

Optimizing Syngeneic Orthotopic Murine Bladder Cancer (MB49)¹

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Abstract

The syngeneic orthotopic murine bladder cancer model MB49 is hampered by unreliable tumor implantation. We optimized this model by a simple modification of the standard implantation technique in three groups of mice. Fifty thousand (group I), 20,000 (group II), or 10,000 (group III) tumor cells were implanted into cauterized bladders by transurethral instillation, and dwell time was prolonged to 3 h. Tumor take, survival, and bladder weights were determined as outcome variables. To verify whether this modification maintained its sensitivity to topical immunotherapy, an initial tumor load of 100,000 MB49 cells was given, and mice were treated intravesically with *Bacillus Calmette-Guérin* or phosphate-buffered saline. The prolonged dwell time of tumor cells resulted in take rates of 100% in all three groups. Survival and bladder weights were significantly correlated with the number of instilled cells. Even with the highest tumor load, *Bacillus Calmette-Guérin* therapy improved survival and reduced bladder weights significantly, as compared to PBS. Thus, the modified model is highly reliable and maintains its susceptibility to topical immunotherapy.

Introduction

Animal models of bladder cancer allow the investigation of aspects of bladder cancer that cannot be studied under clinical conditions, such as evaluation of new chemotherapeutic or immunotherapeutic agents, drug regimens, or other modalities of treatment. Animal models of bladder cancer can also give further insight in basic mechanisms of tumor growth and spread. Currently, three are fundamental murine bladder tumor models: chemically induced bladder cancer (1, 2), the xenograft model (transplantation of human transitional cell carcinoma into immunodeficient mice; Refs. 3–5), and the syngeneic tumor model (transplantation of carcinogen-induced bladder cancer in syngeneic, immunocompetent mice; Refs. 6–9). For the evaluation of immunotherapeutic approaches, the syngeneic murine bladder tumor model seems to be the most appropriate model because of the chance to study the local tumor in an immunocompetent host, which is an absolute necessity for reliable data. Syngeneic tumor cells can be implanted either s.c. (heterotopic tumor) or intravesically (orthotopic). Orthotopic tumor implantation is more difficult; however, the possibility of investigating tumor growth and therapeutic effects in the native organ, where hormonal or immunological processes more closely resemble the clinical situation, making experimental results more reliable, makes this method more attractive. Successful tumor cell implantation of syngeneic MB49 cells, which is the basic principle of the orthotopic bladder tumor model, fails in ~25% of the animals (8–11). Low tumor take rates impair the evaluation of experimental results and lead to higher numbers of animals in the experimental groups. To solve this problem, we developed a modification of

the tumor implantation technique. We hypothesized that a short retention time of instilled cells was a reason for unreliable tumor implantation and tried to increase tumor take rates by prolonging dwell time. Because the orthotopic model is frequently used in the evaluation of intravesical immunotherapy, we confirmed the sensitivity of the modified model to topical BCG³ immunotherapy using an unusually high tumor load.

Materials and Methods

Animals. Sixty 6–8-week-old female C57/BL6 mice, each weighing ~17 g, were purchased from Charles River (Sulzfeld, Germany) and maintained at our animal care facility for 1 week prior to use. The mice were housed five per cage in a limited access area at a room temperature of $20 \pm 1^\circ\text{C}$ and a humidity of $50 \pm 10\%$, with food and water *ad libitum*. All experiments were approved by the Ministry of Environment, Nature and Forestry of Schleswig-Holstein, Germany.

Tumor. Tumor cells used in this study were derived from the 7,12-dimethylbenzanthracene-induced murine bladder cancer MB49 (12). The cells were maintained in *in vitro* culture (DMEM, 10% FCS, and 1% penicillin/streptomycin at 37°C and 5% CO_2). Tumor cells were harvested by trypsinization and suspended in DMEM without L-glutamine, FCS, and antibiotics. Viability was determined by trypan blue exclusion, and only tumor cell suspensions with >90% viable cells were used for tumor implantation. The concentrations of the tumor cell suspensions that we used for implantation were adjusted to 10^6 , 4×10^5 , and 2×10^5 cells/ml for groups I, II, and III (see below), respectively, in the first experiment and to 2×10^6 cells/ml in the second set of experiments.

Tumor Implantation. Intravesical tumor implantation was performed according to a modification of the methods described by Soloway and Masters (8, 13) and Shapiro *et al.* (14) for the MBT-2 model and by Hudson *et al.* (7) for the MB49 model. Briefly, after a short ether inhalation anesthesia, the mice received an i.p. injection of diluted sodium pentobarbital (6 mg/ml) for general anesthesia of a single dose of 0.06 mg/g body weight. After shaving areas of ~1 cm² on the backs of the mice, we inserted a 24-gauge Teflon i.v. catheter (Insyte-W; Becton Dickinson, Heidelberg, Germany) transurethrally into the bladder using a lubricant (Instilla Gel; Farco-Pharma, Köln, Germany). Mice were placed with their backs on the ground plate of the cautery unit. To optimize contact, we used electrocardiogram electrode contact gel. The soft-tipped end of a spring-wire guide of a 24-gauge central venous catheter (Arrow, Erding, Germany) was inserted into the bladder via the Teflon catheter and gently pushed forward until it reached the bladder wall. The guide wire was attached to the cautery unit (Elektrotrom 500; Gebrüder Martin, Tuttingen, Germany), and a monopolar coagulation was applied for 5 s at the lowest setting (5 W). After removal of the guide wire, 0.05 ml of the tumor cell suspension was instilled. Unlike the conventional procedure, in which catheters are removed after instillation, the catheters were pinched off with a clamp, kept locked with a Luer-Lock closing cone, and left in place until the mice awakened. Using this method, we ensured a dwell time of ~3 h. In contrast to others (1, 8, 14), we used DMEM as solvent for instilled tumor cells as a means for improving viability.

Drugs. For intravesical immunotherapy, lyophilized BCG strain Connaught strain was kindly provided by Cytochemia (Ihringen, Germany). Each vial contained 81 mg of lyophilized BCG Connaught with at least 1.8×10^8

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³ The abbreviation used is: BCG, *Bacillus Calmette-Guérin*.

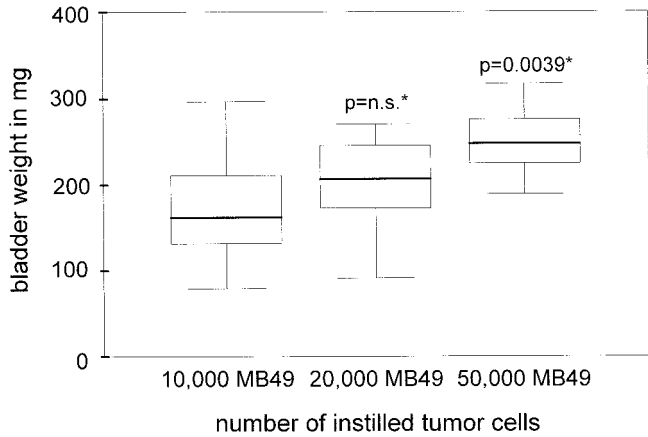


Fig. 1. Effect of number of instilled tumor cells on urinary bladder weight. The distributions of bladder weights are plotted. The upper and lower boundaries of the boxes are the upper and lower quartiles. The length of the box is the interquartile distance, so the box contains 50% of the values falling between the 25th and 75th percentiles in a group. The black line inside the box identifies the group median. The lines extending from each box extend to the smallest and largest observations in a group. *, P s compared to instillation with 10,000 tumor cells, Mann-Whitney U test; n.s., not significant.

colony-forming units. BCG was reconstituted with 3 ml of solvent, according to the manufacturer's recommendation.

Optimization of Intravesical Tumor Implantation. Thirty mice were assigned to three groups, and intravesical bladder tumors were implanted by intravesical instillation of MB49 tumor cells, according to the method described above. Group I mice received 10,000 cells, group II mice received 20,000 cells, and group III mice received 50,000 tumor cells. The animals received no further treatment and were sacrificed after 35 days.

Mice were visited daily to check their viability status and to examine for gross hematuria. Tumor incidence and bladder weights were determined after sacrifice. The presence of intravesical tumors was verified histologically (H&E staining). All animals underwent complete dissection so that manifestations of extravascular tumor growth and pulmonary metastasis could be recorded.

Sensitivity to Intravesical Immunotherapy. Intravesical tumors were implanted into 30 mice using 100,000 tumor cells. The animals were randomized to two groups with 15 animals each: PBS control and BCG therapy. Intravesical instillations were performed on days 1, 8, 15, and 22 after tumor implantation by the technique described above. Considering the catheter's death space, the instilled volume was 0.05 ml in both groups. The BCG dose of a single instillation was 1.35 mg (minimum of 3×10^6 colony-forming units). Due to rapid tumor growth, the animals were sacrificed on day 28 and evaluated in the same way as described for the first experiment.

Statistical Analysis. To compare survival, we used the Kaplan-Meier method and the log-rank test. Comparison of bladder weight and body weight was performed with the Mann-Whitney U test. Statistical significance was determined at $P < 0.05$. Results are given in boxplots, in which the upper and lower boundaries of the boxes represent the upper and lower quartiles, respectively. The box length represents the interquartile distance, so the box contains 50% of the values falling between the 25th and 75th percentile of a group. The black line inside the box identifies the group median, whereas the lines extending from each box extend to the smallest and largest observations in the groups. To compare tumor take rates, the χ^2 test was used. For statistical analysis we used SPSS for Microsoft Windows.

Results

Optimization of Intravesical Tumor Implantation

General Findings. Using a cautious implantation technique, as described above, we observed no transmural bladder injury or bladder perforation. All animals with intravesical tumors showed gross hematuria before day 16. No significant differences with regard to the first onset of hematuria were found (day 12 ± 3). The average body weight of the mice increased from 16.0 g on day 1 to 19.3 g on day 18. Beyond day 18, the animals' body weights decreased. No significant

differences were seen between these groups. Gross internal organ examination revealed pulmonary metastasis in 20, 30, and 70% of the mice instilled with 10,000 (group I), 20,000 (group II), and 50,000 (group III) tumor cells, respectively (group I versus group III and group II versus group III; each $P < 0.05$, χ^2 test). One group II mouse and two group III mice showed pyelonephritis. Upper urinary tract tumor growth in the kidney could be demonstrated in one animal from group III.

Tumor Outgrowth. All mice developed intravesical tumors, indicating a tumor take rate of 100%, independent of the number of instilled tumor cells.

Bladder Weight. A correlation between number of instilled tumor cells and bladder weight was found. Mice receiving 50,000 MB49 cells had the highest average bladder weight (251.6 ± 39.0 mg), whereas mice instilled with 20,000 and 10,000 tumor cells had average bladder weights of 202 ± 54.2 and 172 ± 61.9 mg, respectively ($P = 0.04$ and 0.0039 , respectively, Mann-Whitney U test; Fig. 1).

Survival. Upon termination of the experiment 35 days after tumor implantation, no group III mouse was alive, whereas three group II mice and six group I mice were still living. The Kaplan-Meier curve further illustrates survival as a function of initial tumor load. Instillation with 50,000 tumor cells led to a mean survival of 25.9 days, whereas animals receiving 20,000 and 10,000 cells had significantly longer mean survivals of 30.0 and 32.9 days ($P = 0.04$ and $P = 0.0006$, log-rank test; Fig. 2), respectively.

Sensitivity to Intravesical Immunotherapy

The second set of experiments was initiated to verify that the modified model maintained its well-known sensitivity to immunotherapy.

General Findings. Gross hematuria was demonstrable before day 8 in 90% of the animals. The average body weight of all mice increased from 17.6 g on day 1 up to 18.9 g on day 12, and a reduction of body weight was observed thereafter. No significant differences were seen between the PBS and BCG therapy groups.

We found pulmonary metastases in 66.6% of the mice receiving PBS and 53.3% of the mice treated with BCG (not significant, χ^2 test). Two mice of the PBS and one mouse of the BCG group had hydronephrosis due to extensive intravesical tumor growth. Two mice in each group suffered from suppurative pyelonephritis, which is most probably due to ascending urinary infection.

Tumor Outgrowth. Mice receiving PBS instillations had a tumor take rate of 100%, whereas the animals treated with BCG had tumor outgrowth in 93.3% (not significant, χ^2 test).

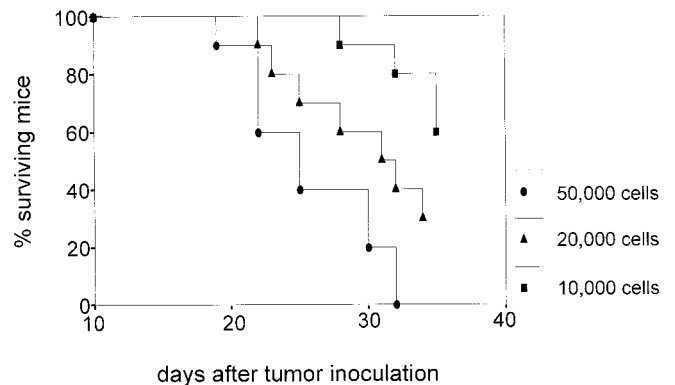


Fig. 2. Kaplan-Meier analysis of murine survival after instillation of different number of MB49 bladder tumor cells. The experiment was terminated at day 35 (10,000 versus 50,000 cells, $P = 0.0006$; 20,000 versus 50,000 cells, $P = 0.04$, log-rank test).

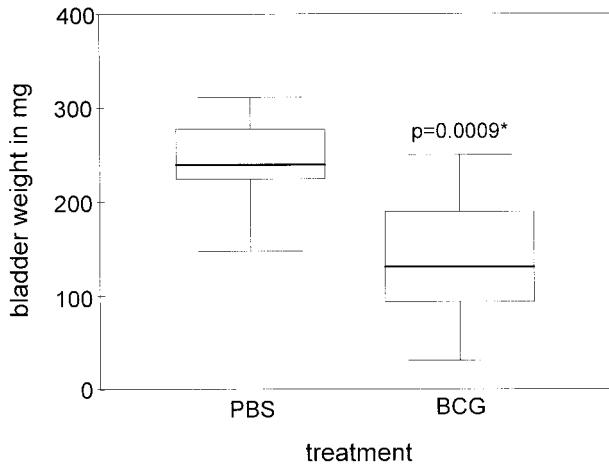


Fig. 3. Effect of intravesical BCG immunotherapy on urinary bladder weights. The distributions of bladder weights are plotted. The upper and lower boundaries of the boxes are the upper and lower quartiles. The length of the box is the interquartile distance, so the box contains 50% of the values falling between the 25th and 75th percentiles in a group. The black line inside the box identifies the group median. The lines extending from each box extend to the smallest and largest observations in a group. *, P compared to PBS treatment, Mann-Whitney- U test.

Effect of BCG Instillations on Average Bladder Weight. The animals of the control group receiving PBS instillations had an average bladder weight of 248 mg. Weekly BCG instillations reduced average bladder weight to 140 mg ($P = 0.0009$, Mann-Whitney U test; Fig. 3).

Effect of BCG Instillations on Survival. Mice treated with BCG showed significantly longer survival as compared to mice receiving PBS (25.7 versus 20.8 days). On day 28, the experiment was terminated due to rapid tumor growth. Ten of 15 mice treated with BCG were alive, whereas only 2 animals of the control group had survived. Kaplan-Meier analysis and log-rank test revealed a significant prolongation of survival in BCG-treated mice ($P = 0.005$, log-rank; Fig. 4).

Discussion

Several animal models of bladder cancer, such as chemically induced bladder cancer (1, 2), the xenograft model (3–5) and s.c. implantation of syngeneic bladder tumor cells, have been used in experimental oncology. For investigation of immunotherapeutic approaches, these tumor models should not be considered. The immunodeficient nude mice used in the xenograft model, for example, are compromised in their ability to develop an adequate immune reaction to an immunological stimulus. Furthermore, the interpretation of experiments using s.c. implanted syngeneic bladder tumors is limited because of different tissue-specific factors. The orthotopic syngeneic bladder tumor model has been successfully used for evaluation of efficacy of intravesical BCG (10, 11), keyhole limpet hemocyanin (15), and chemotherapeutics (11) and for elucidation of the mode of action of intravesical BCG (6, 7). Since the first description of basic features of intravesical tumor implantation by Soloway (8), the orthotopic murine bladder tumor model has undergone several modifications. The common principle behind all modifications is the transurethral intravesical instillation of tumor cells after catheterization and traumatization of the bladder. Tumor cell lines, mice strain, number of instilled tumor cells, technique of bladder traumatization, and response variables are different, however. Currently, the MB49 bladder tumor implanted in C57/BL6 mice and the MBT-2 tumor implanted in C3H/He mice are used for orthotopic tumor implantation. The amount of instilled cells varies from 10^4 cells (7) to 5×10^5 cells (9) using the MBT-2 model. In the MB49 tumor model, the number of instilled

cells is usually smaller and varies between 10^4 and 10^5 cells (7, 16). Shapiro *et al.* (14) investigated the dose response of tumor implantation in the MBT-2 model and found a maximum tumor take of $>90\%$ after instillation of 10^6 tumor cells, whereas instillation with 2.5×10^5 cells led to a take rate of 30%. Therefore, a high number of instilled tumor cells alone cannot guarantee high tumor take rates. Several authors achieved take rates of $\sim 70\%$, even after instillation of 5×10^5 MBT-2 cells (9–11). Such low tumor take rates can compromise the evaluation of experimental results and may increase the number of animals needed for investigation.

Successful tumor implantation is also dependent on adequate traumatization of bladder surface. Instillation of tumor cells in a nontraumatized bladder induces intravesical tumor growth in $<10\%$ of the instilled animals (17). Traumatization of the bladder surface is usually performed with electrocautery after catheterization and transurethral insertion of the cautery wire (7, 10, 15). Traumatization is also achieved by instillations of *N*-methyl-*N*-nitrosourea (8) or hydrochloric acid (18).

Tumor outgrowth, detected by abdominal palpation, tumor take rate, and bladder weight, are the most common used response variables in this tumor model (7, 10, 15). We do not consider mere bladder palpation and tumor take rate to be reliable parameters for assessing treatment effects on tumor growth. Only large tumors (>200 mg) were clearly detectable by abdominal palpation. Using the tumor take as a response variable may give inadequate results because a reduced take rate in one group may be the result of successful treatment or may simply reflect failure of tumor implantation. To overcome these difficulties, other authors tried to monitor intravesical tumor growth and treatment effects by imaging methods like transrectal ultrasound (16) or magnetic resonance (18, 19). These methods proved to be a suitable means for monitoring intravesical tumor growth, but each imaging procedure required anesthesia and catheterization. Almost 20% of the animals died prior to the completion of the treatment protocol due to procedural mishaps (18, 19). Apart from this, imaging methods, especially magnetic resonance imaging, are expensive. Recently a new, invasive technique of intravesical tumor implantation was described, which uses direct submucosal injection of tumor cells after exposing the bladder by a low midline incision. This technique enables tumor take rates of 100% but requires an open surgical procedure and postoperative antibiotic treatment (20). The use of antibiotics might further impair the efficacy of immunotherapies such as BCG.

In our study, we used a different approach and tried to achieve a maximum tumor take rate by a simple modification of the “tradition-

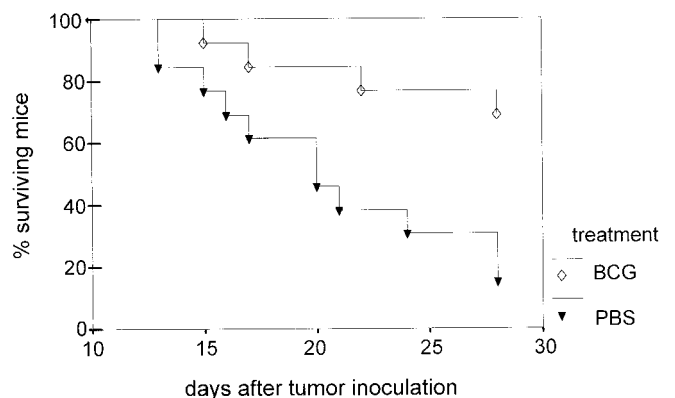


Fig. 4. Kaplan-Meier analysis of murine survival after intravesical BCG immunotherapy. Mice received four intravesical instillation treatments with BCG. The control group received four PBS instillations. The experiment was terminated at day 28 due to rapid tumor growth. $P = 0.005$, log-rank test.

al" procedure. We hypothesized that a prolonged contact time of instilled tumor cells and bladder wall may increase tumor take. This was achieved by leaving locked catheters in the animals' bladder until the animal awakened. Efflux of tumor cells or premature bladder evacuation by voiding could be prevented for ~3 h. We evaluated the response variables survival and bladder weight determined after dissection to describe the treatment effects on existing intravesical tumors.

In our first set of experiments, we investigated whether tumor take was dependent on number of instilled cells or on dwell time. Instillation of 50,000, 20,000, and 10,000 tumor cells each led to tumor take rates of 100%, showing clearly that dwell time was the relevant variable for successful tumor take. On the other hand, the course of the malignant disease was significantly influenced by the number of instilled cells because survival, bladder weight, and rate of pulmonary metastasis were dependent on the number of instilled tumor cells. This opens the possibility of "tailoring" the model to the requirements of different therapeutic approaches. The time slot for therapeutic intervention and the aggressiveness of the disease might be influenced by instillation of different number of tumor cells.

The efficacy of intravesical BCG in murine bladder cancer has been demonstrated in several investigations (8, 10, 11). To confirm that our modified tumor model is still sensitive to BCG, we performed a further set of experiments, using a very high number of tumor cells for instillation. We confirmed the sensitivity of the modified model to intravesical BCG immunotherapy using average bladder weight and survival as response variables. As expected, we saw a rapid progression of tumor growth, indicated by early onset of gross hematuria and early begin of weight loss and death of >85% of the animals of the control group within the observation period. Even with this aggressive course of disease, BCG immunotherapy significantly reduced average bladder weights and could prolong survival as compared to control. Therefore, with these experimental settings, BCG therapy clearly interferes with survival and bladder weight but not with tumor implantation. Our modification is easy to handle and does not need imaging methods or open surgical tumor implantation. Furthermore, the response variables survival and bladder weight are easily determined. Finally, reliably inducing tumor take rates of 100% certainly contributes to a significant reduction in the number of animals per group needed for statistical reliability.

We conclude that our modification is a simple, cheap, and useful method to improve the orthotopic murine bladder cancer model.

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References

- Soloway, M. S. Single and combination chemotherapy for primary murine bladder cancer. *Cancer (Phila.)*, 36: 333-340, 1975.
- Williams, P. D., and Murphy, G. P. Experimental bladder tumor induction, propagation and therapy. *Urology*, 8: 39-42, 1976.
- Hubbell, H. R., Kvalnes-Krick, K., and Carter, W. G. Antiproliferative and immunomodulatory actions of β -interferon and double-stranded RNA, individually and in combination, on human bladder tumor xenografts in nude mice. *Cancer Res.*, 45: 2481-2486, 1985.
- Huland, H., Otto, U., and von Paleske, A. Chemotherapy and human bladder carcinoma transplanted into NMRI *nu/nu* mice. *J. Urol.*, 134: 601-606, 1985.
- Russell, P. J., Raghavan, D., Gregory, P., Philips, J., Wills, E. J., Jelbart, M., Wass, J., Zbroja, R. A., and Vincent, P. C. Bladder cancer xenografts: a model of tumor cell heterogeneity. *Cancer Res.*, 46: 2035-2040, 1986.
- Ratliff, T. L., Gillen, D. P., and Catalona, W. J. Requirement of a thymus-dependent immune response for BCG-mediated antitumor activity. *J. Urol.*, 137: 155-158, 1987.
- Hudson, M. A., Ritchey, J. K., Catalona, W. J., Brown, E. J., and Ratliff, T. L. Comparison of the fibronectin-binding ability and antitumor efficacy of various mycobacteria. *Cancer Res.*, 50: 3843-3847, 1990.
- Soloway, M. Intravesical and systemic chemotherapy of murine bladder cancer. *Cancer Res.*, 37: 2918-2929, 1977.
- Lee, K-E., Weiss, G. H., O'Donnell, R. W., and Cockett, A. T. K. Reduction of bladder cancer growth in mice treated with intravesical *Bacillus Calmette-Guérin* and systemic interleukin 2. *J. Urol.*, 137: 1270-1273, 1987.
- Shapiro, A., Ratliff, T. L., Oakley, D. M., and Catalona, W. J. Reduction of bladder tumor growth in mice treated with intravesical *Bacillus Calmette-Guérin* and its correlation with *Bacillus Calmette-Guérin* viability and natural killer cell activity. *Cancer Res.*, 43: 1611-1615, 1983.
- Shapiro, A., Ratliff, T. L., Oakley, D. M., and Catalona, W. J. Comparison of the efficacy of intravesical *Bacillus Calmette-Guérin* with thiotepa, mitomycin C, poly 1:C/poly-l-lysine and cisplatin in murine bladder cancer. *J. Urol.*, 131: 139-142, 1984.
- Summerhayes, I. C., and Franks, L. M. Effects of donor age on neoplastic transformation of adult mouse bladder epithelium *in vitro*. *J. Natl. Cancer Inst. (Bethesda)*, 62: 1017-1023, 1979.
- Soloway, M. S., and Masters, S. Urothelial susceptibility to tumor cell implantation. Influence of cauterization. *Cancer (Phila.)*, 46: 1158-1158, 1980.
- Shapiro, A., Kelley, D. R., Oakley, D. M., Catalona, W. J., and Ratliff, T. L. Technical factors affecting the reproducibility of intravesical mouse bladder tumor implantation during therapy with *Bacillus Calmette-Guérin*. *Cancer Res.*, 44: 3051-3054, 1984.
- Swerdlow, R. D., Ratliff, T. L., Regina, M. L., Ritchey, J. K., and Ebert, R. F. Immunotherapy with keyhole limpet hemocyanin: efficacy and safety in the MB-49 intravesical murine bladder tumor model. *J. Urol.*, 151: 1718-1722, 1994.
- Alexander, A. A., Liu, J. B., McCue, P., Gomella, L. G., Ross, R. P., and Lattime, E. C. Intravesical growth of murine bladder tumors assessed by transrectal ultrasound. *J. Urol.*, 150: 525-528, 1993.
- Soloway, M. S., Nissenkorn, I., and McCallum, L. Urothelial susceptibility to tumor cell implantation: comparison of cauterization with *N*-methyl-*N*-nitrosourea. *Urology*, 21: 159-161, 1983.
- Chin, J. L., Kadhim, S. A., Batislam, E., Karlik, S. J., Garcia, B. M., Nickel, J. C., and Morales, A. *Mycobacterium* cell wall: an alternative to intravesical *Bacillus Calmette-Guérin* (BCG) therapy in orthotopic murine bladder cancer. *J. Urol.*, 156: 1189-1193, 1996.
- Chin, J., Kadhim, S., Garcia, B., Kim, Y. S., and Karlik, S. Magnetic resonance imaging for detecting and treatment monitoring of orthotopic murine bladder tumor implants. *J. Urol.*, 145: 1297-1301, 1991.
- Jiang, F., and Zhou, X-M. A model of orthotopic murine bladder (MBT-2) tumor implants. *Urol. Res.*, 25: 179-182, 1997.