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Keyhole limpet hemocyanin immunotherapy of murine bladder cancer

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Summary. The current treatment of choice for superficial bladder cancer, bacillus Calmette-Guérin, has significant adverse side effects. We have compared two alternative immunotherapies – crude keyhole limpet hemocyanin (KLH) and Immucothel, a KLH modified for clinical use (Biosyn) – in an intralesional mouse model of bladder cancer (MBT2). Crude KLH required either immunization before tumor transplant or frequent intralesional therapy after transplantation to be effective. In addition, Immucothel required pre-immunization to be effective, and increasing the frequency and dosage of post-transplant immunization was not effective without pre-immunization. Preliminary investigations into the KLH-induced anti-tumor mechanism(s) suggest that natural killer cell activity may be involved. Both crude KLH and Immucothel appear to be effective immunotherapies of use in the treatment of transitional cell carcinoma.

Key words: Bladder neoplasm – Immunotherapy – Keyhole limpet hemocyanin

The current treatment of choice for carcinoma in situ and recurrent superficial bladder cancer is intravesical bacillus Calmette-Guérin (BCG) immunotherapy. Unlike conventional chemotherapy, BCG appears to reduce disease progression significantly and improve survival [9]. Local irritative bladder symptoms are a common, annoying side effect of BCG therapy. Systemic reactions such as sepsis or hypersensitivity are rare but can be fatal [2, 14]. The experience with BCG has stimulated efforts to develop less injurious immunotherapies.

Keyhole limpet hemocyanin (KLH), a highly antigenic oxygen-carrying protein of the sea mollusk *Megathuria crenulata,* was used by Curtis et al. to show that humoral and cellular immune responses to KLH were depressed in tumor-bearing patients compared with normal controls [3]. Using skin tests to measure immune responsiveness to KLH antigen before and after bladder tumor resection, Olsson surprisingly found a correlation of KLH immunization with a reduced tumor recurrence rate ($P < 0.005$) [16–18]. Two independent, European phase III trials have since shown that a proprietary form of KLH (Immucothel, IM) used intravesically is equal or superior to Epodyl and mitomycin C (MMC, $P < 0.05$) and is without significant side effects [7, 11]. Since KLH may be a promising alternative to BCG, we compared the anti-tumor effects of two types of KLH (IM and crudely purified KLH, C-KLH) against BCG in the intralesional MBT2 mouse bladder tumor model.

The MBT2 murine model of transitional cell carcinoma resembles the human disease histologically and has proven to be a remarkably accurate predictor of clinical response to both cytotoxic chemotherapy and immunotherapy [12, 21]. Lamm et al. found both a reduction in MBT2 tumor growth and prolongation of survival using 200 µg KLH s.c. 21 days before tumor transplantation, followed by two 50-µg intralesional treatments on days 1 and 7 after transplantation [13]. The MBT2 murine model was used here (1) to compare the anti-tumor responses elicited by intralesional C-KLH, IM, and BCG; (2) to determine the necessity for pre-transplant C-KLH and IM immunization; (3) to modify the post-transplant KLH immunization schedule and dose to increase efficacy; and (4) to evaluate the toxicity of KLH relative to BCG.

Materials and methods

Reagents

C-KLH (crystal, Calbiochem, La Jolla, Calif.), IM (lyophilized, Biosyn, Stuttgart, FRG), and BCG (lyophilized Tice, Organon-Teknika, Rockville, Md.) were resuspended in sterile, endotoxin-free phosphate-buffered saline (PBS, 0.1 ml/dose).

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Cell lines

All cell lines were maintained in RPMI-1640 (Whittaker, Walkersville, Md.) with 10% fetal calf serum (RPMI-FCS). The MBT2 mouse bladder tumor cell line was obtained from Dr. Timothy Ratliff (St. Louis, Mo.) in vivo, and was cultured from enzymically dissociated tumor. The YAC-1 mouse T lymphoma cell line was purschased from the American Type Culture Collection (Rockville, Md.). Cell lines were not routinely screened for mycoplasma.

Animals

C3H/HeN mice obtained from Harlan Sprague-Dawley (Indianapolis, Ind.) were used in anti-tumor efficacy experiments. Endotoxin-insensitive C3H/HeJ mice were obtained from Jackson Labs (Bar Harbor, Me.) for mechanism studies.

Antibody responses

C3H/HeJ mice (5 per group, three groups) received: (1) 1 intradermal footpad immunization (200 μg C-KLH), (2) 3-weekly s.c. (thigh) immunizations (50 μg C-KLH), or (3) 10^3 MBT2 cells s.c. (thigh) followed 24 h later by the first of three weekly intralesional immunizations (50 μg C-KLH each). Each mouse was bled weekly and plasma was frozen for later enzyme-linked immunoadsorbent assay (ELISA).

Anti-KLH

KLH was adsorbed to the plate (25 μg/ml) and subsequently washed prior to incubating with dilutions of mouse sera. After washing, the wells were incubated with horseradish-peroxidase-conjugated goat anti-mouse anti-immunoglobulin (1/500 dilution). Unreacted conjugate was washed off; bound conjugate developed by addition of appropriate enzyme substrate and absorbance was read at 415 nm [1].

Natural killer cell assay

Splenocytes from control and C-KLH-immunized HeJ mice were washed 3 times, resuspended in RPMI-FCS and plated at ratios of 200, 100, 50, 25, 12.5:1 against ^{51}Cr-labelled YAC-1 target cells. YAC-1 cells are the standard targets for measuring murine natural killer (NK) activity. Cromium-51 release was measured after 3 h of incubation (37°C, 5% CO_2) by harvesting (Skatron, Sterling, Va.) and counting (Packard 1550 Gamma Counter, Downers Grove, Ill.). Results (% kill) were calculated as follows:

$$\left[\frac{(\text{mean exp. group cpm}) - (\text{mean spontaneous release cpm})}{(\text{mean max. release cpm}) - (\text{mean spontaneous release cpm})}\right] \times 100$$

MBT2 murine model

The MBT2 mouse bladder tumor is a transplantable, poorly differentiated transitional cell carcinoma. Tumor transplants were accomplished by mechanical dispersion of tumor tissue in RPMI, followed by s.c. inoculation of a single-cell suspension of 10^3 cells into the thigh. Tumor incidence and dimensions (length, width) measured using calipers were recorded three times weekly. Tumor volume was calculated according to the formula for the volume of an ellipse: $V = 0.4\,LW^2$.

Statistics

The pooled *t*-test was used to compare tumor volumes at day 35. Kruskal-Wallis one-way analysis of variance was used to determine the significance of differences in mean tumor volume between groups (BMDP, Biomedical Data Processing Statistical Software, Los Angeles, Calif.). Fisher's exact test was used to compare tumor incidence (day 18) and survival (day 55) against the saline and BCG control values. NK cell data were analysed by the non-parametric, rank-sum Mann-Whitney method.

Results

In vivo comparison of C-KLH and IM anti-tumor immunotherapy (trials 1 and 2)

Trial 1 involved 60 C3H/HeN mice randomized into six groups (10 mice per group). Trial 2 involved 90 mice randomized into the same six treatment groups (15 per group). Subcutaneous KLH immunizations with assigned treatments were given 21 days before tumor transplant (day −21) and consisted of 200 μg C-KLH, 200 μg Immucothel (IM-200), 50 μg Immucothel (IM-50), and no Immucothel pre-treatment (IM-0). Post-transplant intralesional KLH therapy (50 μg/100 μl dose) and BCG (10^7 cfu/dose) were given on days 1, 7, and 13 or 14 after transplantation.

BCG and C-KLH were equivalent in their anti-tumor effect. Pre-immunization with IM (50 or 200 μg) was as good as BCG in reducing tumor incidence and increasing survival, but was less effective in reducing tumor growth (Table 1, trial 1). Failure to pre-immunize (IM-0) produced results indistinguishable from those in untreated (saline) control, in spite of weekly post-transplant immunizations. The results in trial 2 were similar, except that BCG was less effective, perhaps due to a slightly higher inoculum of tumor cells (Table 1).

Although BCG does not induce toxicity in this system, a large proportion of the animals develop granulomas at the site of BCG injection. No evidence of systemic toxicity was observed with KLH or BCG injection. While this model is not specifically designed to measure toxicity we have previously observed significant and even lethal toxicity due to frequent immunization with two other biological response modifiers (garlic extract and alpha-interferon, unpublished data). No visible toxicity or granuloma was observed with any KLH preparation.

Enhancement of post-transplant immunotherapy (trial 3)

Eightyfour mice were randomized into seven groups (12 per group) to receive post-transplant saline, BCG (10^7 cfu, weekly ×3), crude KLH (C-KLH, 50 μg biweekly ×3 weeks) or Immucothel (5, 50, 100, or 200 μg biweekly ×3 weeks).

The importance of pre-immunization in achieving an effective anti-tumor response in this model suggested an immune mechanism. Frequent post-transplant immunization (H) with C-KLH (50 μg) induced anti-tumor responses equivalent to weekly BCG and those achieved in

Table 1. In vivo trials comparing BCG, Calbiochem KLH (C-KLH) and Immucothel (IM) dose-response to pre-immuzination and three weekly post-transplant immunizations

Treatment	Day number										
	−21 dose	+1, +7 +14 dose	18 (Fisher's exact)			35 Pooled *t*-Test			55 (Fisher's exact)		
	SC	IL	Inc.	*P* (Saline)	*P* (BCG)	Vol. ± SE (mm^3)	*P* (Saline)	*P* (BCG)	Surv.	*P* (Saline)	*P* (BCG)
Trial 1[a]											
Saline	S	SW	80%	–	*	3362 ± 1887	–	*	30%	–	*
BCG	–	10^7 W	20%	*	–	71 ± 111	*	–	90%	*	–
C-KLH	200	50W	0	*	NS	233 ± 476	*	NS	100%	*	NS
IM-200	200	50W	30%	*	NS	1547 ± 480	*	*	80%	*	NS
IM-50	50	50W	0	*	NS	752 ± 194	*	*	90%	*	NS
IM-0	0	50W	60%	NS	*	4279 ± 1089	NS	*	30%	NS	*
Trial 2[a]											
Saline	S	SW	87%	–	NS	3881 ± 549	–	*	13%	–	NS
BCG	–	10^7W	80%	NS	–	1094 ± 177	*	–	80%	*	–
C-KLH	200	50W	27%	*	NS	770 ± 215	*	NS	80%	*	NS
IM-200	200	50W	40%	*	NS	751 ± 285	*	NS	80%	*	NS
IM-50	50	50W	47%	*	NS	1375 ± 363	*	NS	67%	*[b]	NS
IM-O	0	50W	80%	NS	*	3022 ± 419	NS	*	20%	*[b]	NS

[a] Trials 1 and 2: Subcutaneous immunizations were given 21 days before tumor transplant (day −21) and consisted of 200 μg C-KLH, 200 μg Immucothel (IM-200), 50 μg Immucothel (IM-50), and no Immucothel pre-treatment (IM-0). Post-transplant intralesional therapy with KLH (50 μg/100 μl dose) or BCG (10^7 cfu dose) was given on days 1, 7, and 13 after transplant (day 0). SC, Subcutaneous, day −21; *IL*, intralesional, post-transplant; *B*, Tice BCG, 1×10^7 colony forming units; *S*, saline; *W*, weekly; *Inc*, incidence of tumor; *Vol.*, tumor volume; *Surv.*, survival; *NS*, not significant

[b] Fishers exact comparing survival of IM-50 vs IM-0 in trial 2: $P = 0.0127$.

Chi square comparing survival of all groups at day 55: $P < 0.0001$. Pooled *t*-test of tumor volume at day 35. Kruskal-Wallis one-way analysis of variance test of tumor volume at day 35: $P = 0.0001$

* $P \leq 0.05$

Table 2. Enhancement of post-transplant immunotherapy

Treatment	Day number										
	−21 dose	+1 dose	21 (Fishers's exact)			35 (Pooled *t*-test)			54 (Fisher's exact)		
	SC	IL	Inc.	*P* (Saline)	*P* (BCG)	Vol. ± SE (mm^3)	*P* (Saline)	*P* (BCG)	Surv.	*P* (Saline)	*P* (BCG)
Trial 3[a]											
Saline	ND	SH	12/12 (100%)	–	*	8245 ± 448	–	*	0/12 (0%)	–	*
BCG	ND	BW	6/12 (50%)	*	–	2332 ± 773	*	–	6/12 (50%)	*	–
C-KLH	0	50H	3/12 (25%)	*	NS	1283 ± 604	*	NS	8/12 (67%)	*	NS
IM-200	0	200H	10/12 (83%)	NS	NS	5367 ± 573	*	*	1/12 (8%)	NS	*
IM-100	0	100H	11/12 (92%)	NS	*	4724 ± 897	*	*	3/12 (25%)	NS	NS
IM-50	0	50H	9/12 (75%)	NS	NS	5298 ± 521	*	*	1/12 (8%)	NS	*
IM-5	0	5H	12/12 (100%)	NS	*	4300 ± 951	*	NS	2/12 (17%)	NS	NS

[a] Trial 3: No pre-immunzations were given. C-KLH and IM were given intralesionally (200 μg, or 5 μg/dose) on days 1, 3, 8, 10, 15 and 17 post-transplant. BCG was given as in trials 1 and 2. *SC*, Subcutaneous; *IL*, intralesional; *S*, Saline; *H*, frequent immunization schedule; *W*, weekly; *Inc.*, incidence of tumor; *Vol.*, tumor volume; *Surv.*, survival; *NS*, not significant

* $P \leq 0.05$

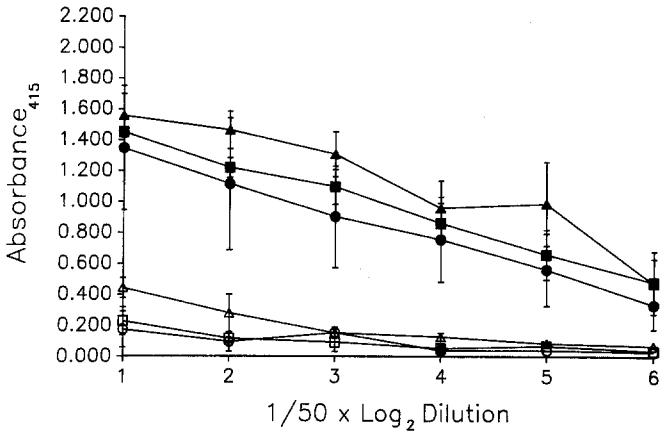

Abb. 1. Induction of anti-KLH antibody responses in MBT2-bearing mice (5 per group). These mice received 10^3 MBT2 cells inoculated subcutaneously in the thigh, followed by three weekly intralesional immunizations with 50 µg C-KLH □—□ HeN wk 1; ■—■ HeN wk 3; △—△ HeJ wk 1; ▲—▲ HeJ wk 3; 50 µg ○—○ HeJ wk 1 with MBT2; ●—● HeJ wk 3 with MBT2

Table 3. Effect of C-KLH immunization on natural killer cell activity (% kill for 100:1 effector to target ratio)

Animal	Control	Group 1 (200 µg)	Group 2 (50 µg×3)
A	7.1%	9.2%	17.4%
B	5.1%	6.5%	16.2%
C	16.1%	9.6%	44.1%
D	13.0%	6.3%	19.0%
E	11.1%	8.2%	16.8%
F		1.0%	
G	2.8%	5.8%	
H	3.2%		
I	4.1%		
J	2.0%		

Mann-Whitney non-parametric test. Group 1, $P=0.87$; group 2, $P=0.003$

trials 1 and 2 by C-KLH pre-immunization followed by weekly C-KLH. Frequent immunization consisted of two immunizations per week, which provided a cumulative post-transplant dose of 300 µg. Frequent immunization with Immucothel was ineffective, regardless of dosage escalation (Table 2).

Antibody responses (Fig. 1)

The quantity and class of antibody produced were schedule- and dose-related. Two hundred micrograms of C-KLH induced high levels of anti-KLH IgG within 1 week in normal mice, while 50 µg in normal and tumor-bearing mice initially produced low levels of IgM and IgG but achieved similar and high levels of IgG after three weekly (50W) immunizations. The weekly 50 µg post-transplant dose in tumor-bearing mice was able to induce humoral anti-KLH responses of equivalent level and class to those in normal mice, yet did not reduce the rate of tumor growth or increase survival.

Direct antiproliferative, cytotoxic effects, and NK activity (Table 3)

Neither C-KLH nor IM was directly cytotoxic to MBT2 or 3T3 fibroblasts as measured by viability assays. C-KLH (50 µg/ml) has a slightly anti-proliferative effect only on MBT2 and only after 96 h (data not shown). Murine anti-KLH (from either immunization schedule) with complement failed to lyse MBT2 in culture (data not shown). NK cell activity in splenocytes from normal C-KLH immunized mice was elevated only in the group receiving the three weekly 50 µg immunizations and not in the group receiving one 200 µg immunization (Table 3). NK activity in tumor-bearing mice immunized with C-KLH was not tested.

Discussion

The retrospective correlation [17] of subcutaneous KLH immunizations with a reduced rate of bladder tumor recurrence was made in patients participating in an immune responsiveness study [16]. In a subsequent study of patients with previously resected tumors, only 1 of 9 KLH-immunized patients had a recurrence of disease over a combined observation period of 204 months (0.49/100 patient-months), while 7 of 10 non-immunized patients had disease recurrence over 228 months (7.9/100 patient-months, $P<0.005$) [18]. European clinical trials with intravesically administered IM have shown it to be as effective as or superior to mitomycin C [5, 20], yet without significant side effects. Twenty-three patients randomized to mitomycin C experienced a higher disease recurrence rate (9.38/100 patient-months, 39% recurring) than did 21 patients randomized to IM (3.26 recurrences/100 patient-months, 14% recurring, $P<0.05$) [11].

Trials 1 and 2 showed that IM required pre-immunization for induction of anti-tumor responses. Increasing the dose and frequency of IM did not compensate for lack of pre-immunization (trial 3). C-KLH, on the other hand, was able to induce a successful anti-tumor response without pre-immunization when the post-transplant frequency of administration and/or the cumulative dose was increased. C-KLH may therefore contain additional components which potentiate the anti-tumor effect.

The mechanism of tumor modulation by C-KLH or IM has not been determined. No antigenic cross-reactivity between KLH and MBT2 was indicated, since anti-KLH with complement failed to lyse MBT2 in culture. Humoral immunity (IgG) to C-KLH was probably not directly involved, since it occurred without a successful anti-tumor response. These experiments suggest that KLH does not act via antigenic mimicry or direct humoral cytotoxicity, and points to two mechanisms – induced immune response and NK cell activity – which may be responsible for the anti-tumor effect. Clinical data supports involvement of T and NK cells. Jurincic described bladder infiltrates

with increased ratios of T helper cells to suppressor/cytotoxic cells (T4:T8) in 7 of 10 patients treated with intravesical KLH after just 2 weeks of therapy [11]. Our experiments support the enhancement of NK activity by KLH as a potential mechanism of anti-tumor response, since three weekly 50 μg C-KLH immunizations increased NK activity. One 200 μg C-KLH immunization failed to elevate NK activity. This may have been due to measurement of NK activity 3 weeks after immunization, while the 50 μg group was measured 1 week after the third weekly immunization. Sampling time relative to immune stimulation may therefore account for the low NK activity in the 200 μg C-KLH group. Frequent exposure to KLH may have contributd to the anti-tumor effects observed in the C-KLH group (Table 2) receiving two 50 μg immunizations per week. No NK measurements were conducted using Immucothel, and thus we cannot say whether the lack of response to frequent immunization (Table 2) is due to an inability to induce NK activity. Depressed NK activity in bladder cancer patients (peripheral blood) was normalized by co-incubation with Immucothel for 2 h, 15 h, or 5 days in vitro [15]. This cytotoxicity was increased in a time-dependent (not dose-dependent) manner. NK cells have not been unequivocally shown to make a major contribution to the BCG mechanism, and the increasing NK activity induced by KLH may be coincident rather than causal. Increasing NK activity correlating with anti-tumor effect has been observed in both the MBT2 model [19] and in clinical trials Lamm, (unpublished data). However, the elimination of NK cells by anti-asialo GM-1 antibody did not abrogate the anti-tumor activity of BCG.

We observed no adverse reaction with any KLH preparation at any dosage or frequency. A reproducible anti-tumor response comparable to that elicited by BCG was observed. Our data suggest that both crude KLH and immucothel may prove to be safe and effective additions to our growing armamentarium of immunotherapeutic agents in the treatment and prophylaxis of superficial bladder cancer.

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