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Keyhole-Limpet Haemacyanin and Immune Ribonucleic Acid Immunotherapy of Murine Transitional Cell Carcinoma

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Summary. Transitional cell carcinoma is known to be an immunogenic tumour. This immunogenicity has been the basis of a search for effective immunotherapeutic agents and for the evaluation in this study of two additional agents, keyhole-limpet haemacyanin (KLH) and immune ribonucleic acid (RNA) extract. The results in this animal model showed KLH to be a potent non-specific stimulant of the immune response which caused both a reduction in tumour growth and a prolongation of animal survival ($p = 0.01$). No anti-tumour effects were observed with either local or systemic RNA.

Key words: Bladder cancer, Immunotherapy, Keyhole-limpet haemacyanin (KLH), Ribonucleic acid (RNA).

INTRODUCTION

Since the original demonstration by Bubenik (1) that transitional cell carcinoma is an immunogenic tumour, many authors have confirmed the presence of cellular and humoral immune responses in patients with bladder carcinoma (2). The immunogenicity of bladder cancer has prompted a search for effective immunotherapeutic agents in the treatment of bladder cancer. Various immunotherapeutic agents including the chemical immunostimulant levamisole (3, 4), the interferon inducer poly I:C (5), and the bacterial immunostimulants C. parvum (6) and Bacillus Calmette-Guerin (BCG) (7, 8, 9, 10, 11) have been evaluated in both animals and man. To date the most promising results with immunotherapy have been seen in response to the non-specific agent BCG. This study was designed to evaluate additional immunotherapeutic agents of potential value in patients with bladder cancer.

MATERIAL AND METHODS

Tumour Model

A poorly differentiated transitional cell carcinoma (MBT2) originally induced in C3H mice by chemical carcinogen (FANFT)[1] feeding was used. MBT2 is maintained by serial transplantation in syngenetic C3H mice (Jackson Laboratories, Bar Harbor, Maine). Subcutaneous tumour was excised, minced, and forced through a stainless steel screen. Cells were centrifuged and washed and the number of viable cells estimated by trypan blue exclusion. A suspension of 8×10^5 MBT2 cells in 0.05 ml of TC199 was injected intradermally into the right thigh. Animals were then examined three times a week and the resulting tumours were measured along two perpendicular axes by an observer who had no knowledge of the treatment given.

Immunotherapy Keyhole-Limpet Haemacyanin (KLH)

Three weeks prior to tumour transplantation 15 C3H mice received an injection of 50 mcg KLH (Pacific Biomarine) in 25 λ of phosphate buffered saline into each of four foot pads. On day 1 and day 7 following tumour transplantation these animals received a 50 λ intralesional injection of 50 mcg KLH.

Immune RNA

Twenty Hartley strain guinea pigs were sensitized to MBT2 by the administration of a one to

[1] Supplied by Dr. Mark Soloway, Memphis, Tennessee

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four slurry of MBT2 in TC199 plus an equal volume of Freund's complete adjuvant given intradermally into each pad and the nape of the neck. Immunisation was repeated one month later with MBT2 in incomplete Freund's adjuvant. Seven days after the second immunisation the animals were sacrificed; lymph nodes and spleens were removed and the immune RNA extracted as described by Paque and Dray (12). Briefly, frozen tissues were added to freshly distilled phenol saturated with 0.01M sodium acetate at pH 5.0, 0.1% 8-hydroxyquinoline, 0.5% sodium dodecylsulfate, and 4 mcg per ml polyvinyl sulphate. After homogenisation, an equal volume of 0.1M sodium acetate containing 0.5 mg/ml bentonite was added and the mixture was heated to 55°C. After cooling to 4°C the mixture was centrifuged at 15,000 RPM for 15 min and the aqueous phase separated. The extraction was repeated four times on the aqueous phase and the RNA precipitate was then centrifuged, dissolved in 0.3M sodium acetate, and reprecipitated in 95% ethanol at -20°C. Dextran sulphate (5,000 MW) was added to the RNA preparation and adjusted to a final concentration of 3 mg/ml to serve as RNAase inhibitor.

Thirty animals were given immune RNA immunotherapy: 15 had systemic immunotherapy and 15 local RNA immunotherapy. Systemic immunotherapy consisted of 0.25 ml injection into each of four foot pads using RNA solution containing 1 mg of RNA and 10 mg dextran sulphate per ml. Injections were given 3 days prior to tumour transplantation, on the day of tumour transplantation, and 2, 4, 6, and 8 days thereafter. Local RNA immunotherapy consisted of the intralesional injection of 2.5 mg immune RNA; 0.6 mg of tumour antigen (extracted from MBT2 cells using 3 M KCL); and 10^7 non-immune peritoneal exudate cells obtained from C3H mice after the intraperitoneal injection of 2 ml 3% thioglycolate broth. Intralesional RNA immunotherapy was repeated 7 days after tumour transplantation.

Saline Control

Fifteen animals severd as controls and were given an intralesional injection of 0.05 ml of normal saline intralesionally within the transplanted tumour 1 and 8 days after inoculation.

RESULTS

Figure 1 illustrates the tumour growth curves for the 4 groups. Tumour growth is virtually identical in the systemic RNA treated group, the local RNA treated group, and the control. A reduction in tumour growth is apparent in the KLH treated animals. This reduction in tumour growth is statistically significant at the $p < 0.01$ level (Dunnet's t-test). This reduced tumour growth in the KLH treated animals versus controls resulted in significant prolongation of animal survival. As illustrated in Fig. 2, KLH treated animals survived longer than saline treated controls. This prolongation of survival was significant at a p value of 0.01 at day 40 (Life tables, Mantel). Although a

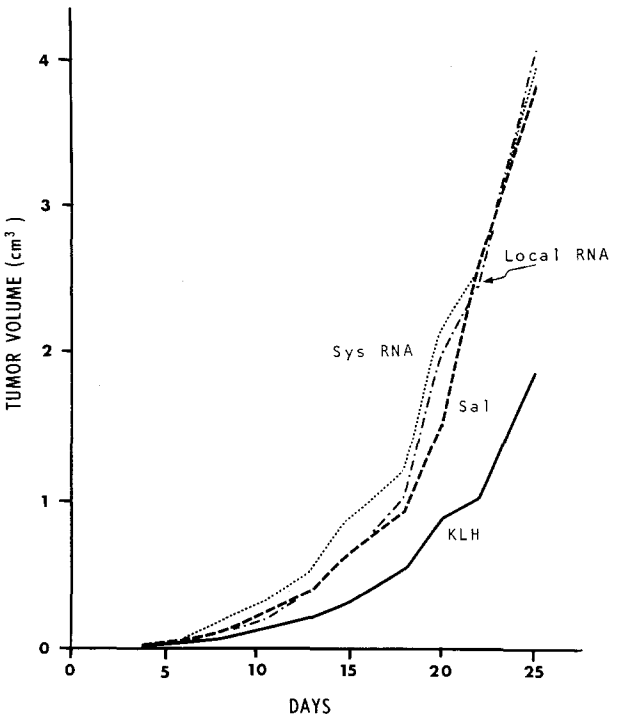

Fig. 1. Tumour growth curves for the four groups demonstrate remarkably similar growth in the control (saline treated), local RNA, and systemic RNA groups. Inhibition of tumour growth in the KLH treated animals is apparent and statistically significant ($p < 0.01$ Dunnet's t-test)

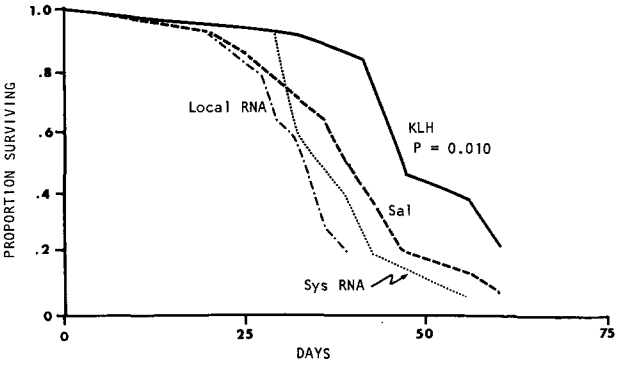

Fig. 2. The inhibition of tumour growth was associated with statistically significant ($p = 0.010$, Mantel, life tables) prolongation of survival in KLH treated animals

reduction of tumour growth and resulting in prolongation of survival was apparent with KLH immunotherapy in this model, immunotherapy was not observed to prevent the occurrence tumour or the subsequent eventual death of the animal.

DISCUSSION

Keyhole-limpet haemacyanin (KLH) is a large molecular weight protein antigen collected from the haemolymph of the keyhole-limpet. KLH has been used extensively to evaluate primary cellular and humoral immune responses in humans. In 1974 Olsson (13) and associates observed a statistically significant ($p < 0.005, \chi^2$) reduction of the recurrence rate of bladder cancer patients immunised with 5 mg of KLH compared with controls. Only one tumour was found in 9 KLH treated patients whereas 7 of the 10 control patients had 18 separate bladder tumours during the observation period. To our knowledge no further evaluation of the immunotherapeutic potential of KLH has been performed. The current study supports the hypothesis that KLH does in fact favourably influence the course of bladder cancer. The mechanism of action of this immunotherapeutic agent in unknown. KLH has been demonstrated to induce a primary cell-mediated delayed hypersensitivity response as well as a primary antibody response in humans (14). This potent antigen may therefore act as a non-specific stimulant of the immune response. Alternatively, antigenic cross-reactivity between KLH and bladder tumour antigens could explain the anti-tumour response evoked by KLH immunisation. Evidence for antigenic cross-reactivity with KLH does exist: several investigators have demonstrated the presence of humoral anti-KLH antibodies (15, 16) as well as specific binding of KLH to lymphocytes (17) in patients who have had no previous exposure to the keyhole-limpet.

Although no anti-tumour effect using local or systemic immune RNA was observed in this experiment, systemic RNA immunotherapy has been found to very significantly inhibit tumour growth and prolong survival in experimental animals (18, 19) and preliminary uncontrolled clinical trials have confirmed the safety, though not the efficacy, of immune RNA (19). Local immunotherapy similar to that performed in the present experiment has resulted in complete regression of transplanted tumours in guinea pigs (20). Although the potential for immune RNA immunotherapy is indeed exciting, the absence of measurable beneficial effect in this controlled experiment underlines the importance of defining the important variables such as immunisation and RNA extraction procedures, as well as the dose, route, timing, and effective technique of immune RNA administration in the experimental animal before embarking on clinical trials.

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