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PROTECTIVE EFFECT OF *RAPANA VENOSA* HEMOCYANIN (RvH) ON SURVIVABILITY OF HAMSTERS WITH TRANSPLANTED MYELOID GRAFFI TUMOURS

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Abstract

The application of *Rapana venosa* hemocyanin (RvH) on the survivability of hamsters with transplanted myeloid Graffi tumours was studied. It was found that the preparation induced elongation of mean survival time, inhibition of tumour growth and decrease of mortality. The effect was similar to that of a commercial preparation of *keyhole limpet* hemocyanin (KLH) which is used as a component in dendritic cells-based anti-tumour vaccines in human immunotherapy.

Key words: tumours, immunotherapy, hemocyanins, dendritic cells

Introduction. Hemocyanins are Cu-containing respiratory proteins with large molecular mass of marine molluscs. *Keyhole limpet* hemocyanin (KLH) is a commercial preparation with an increasing significance in immunotherapy of cancer. It is used in dendritic antitumour vaccines as a component accelerating the maturation of dendritic cells (DCs) resulting in stimulation of Th1 immune response and T-cytotoxic activity as shown by in vitro experiments [1]. Clinical trials demonstrated the protective effect of intradermal application of KLH near the dendritic cell vaccine for therapy of urological cancers [2]. Griffoen et al. [3] reported that some melanoma patients vaccinated with DCs, pulsed with tumour lysates and KLH, showed specific CD8 T-cell response to melanosomal peptides as well as to If-gamma-releasing (γ- If-) KLH-specific T-cells. Curti et al. [4] found that peripheral blood CD14+ monocytes from multiple myeloma patients can be induced to differentiate into CD83+ dendritic cells which are highly efficient in secretion of Il-12 and have a potential stimulating activity on T-cells. Sorg et al. [5] collected leukocytes by leukaferesis from patients with urological cancers. The cells were subsetted and cultivated in the presence of interleukines (GM-CSF, Il-4, Il-1-β, Il-6, TNF-α and PGE). The cells showed a mature DC morphology and phenotype.

On the other hand, KLH is used as a carrier of antigen molecules in synthetic vaccines against some tumours. Lambert et al. [6] found that immunization with tumour-associated protein TCR, conjugated to the immunogenic protein KLH, protects mice from tumour challenge with murine T-cell lymphoma C6VL. Rasupathi et al. [7] reported that the vaccine KLH-conjugated ganglioside CD2 plus adjuvant QS-21 induce

antibodies against cell surface tumour antigens and induce complement-dependent cytotoxicity in the majority of patients with melanoma. HERSEY et al. [8] found that autologous lysates may be more effective than melanoma peptides as antigens for DC vaccines supplemented by KLH. TOLLER et al. [9] developed a polyclonal rabbit antiserum using KLH for peptide conjugation for detection of isoforms of receptors for human growth releasing factors in tumours.

Having in mind the stimulating effect of hemocyanins on the maturation of dendritic cells, Th-1 helper and T-cytotoxic lymphocyte populations, in the present work we aimed to investigate the effect of hemocyanin isolated from *Rapana venosa* on the survivability and tumour growth of hamsters with transplanted myeloid Graffi tumours.

Materials and methods. EXPERIMENTAL ANIMALS. Ninety Golden Syrian hamsters weighing 80–100 g. two months of age of both sexes were used for the experiments. The animals were obtained from the animal house of the Institute of Oncology, Sofia, Bulgaria. They were grown up and bred under standard conditions accepted from the National Veterinary Health Service, Bulgaria, in the animal house of the Institute of Microbiology, Bulgarian Academy of Sciences.

The animals were separated in nine groups as follows and treated by different way:

1. Hamsters injected by *Rapana venosa* hemocyanin (RvH) (0.25mg i. t.).
2. Hamsters injected with RvH (0.5 mg i. t).
3. Hamsters injected with RvH (0.25 mg s. c.).
4. Hamsters injected with RvH (0.5 mg s. c.).
5. Hamsters injected with KLH (0.25 mg i. t.).
6. Hamsters injected with KLH (0.5 mg i. t.).
7. Hamsters injected with KLH (0.25 mg s. c.).
8. Hamsters injected with KLH (0.5 mg s. c.).
9. Hamsters with tumours without treatment – control (TBH).

TUMOUR AND TRANSPLANTATION. The myeloid Graffi tumours were induced by Graffi virus in mice and adapted for hamsters by YAKIMOV et al. [10]. The tumours were maintained in vivo in the Institute of Experimental Pathology and Parasitology, Sofia, Bulgaria, by subcutaneous inoculation of 10^6 viable tumour cells.

In the present experiments, 100% transplantability of tumours was achieved by s. c. inoculation of 2×10^4 viable tumour cells into the interscapular field of the animals. By this quantity of cells, 100% obligatory transplantability and mortality of animals in our previous studies was established [11].

HEMOCYANIN FROM *Rapana venosa* (RvH). Marine snails, *Rapana venosa* grosse, were caught near the Bulgarian coast of the Black Sea. Hemolymph was collected from specimens of 20–35 g. Hemocyanin was isolated by preparative ultracentrifugation using UZ rotor Ti45 with Beckman L-80 Ultracentrifuge at speed 24000 rpm, for 4 h at 4 °C [12].

Keyhole limpet hemocyanin (KLH) was used as control. KLH was obtained from Biosyn (Fellbach, Germany) with a protein concentration of 6.9 mg/ml in Tris/HCl buffer, pH 7.4. The sample was dialysed for 24 h against 0.1 M Tris/HCl buffer, pH 6.5, containing 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$.

IMMUNOMODULATION. The protective effect of RvH application on the survivability of hamsters with transplanted myeloid tumours was examined. Experimental groups of 10 hamsters were injected by 0.5 mg or 0.25 mg of RvH or of KLH, subcutaneously (s. c.) or intratumourally (i. t.) at day 14 after tumour transplantation. Experimental groups see above.

BIOMETRIC EXAMINATIONS ON TUMOUR DEVELOPMENT. The parameters of mean survival time (MST), mortality %, as well as the inhibition of tumour growth (ITG)(%) were followed. MST was calculated as a mean arithmetical value of survival (days) of

all animals in each experimental group. % mortality was determined at day 40 after tumour transplantation. The inhibition of tumour growth was calculated as follows:

$$\text{ITG} = \frac{\text{Tumour size (mm) of control} - \text{Tumour size (mm) of experimental}}{\text{Tumour size (mm) of control animals}} \times 100$$

Results. It was established that the application of RvH and KLH in hamsters with transplanted myeloid Graffi tumours resulted in elongation of MST for 2–6 days compared to the control (untreated animals). MST of the untreated tumour-bearing hamsters (TBH) was 30.0±1.5 days. The most pronounced effect was obtained by s. c. application of 0.5 mg for both preparations. The way of application of KLH of both preparations did not change the survivability of animals (Fig. 1).

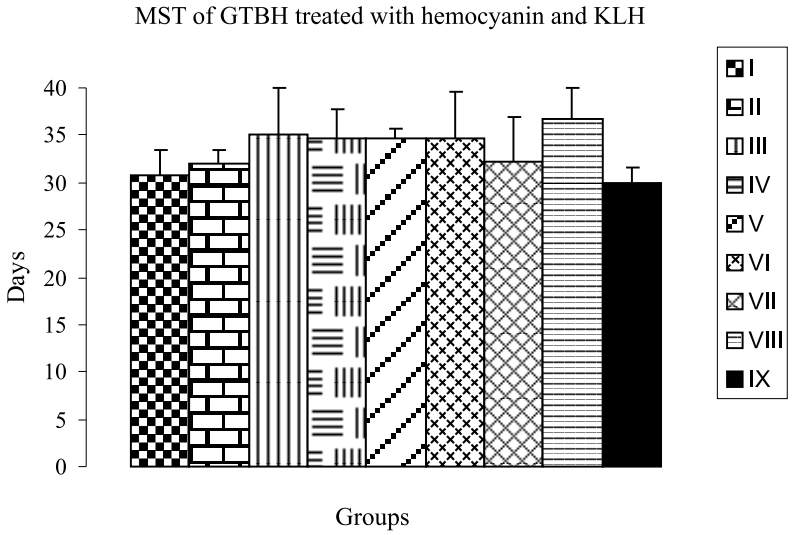

Fig. 1. Mean survival time (MST) of hamsters with transplanted myeloid Graffi tumours, treated by *Rapana venosa* hemocyanine (RvH) or *keyhole limpet* hemocyanin (KLH) (Sigma). Experimental groups: Group *1* – hamsters injected i. t. with 0.25 mg RvH; group *2* – hamsters injected i. t. with 0.5 mg RvH; group *3* – hamsters injected s. c. with 0.25 mg RvH; group *4* – hamsters injected s. c. with 0.5 mg RvH; group *5* – hamsters injected i. t. with 0.25 mg KLH; group *6* – hamsters injected i. t. with 0.5 mg KLH; group *7* – hamsters injected s. c. with 0.25 mg KLH; group *8* – hamsters injected s. c. with 0.5 mg KLH; group *9* – tumour-bearing hamsters without treatment

Using two concentrations of RvH (0.25 and 0.50 mg) the mortality was decreased on the 35th day about 72% while only the higher concentration of KLH had effect on the mortality. The percentage of mortality was significantly decreased in the group of animals treated with 0.25 mg RvH (75% mortality determined at day 40, control untreated being 100% mortality on the same day of observation) (Fig. 2).

It was found that both preparations inhibited the tumour growth of TBH till the 31st day of the observation. Treatment of TBH with different concentration of RvH and KLH intratumourally almost had no effect and the inhibition of tumour growth was very little. They were more effective when applied s. c. at a dose of 0.25 mg for RvH

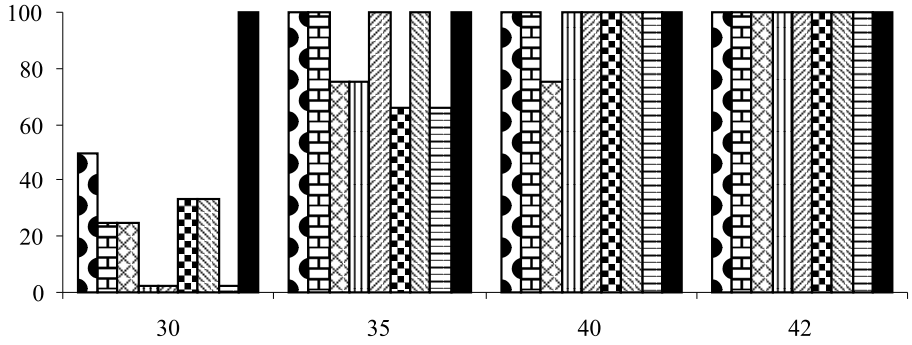

Fig. 2. Mortality % at day 40 of investigation of hamsters with transplanted myeloid tumours treated with RvH or KLH. Experimental groups as in Fig. 1

and KLH as well. The inhibition of tumour growth was the best expressed in group 3 of TBH (injected with 0.25 mg RvH s. c.) (ITG – 66.6%, 67.13%, 59.93%, 34.50% and 41.30% determined at days 17, 12, 24, 28 and 31 respectively) and in group 7 of TBH (injected with 0.25 mg KLH s. c.) (ITG – 66.6%, 70.67%, 55.12%, 31.25% and 33.3% established on the same days of investigation) (Fig. 3).

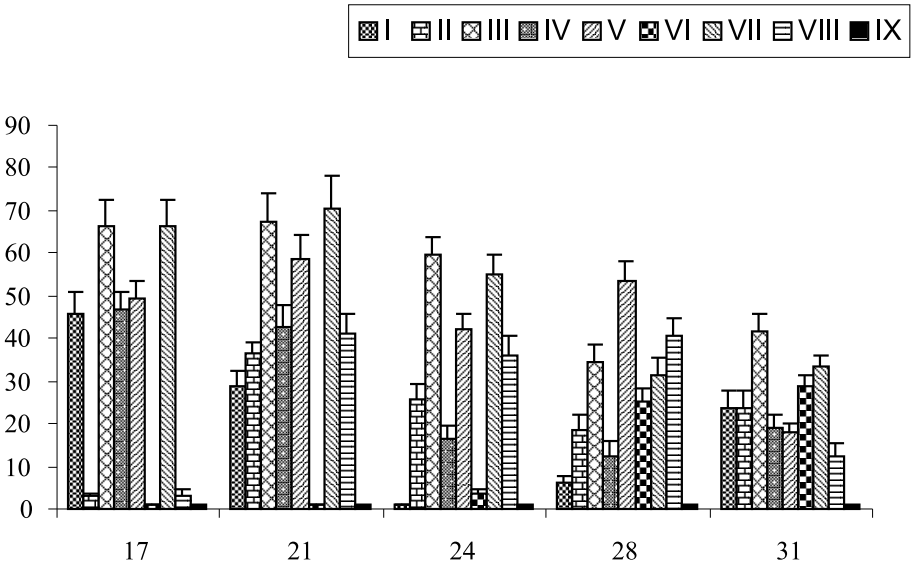

Fig. 3. Inhibition of tumour growth (%) in hamsters with transplanted myeloid tumours treated with RvH or KLH. Experimental groups: as in Fig. 1

Discussion. Analysis of the results of investigation showed that both preparations RvH and KLH produced similar protective effect on the survivability of hamsters with transplanted myeloid Graffi tumour and at the 31st days of transplantation RvH showed about 8% higher inhibition effect on tumour (Fig. 1). The s. c. way of application of both preparations produced more pronounced inhibition of the tumour growth (Fig. 3).

The protective effect of new isolated RvH on survivability of TBH could be explained by stimulation the antitumour immune response via the mechanism of action of hemocyanins. Probably both preparations induce acceleration of maturation

of dendritic cells, stimulation of Th-1 response and T-cytotoxic anti-tumour immune response, as previously shown [1]. Comparison of the protective action of both preparations on survivability, tumour growth and mortality of hamsters with transplanted myeloid tumours showed similar effects. These data will be the basis for our next investigation concerning the effect of RvH on the immune status of TBH.

Many authors have reported the immunoprotective effect of hemocyanins on the development of experimental tumours and on the survival of cancer patients in clinical trials. The investigation performed by McFadden et al. [13] shows that KLH directly inhibits the growth of human Barett's oesophageal carcinoma and has antiproliferative effects against breast, prostate and pancreas cancers. Riggs et al. [14] established a significant in vitro growth inhibition effect of KLH on breast, pancreas and prostate cancer cell lines. It was found that intralesional application of KLH in the mouse bladder tumour model significantly reduced tumour incidence, growth rate and mortality [15]. Krug [16] reported about a vaccine therapy of small cell lung cancer with a polyvalent KLH-conjugate vaccine containing tumour antigens GM-2, Globo H, fucosyl GM-1 and polysialic acid. Also positive results were achieved by dendritic cell vaccination of patients with medullary thyroid carcinoma [17]. Mature DCs were selected from peripheral blood monocytes by CD14 magnetic bead selection and cultured in the presence of GM-CSF, IL-4 and TNF-α. DCs were loaded with tumour lysates and further injected in groin lymph node. Positive immunoanalogue response was announced by delayed type hypersensitivity [16]. Hold et al. [18] and Arrayo et al. [19] showed the preferences of dendritic cell – KLH-based immunotherapy for patients with advanced renal carcinoma.

Our experimental data give basis for extending the spectrum of hemocyanins which could be useful for human antitumour immunotherapy.

REFERENCES

[1] Schnurr M., P. Calambus, F. Then, M. Dauer, S. Enders, A. Eigler. Cancer Res., **61**, 2001, 6445–6450. [2] Pandha H. S., R. L. John, J. Hutchinson, N. James, M. Whelan, C. Corbishley, A. G. Dalfiesh. BJU Int., **94**, 2004, 412–418. [3] Griffoen M., M. Borghi, P. I. Schreier, S. Osanto, D. Schadendorf. Cancer Immunol. Immunother., **53**, 2004, 715–722. [4] Curti A., A. Isidori, E. Ferri, C. Terragna, P. Neyroz, C. Cellini, M. Ratta, M. Baccarini, R. M. Lemoni. Leuk. Lymphoma, **45**, 2004, 1419–1428. [5] Sorg R. V., Z. Ozcan, T. Brefort, J. Fischer, R. Ackerman, M.Muller, P. Wernet. J. Immunother., **26**, 2003, 374–383. [6] Lambert S. L., C. Y. Okada., R. Levy. J. Immunol., **172**, 2004, 929–936. [7] Rasupathi G., P. O. Livingston, C. Hood, J. Gathurn, S. E. Krown, P. B. Shapman, J. D. Wolchok, L. J. Williams, R. C. Oldfield, W. G. Hwu. Clin. Cancer Res., **9**, 2003, 5214–5220. [8] Hersey P., S. W. Menzies, G. M. Halliday, T. Nguen, M. L. Farelly, C. De Silva, M. Lett. Cancer Immunol. Immunother., **53**, 2004, 125–134. [9] Toller G. L., J. E. Horvath, A. V. Schally, G. Halmos, J. L. Varda, K. Groot, D. Chism, M. Zarandi. Proc. Natl. Acad. Sci., USA, **101**, 2004, 15160–65. [10] Yakimov M., Z. Mladenov, A. Konstantinov, I. Yanchev. Gen. Comp. Pathol., **6**, 1979, 24–35. [11] Toshkova R. Immunity and experiments for immunomodulation in hamsters with experimental tumour preliminary induced by Graffi virus. PhD Thesis, Sofia, Bulgaria, 1995 [12] Dolashka-Angelova, P., M. Schick, S. Stoeva. Int. J. Biochem. and Cell Biology, **32**, 2000, 529–538. [13] McFaden D. W., D. R. Riggs, B. J. Jackson, L. Vona-Davied. Am. J. Surg., **186**, 2003, 552–555. [14] Riggs D. R., B.Jackson, L. Vona-Davis, D. McFadden. J. Surg. Res., **108**, 2002, 279–284. [15] Lamm D. L., J. I. De Haven, D. R. Riggs, R. F. Ebbert. J. Urol., **149**, 1993, 648–652. [16] Krug L. M. Vaccine therapy for small cell lung cancer. Semin. Oncol., **31 (suppl. 1)**, 2004, 112–116. [17] Stight A., M. Sachet, R. Yagubian, C.Bitterman, P. Dubsky,

C. Brostjan, R. Pfranger, B. Niederle, R. Jakesz, M. Gnant, J. Friedl. Clin. Cancer Res., **10**, 2004, 2944–2953. [18] Hold L., C. Reiser, C. Papesh, R. Ramoner, M. Herold, H. Klocker, C. Radmayer, A. Stenzl, G. Bartsch, M. Thurner. J. Urol., **161**, 1999, 777–782. [19] Arroyo J. C., F. Gabilondo, L. Lorente, M. A. Meraz-Rioz, C. Sanchez-Torez. J. Clin. Immunol., 24, 2004, 86–96.

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