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Keyhole-Limpet Haemocyanin (KLH) Immunotherapy of Murine Transitional Cell Carcinoma

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Summary. The antigenicity of transitional cell carcinoma of the bladder has stimulated the search for effective immunotherapeutic agents in the treatment of this disease. Non-specific immunotherapy with local (intravesical/intralesional) and systemic Keyhole Limpet Haemocyanin (KLH) in a FANFT induced murine bladder tumor model was studied. Results showed no difference between control or treated groups in either tumor growth or animal survival.

Key words: Bladder carcinoma, Keyhole limpet haemocyanin (KLH), Immunotherapy.

Introduction

Bladder cancer is responsible for approximately 10,000 deaths annually in the United States [17]. The majority of the 35,000 patients who develop bladder cancer annually present with disease localized to the bladder. More than two-thirds of these patients will suffer recurrence of their tumor following surgical excision, while one-third will eventually die of the disease.

As transitional cell carcinoma of the bladder has been shown to be an antigenic tumor in both animals [6, 20] and man [1, 15, 19] there has been a renewal of interest in the role of immunotherapy in treating bladder cancer. The prevention of bladder tumor recurrences with a non-bacterial agent such as Keyhole Limpet Haemocyanin (KLH) could decrease the incidence of disease progression and thus improve patient survival. KLH was recently studied by Lamm and associates [11] who found that intralesional administration of this antigen retarded transitional cell carcinoma (TCC) growth in a murine model. The following studies were designed to further evaluate the ability of KLH to interfere with the growth of a FANFT induced bladder tumor.

Materials and Methods

A FANFT (N-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide) induced transitional cell carcinoma of the mouse bladder was carried as a subcutaneous implant in female C3H/HE mice for more than a dozen generations in our laboratory. This bladder tumor (MBT 2) is moderately well differentiated and represents one of the established lines of Soloway. In these experiments, this tumor was implanted either subcutaneously, as a free-cell preparation, or intravesically after pre-treatment of the recipient bladder with MNU (N-methyl-N-nitrosourea) according to the method of Weldon and Soloway [21].

Bladder catheterization was accomplished by inserting a P.E. 10 polyethylene catheter per urethra. The catheters were left in situ for approximately 10 min to keep the solutions within the bladder.

In the first experiment, 60 female C3H/HE mice were anesthetized with intraperitoneal Nembutal (55 mg/kg). A solution of 1.5 mg MNU in 0.1 cc sodium acetate buffer (0.15 M) was instilled via urethral catheterization into each bladder. 48 h later a fresh tumor cell suspension was prepared. Fresh tumor was excised and free cells were prepared by stirring for 2 h with trypsin and versene. The final tumor cell suspension contained 21×10^6 cells per ml. The number of viable cells determined by Trypan Blue exclusion exceeded 95%. The animals were anesthetized with Nembutal and implanted intravesically with 2.1×10^6 tumor cells in 0.1 ml.

On the day of implant, the mice were divided into two groups: Group I consisted of 30 mice which were injected with 50 mcg KLH (Calbiochem, San Diego) in 25 µl phosphate buffered saline (PBS) in each of the four foot pads. Group II consisted of 30 mice which were injected similarly with the same volume of saline. On days 14 and 21 after implant, the treated group received 200 mcg KLH in 0.1 cc PBS intravesically. Again the controls received the same volume of saline. Survival of both groups was monitored. The surviving mice were sacrificed 4 weeks after implantation and the bladders removed, weighed, and observed for gross tumors. The whole bladders were fixed in 10% formalin and H and E stained sections obtained.

In the second experiment, 36 female C3H/HE mice were divided into three groups: Group I consisted of 12 animals which served as controls receiving 25 µl PBS in each of the four foot pads. Group II and III each consisted of 12 mice which were pre-sensitized with 50 mcg KLH in 25 µl PBS in each foot pad. Twenty days later, a fresh tumor cell suspension was prepared. All animals received an injection of 2.5×10^6 tumor cells (85% viable) in 0.1 ml medium, subcutaneously. On days 1, 8 and 18 post implant, Group II re-

Table 1. The intravesical tumor growth 4 weeks after implantation is shown. Intravesical KLH failed to reduce the number of bladders containing tumors or to reduce the size of these tumors

Tumor Status	Control	KLH
Gross tumor	11/16	13/16
Microscopic tumor	13/16	15/16
Mean bladder weight	0.94 ± 0.19 gm	1.15 ± 0.31 gm

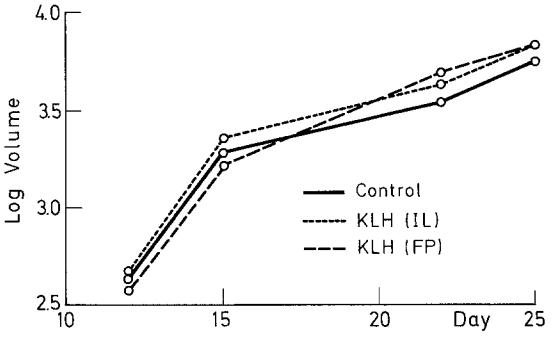

Fig. 1. Tumor growth curves for the three groups demonstrating similar growth for the control (saline treated) systemic KLH and local KLH treated groups

ceived 50 mcg KLH in 25 μl PBS intralesionally, whereas Group III was treated systemically with 50 mcg KLH in 25 μl PBS per foot pad. Controls received intralesional PBS. The effect of treatment upon growing subcutaneous tumors was judged by computing tumor volumes using three orthogonal diameters.

Results

Thirty-two of the 60 animals that received intravesical TCC cells were alive at the termination of the first experiment, 16 of 30 in the KLH treated group and 16 of 30 controls. Nine animals (5 in Group I and 4 in Group II) died several hours following the second administration of Nembutal. Laparotomies performed in all animals were negative. Thirty-seven mice were alive 21 days post implantation, 19 in Group I and 18 in Group II. Four of the six mice that died in Group I and seven of the eight mice in Group II were found to have bladder tumors on histological section. Another five animals died prior to termination of the experiment; 3 in Group I and 2 in Group II. All 5 animals had gross bladder tumors at laparotomy. The bladders from the surviving animals were evaluated for the presence of tumor by gross inspection, histologic section and determination of mean bladder weights for each group. No difference between KLH treated and control groups was found (Table 1).

Thirty-one of the 36 animals with subcutaneous tumors were alive at the completion of the second experiment; 11 of the 12 in Group I (control), 11 of 12 in Group II (KLH intralesional) and 9 of 12 animals in Group III (KLH peripheral). One animal in each of the three groups died within eleven days of implantation. The inadvertent injection of the tumor cells intraperitoneally was the apparent cause of death. An additional 2 animals in Group III carrying flank tumors died four days prior to termination of the experiment. Systemic and local treatment with KLH was ineffective in retarding the growth of the murine transitional cell carcinoma with tumor doubling times of 4.20, 3.36 and 3.70 for Groups I, II and III respectively (Fig. 1). Review of the histological sections in both experiments revealed the tumor to be a moderately well differentiated transitional cell carcinoma.

Discussion

Since one-third of the patients who initially present with low grade low stage TCC of the bladder eventually die of their disease, an effective method of preventing these patients from developing recurrent cancer after their initial resection needs to be identified.

To date, intravesical chemotherapy is the standard method of treatment for these patients who are determined to be at high risk of developing recurrent superficial disease. However, in many cases this form of therapy is ineffective, whereas in others the treatment appears at best to merely prolong the interval to cystectomy. Thus, the antigenicity of bladder cancer has stimulated the search for effective immunotherapeutic agents in the treatment of this disease. In animals, BCG immunization using cell wall skeletons [12] or living organisms [13] has significantly inhibited the growth of chemically induced and transplanted transitional cell carcinoma. Morales et al. [16] examined the effects of intravesical and intradermal BCG immunotherapy on superficial bladder cancer in 16 patients. They found a decreased rate of bladder tumor recurrence compared with the rate of recurrence prior to treatment. Subsequent reports [2, 14] seem to demonstrate clearly that this form of immunotherapy significantly reduces the recurrence rate of superficial bladder cancer.

Other immunogenic agents such as Poly I.C. are ineffective against bladder cancer [8] whereas the effectiveness of streptococcal (OK-432) [9] and passive immunotherapy with immune pig lymphocytes [7] needs to be further documented before this form of therapy can be recommended.

Keyhole Limpet Haemocyanin (KLH) is a large molecular weight protein extract of the hemolymph of an inedible mollusk. This potent antigen has been used extensively to evaluate the primary humoral and cellular immune response in humans [3–5].

In 1974, Olsson and associates [18] observed a statistically significant reduction of the recurrence rate of bladder cancer in patients immunized with KLH compared to controls. Only 1 tumor was found in 9 KLH treated patients whereas 7 of the 10 control patients had 18 separate bladder tumors during the observation period. These investigators were not treating actually existing tumors but rather were using KLH as an immunoprophylactic agent to prevent tumor recurrences. They suggested that, besides non-specific stimulation of the immune system and antigenic cross reac-

tivity, coupling of KLH to the bladder tumor antigens could explain the observed anti-tumor response. Thus, the ability of the host to respond immunologically to KLH could convey protection from further tumor recurrences. Klippel and associates [10] observed that the submucosal injection of KLH into the bladder of sensitized rats evoked a local inflammatory reaction with a predominant leucocytic infiltration. This resulted in a more predictable inflammatory response than did the use of BCG.

These studies together with the recent report from Lamm [11] in which he showed that intralesional KLH in pre-sensitized mice resulted in a significant reduction of tumor growth with prolongation of animal survival stimulated these present studies. We presumed from the above work that KLH would be effective and thus rather than re-evaluating it again with a subcutaneous tumor nodule, as Lamm had done, we decided to test KLH against an in situ bladder cancer model. The technique of tumor implantation obviously was satisfactory since, at sacrifice, 28 of 32 animals had bladder tumors. However, no effect had been demonstrated in terms of reducing tumor take or size in the KLH treated animals. It was our feeling that since KLH had failed intravesically to be effective that this might represent a local factor, such as the animals not retaining the KLH for a sufficiently long period. However, the possibility also existed that the KLH was ineffective when used against in situ transitional cell carcinoma. A further possibility was that against our strain of TCC, KLH might be ineffective regardless of the site of the tumor.

In order to at least partially answer these questions, the second experiment, in which the tumor was placed subcutaneously, was carried out. Again KLH proved ineffective in retarding tumor growth. In case the consignment of KLH was at fault, the subcutaneous experiment was repeated (results not shown) using a different lot of KLH. Again it was ineffective.

Whether the negative results obtained in these experiments rest with the tumor used we do not know. However, regardless of the reason, it does appear that KLH is not effective in preventing the subcutaneous or intravesical growth of all FANFT induced bladder tumors.

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