the parental tumor was first demonstrated by Van Pel and Boon (8), and subsequently extended to other tumor systems (20–24, 32). Recent studies have shown that this paradigm may also apply to peripherally expressed self-antigens, providing a plausible explanation as to the maintenance of peripheral tolerance (33, 34).

In conclusion, these observations support the view that active immunization of cancer patients, including prostate cancer patients, deserves consideration despite lack of demonstrable immunogenicity in many human tumors.

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REFERENCES


Immunotherapy of Prostate Cancer in the Dunning Rat Model: Use of Cytokine Gene Modified Tumor Vaccines


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