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Effect of *Rapana venosa* Hemocyanin on Antibody-Dependent Cell Cytotoxicicity (ADCC) and Mitogen Responsibility of Lymphocytes from Hamsters with Progressing Myeloid Tumors

[1]*Reneta Toshkova,* [2]*Emilia Ivanova,* [3]*Rumyana Hristova,*
[4]*Wolfgang Voelter and* [3]*Pavlina Dolashka-Angelova*

[1]Institute of Experimental Pathology and Parasitology,
Bulgarian Academy of Sciences, G. Bonchev, Sofia 1113, Bulgaria
[2]Institute of Microbiology, Bulgarian Academy of Sciences, G. Bonchev 26, Sofia 1113, Bulgaria
[3]Institute of Organic Chemistry, Bulgarian Academy of Sciences, G. Bonchev 9, Sofia 1113, Bulgaria
[4]Abteilung für Physikalische Biochemie des Physiologisch-Chemischen Instituts der Universität Tübingen,
Hoppe-Seyler-Straße 4, 72076 Tübingen, Germany

Abstract: In the present work the effect of application of RvH on ADCC as well as on mitogen responsibility of spleen lymphocytes in hamsters with progressing myeloid Graffi tumors are followed during 25 days after tumor transplantation. The *in vitro* ADCC tests were performed in presence of serum from Tumor-bearing Hamsters (TBH), rabbit anti-tumor Graffi serum or of normal hamster serum. RvH was injected s. c. twice in a dose of 0.5 mg per animal, determined previously as the optimal protective dose and Keyhole Limpet Hemocyanin (KLH) was used as a control. It was found that spleen lymphocyte ADCC decreased during tumor progression, while ADCC of spleen lymphocytes against own tumor cells increased about twofold in comparison to that of lymphocytes from untreated TBH after treatment of animals with KLH or RvH. The lymphocytes isolated from normal animals without treatment showed two times lower cytotoxic activity compared to those from RvH-or KLH-treated controls. RvH induced 3-5% higher ADCC compared to KLH in all combinations of sera and lymphocytes. Both, RvH and KLH showed stimulating effects on spleen lymphocytes from TBH to the B-cell mitogen LPS. Three mechanisms for the stimulation effect of RvH on lymphocytes in TBH can be hypothesized: i/hemocyanins stimulate the maturatiion of dendritic cells, the proliferation of Th1 and specific T-cytotoxic lymphocyte populations; ii/direct action of RvH on tumor cells by an apoptotic way; iii/indirect action of RvH by stimulation of cytokines.

Key words: Antibody-dependent cell cytotoxicity · mitogen responsibility · hemocyanins · tumors · lymphocytes

INTRODUCTION

Biomedical interest in the high molecular mass hemocyanin of the marine mollusc keyhole limpet *Megathura crenulata* (KLH) goes back to more than 30 years, when this copper-containing extracellular respiratory protein was first found to posses remarkable immunostimulatory properties [1, 2].

Many clinical trials showed the inhibitory effect of KLH against human cancers. Several mechanisms of antitumor action of hemocyanins are proposed. During the last years, KLH is used for the preparation of dendritic antitumor vaccines for cancer patients. These kinds of vaccines are based on *in vitro* cultivated dendritic cells in presence of KLH and interleukines, as well as tumor cell lysates from cancer patients [3-8]. Schnurr *et al*. [9] were the first who showed that T-cells, specific for pancreatic carcinoma cells, could be generated *in vitro* by lysate pulsed dendritic cells (DCs) in presence of KLH.

Other authors showed that KLH directly inhibits the growth of human Barrett's esophagus cancer [10], breast and pancreatic cancer [11] and renal carcinoma [12] by apoptotic and non-apoptotic mechanisms. Non-apoptotic mechanisms include cytokine production [11] as well as induction of proteins typical for oxidative stress and attenuation of metabolic processes in tumor cells [13].

Corresponding Author: Dr. Pavlina Dolashka-Angelova, Acad. G. Bonchev Street, bl. 9, 1113, Sofia, Bulgaria

The aim of the present work is to study the effect of application of hemocyanin, isolated from *Rapana venosa* (RvH), on the antibody-dependent cell cytotoxicity (ADCC) and mitogen responsibility of spleen lymphocytes from hamsters with transplanted myeloid Graffi tumors.

MATERIALS AND METHODS

Experimental animals: "Golden Syrian" hamsters from both sexes, weighing 80-100 g, 2 months in age, were used for the experiments. The animals were bred and grown at conditions accepted by the Bulgarian Veterinary Health Service in the Animal house of the Institute of Microbiology, Bulgarian Academy of Sciences.

Tumor and transplantation: Mice Graffi virus was adapted for hamsters by Yakimov *et al*. [14]. The tumor was maintained in hamsters by s. c. inoculation of 1-2.10^6 viable tumor cells. For the experiments, the myeloid Graffi tumor was transplanted in hamster by s.c. injection of 2x10^4 viable trypan blue excluded tumor cells in the field of interscapular space. Our previous studies showed that such quantity of tumor cells induced 100% transplantability and 100% mortality in hamsters.

Purification of rapana venosa hemocyanin (RvH): Marine snails, *Rapana venosa* grosse, were caught near to the Bulgarian coast of the Black Sea. Hemolymph was collected from specimens of 20±35 g. Hemocyanin was isolated by preparative ultracentrifugation, using an UZ rotor Ti 45 of a Beckman L-80 ultracentrifuge and a speed of 24 000 RPM for 4 hours at 4°C [15]. KLH, used as a control, was a gift from Biosyn company, Fellbach, Germany.

Treatment with hemocyanins: For the study, 60 animals were separated in 6 experimental groups.

1st group: Hamsters were treated s.c. two times with RvH (0.5 mg per animal)-7 days before (-7day) and on the day of tumor transplantation (day 0)-and inoculated s.c. with 2.10^4 viable tumor cells in the interscapular area on day 0.

2nd group: Hamsters were treated s.c. two times with KLH (0.5 mg per animal)-7 days before (-7day) and on the day of tumor transplantation (day 0)-and inoculated s.c. with 2.10^4 viable tumor cells in the interscapular area on day 0.

3rd group: Hamsters were inoculated only with 2.10^4 viable tumor cells on day 0.

4th group: Healthy hamsters were treated twice with RvH (0.5 mg per animal) s. c. in the interscapular field on day-7 and day 0.

5th group: Healthy hamsters were treated twice with KLH (0.5 mg per animal) s. c. in the interscapular field on day-7 and day 0.

6th group: Healthy hamsters were observed without any treatment.

Specific immune sera against myeloid graffi tumor cells: Rabbit immune anti-serum against myeloid Graffi tumor-associated antigens, used for the ADCC test, was obtained according to a method described previously [16]. Briefly, rabbits were injected 6 times with myeloid Graffi tumor cells according to the scheme:

Day 1-s.c. injection by 3x10^7 tumor cells/0.5 ml PBS mixed with 0,5 ml Complete Freund's adjuvant (Difco Lab.)(CFA);

Day 9-i.m. injection by 5x10^7 tumor cells/1 ml PBS;

Day 16-i.m. injection by 5x10^7 tumor cells/1 ml PBS.

Thirty days later two i.v. booster injections of 5x10^7 tumor cells/1 ml PBS were given at weekly intervals.

The antiserum was collected 1 week after the last injection.

Absorptions of the serum were consequently performed with suspensions of hamster embryonic cells, hamster liver, kidney, spleen, bone marrow, lung and hamster serum and plasma, as well as sheep erythrocytes. Specificity of the absorbed antiserum against tumor-associated antigens was only proven by the immune-precipitation test according to Ouchterlony, immunefluorescence microscopy and immuneelectron microscopy [16]. The absorbed antiserum was used in a dilution of 1:10 in PBS.

Tumor-specific antisera obtained from tumor-bearing hamsters at day 25 after tumor transplantation were comparatively used in the ADCC tests with spleen lymphocytes. Normal serum from healthy hamsters was used as control in the ADCC test.

Spleen lymphocytes: A single cell lymphocyte suspension from aseptically-removed and mechanically-desintegrated spleens was prepared. The spleen cells were diluted in 3 ml Phosphate Buffered Saline (PBS) in a

ratio of 1:2 and were layered on 3ml Ficoll-Paque (Pharmacia, Uppsala, Sweden). The gradient was centrifuged at 1850 rpm for 40 min at 20°C. The lymphocytes were collected from the interphase and washed three times with RPMI-1640 medium (Fluka), supplemented by 2 mM L-glutamine, 100 E ml^{-1} penicillin, 100 µg ml^{-1} streptomycin and 10% fetal calf serum (Fluka). The viability of lymphocytes tested by trypan blue exclusion test was about 95%. Cell suspensions with concentrations of $6x10^6$ and 10^6 cells ml^{-1} were adjusted and subsequently used for ADCC and mitogen responsibility tests, respectively.

Antibody-Dependent Cell Cytotoxicity (ADCC) of spleen lymphocytes: ADCC of the spleen lymphocytes of hamsters was assessed at days 7, 14 and 25 after tumor transplantation according to the method of Pearson [17]. Briefly, myeloid Graffi tumor cells, obtained from non-necrotic tumor tissue 15-20 days after tumor appearance, were used as target cells. Aliquots of 0.1 ml myeloid tumor cells in RPMI-1640 medium of the concentration of $1.5x10^4$ were distributed in 96-wells plates. The cells were pre-incubated with 0.1 ml of serum at a dilution of 1:10 at 37°C for 30 min. Subsequently, 0.1 ml of 6.10^6 cells ml^{-1} lymphocyte suspension was added to each well (ratio of target cells: lymphocyte cells=1:40). The interacting cell suspension was incubated at 37°C in a 5% CO_2 incubator for 20 h. At the end of interaction, the cells were centrifuged and 0.05 ml of a 0.2% trypan blue solution was added to each well and the percentage of dead tumor cells was determined. All experiments were done in triplicate and the ADCC of the spleen lymphocytes was calculated as follows:

$$\text{ADCC (\%)} = \frac{\begin{array}{l}\text{\% surviving tumor cells after incubation with control serum}\end{array} - \begin{array}{l}\text{\% surviving tumor cells afterincubation with test anti-sera}\end{array}}{\begin{array}{l}\text{\% surviving tumor cells after incubation with control serum}\end{array}} \times 100$$

***In vitro* proliferation of spleen lymphocytes in presence of mitogens:** The proliferation responses of spleen lymphocytes in presence of mitogens were determined on days 7, 14 and 25 after tumor inoculation by the method described by Masson and Gwanzura [18]. Suspension of 1 $x10^5$ of live cells in 0.1 ml of complete RPMI-1640 medium was distributed in triplicate in 96 wells flat-bottom tissue culture plates. The cells were stimulated by phytohemagglutinin (PHA, Sigma, 20 µg ml^{-1}) or *Escherichia coli* lipopolysaccharide (LPS; Sigma; 20 µg ml^{-1}). The cells were cultivated in a CO_2 incubator at 37°C for 72 hrs. 3^H Thymidine (1 µ Ci) was added to each well 18 h before the end of incubation. The cells were collected on nitrocellulose filters by a semiautomatic manual cell harvester and were distributed in scintillation flasks. After supplementation with 5 ml scintillation mixture, the isotope uptake of each sample was determined by a Beckman scintillation counter. The stimulation index (SI) of lymphocytes was calculated according to the formula:

$$\text{SI} = \frac{\begin{array}{l}\text{Number of cpm of lymphocytes}\\ \text{incubated with mitogen}\end{array}}{\begin{array}{l}\text{Number of cpm of lymphocytes}\\ \text{incubated without mitogen}\end{array}} \times 100$$

Statistical analysis: The results from the experiments were analyzed by the Student's t-test. Data are presented as mean arithmetical values ±SD and $p<0.05$ was accepted to be significant.

RESULTS

The effects of the molluscan hemocyanin, isolated from the marine snail *Rapana venosa* (RVH), in comparison to keyhole limpet hemocyanin on ADCC and mitogen responsibility of spleen lymphocytes in hamsters with progressing myeloid Graffi tumors were followed during 25 days after tumor transplantation.

The results show that ADCC of spleen lymphocytes from hamsters with transplanted myeloid Graffi tumors (group 3) decreased during the tumor progression (Fig. 1-3). The cytotoxic indices (CI) in presence of TBH immune serum or rabbit specific anti-tumor serum after 7 days of tumor transplantation were determined to be 22.75% and 23.25%, respectively (Fig. 1, group 3). A drastic decrease of CI after incubation of both, TBH immune serum or rabbit specific anti-tumor serum, was observed on day 14 (14.5% and 15.75%, respectively) and day 25 (5.25% and 5.75%, respectively) after tumor transplantation (Fig. 2, 3, group 3). The CI values of lymphocytes against tumor cells (group 3) were lower compared to those determined in presence of TBH or rabbit specific immune sera at all days of investigation.

Lymphocytes from normal hamsters (control group) in presence of normal hamster serum showed low cytotoxic activity against tumor cells: 7.75% at day 7, 10.75% at day 14 and 5.25% at day 25 (Fig. 1, 2, 3; group 6). At the end of the investigation, the lymphocyte cytotoxicity of TBH from group 3 is lower compared to that of normal hamsters (group 6) in presence of the three kinds of sera (Fig. 3).

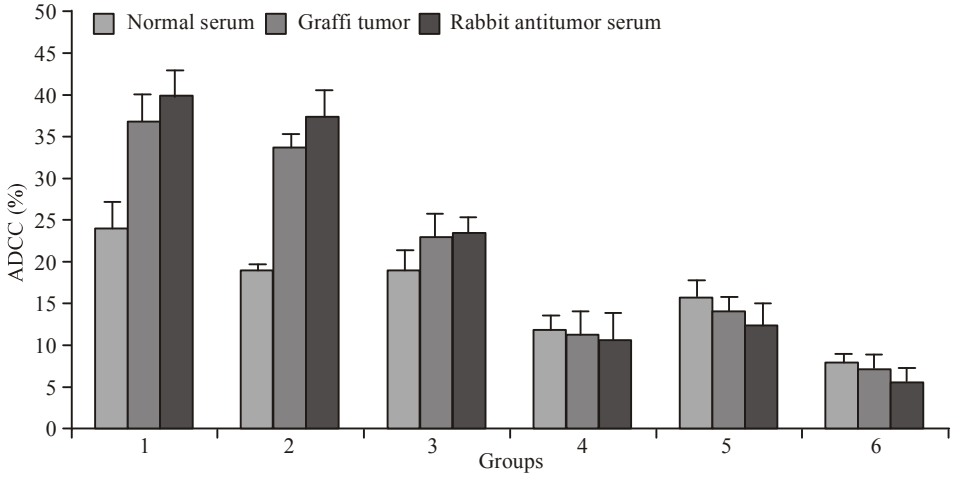

Fig. 1: Effect of molluscan *Rapana venosa* (RvH) and keyhole limpet (KLH) hemocyanins on antibody-dependent cell cytotoxicity (ADCC) in hamsters with myeloid Graffi tumors 7 days after tumor transplantation. Cytotoxic indices (CI) were determined in presence of TBH immune serum, rabbit specific anti-tumor serum and normal hamster serum

Experimental groups: 1-tumor bearing hamsters treated two times with RvH; 2-tumor bearing hamsters treated two times with KLH; 3-tumor bearing hamsters; 4-healthy hamsters treated two times with RvH; 5-healthy hamsters treated two times with KLH; 6-control, healthy hamsters without any treatment

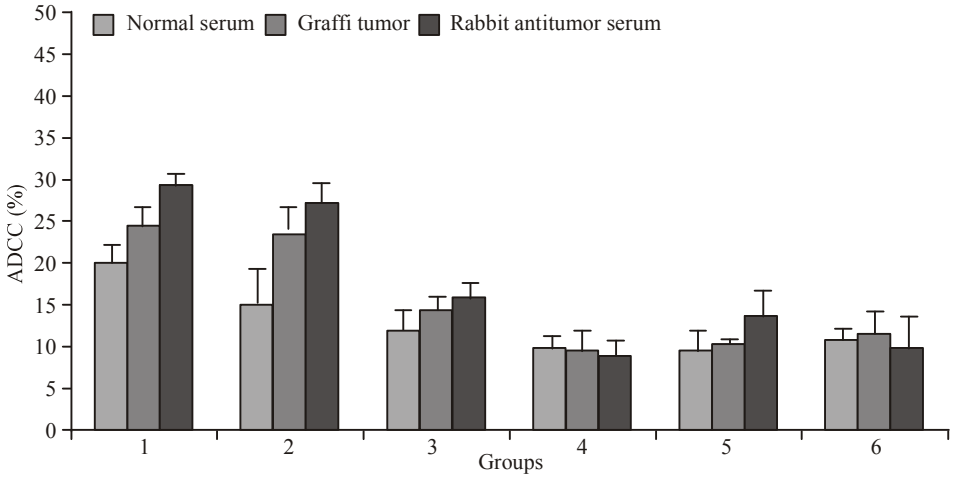

Fig. 2: Effect of molluscan *Rapana venosa* (RVH) and keyhole limpet (KLH) hemocyanins on antibody-dependent cell cytotoxicity (ADCC) in hamsters with myeloid Graffi tumors after 14 days of tumor transplantation. Cytotoxic indices (CI) were determined in presence of TBH immune serum, rabbit specific anti-tumor serum and normal hamster serum. For experimental groups see legend to Fig. 1

Treatment of TBH with RvH (group 1) induced a 2.0-3.0 fold increase of spleen lymphocyte ADCC compared to all other groups. At days 7 and 14, CI enhanced about two times in presence of the two kinds of anti-tumor sera. CI values for TBH treated with RvH (group 1) were 36.75% and 39.75% at day 7, while those for TBH without treatment (group 3) were found to be 22.75 and 23.25%. At day 14 the CI of TBH treated with RvH (group 1) in presence of both kinds of anti-tumor sera decreased to 24.5 and 29.25%, respectively, while for TBH (group 3) percentages of 14.5 and 15.75, respectively, were found (Fig. 1, 2; groups 1, 3). The CI values for TBH treated by RvH (group 1) are relatively higher at the end of the investigation, at day 25 were measured to be 15.0 and 11.5%, while those for TBH without treatment (group 3) were 5.25 and 5.75% only in presence of TBH or rabbit immune sera, respectively (Fig. 3).

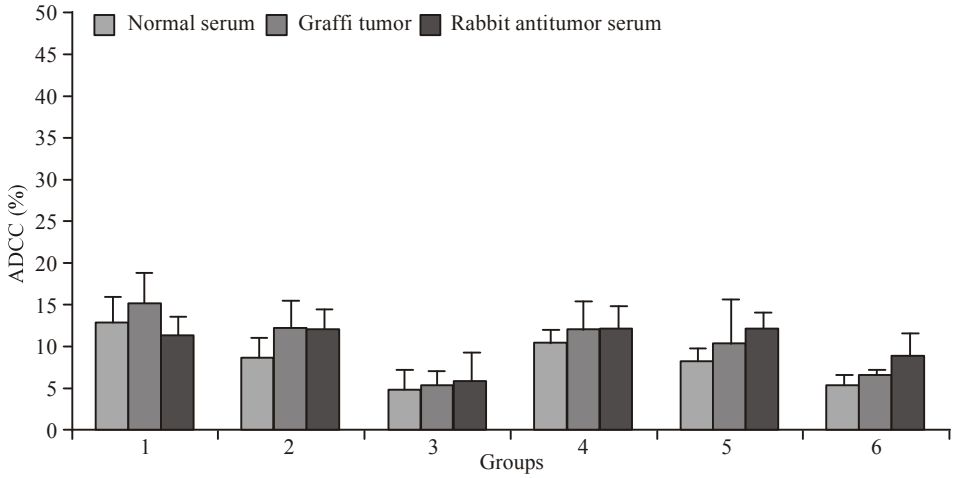

Fig. 3: Effect of molluscan *Rapana venosa* (RVH) and Keyhole Limpet (KLH) hemocyanins on antibody-dependent cell cytotoxicity (ADCC) in hamsters with myeloid Graffi tumors after 25 days of tumor transplantation. Cytotoxic Indices (CI) were determined in presence of TBH immune serum, rabbit specific anti-tumor serum and normal hamster serum. For experimental groups see legend to Fig. 1

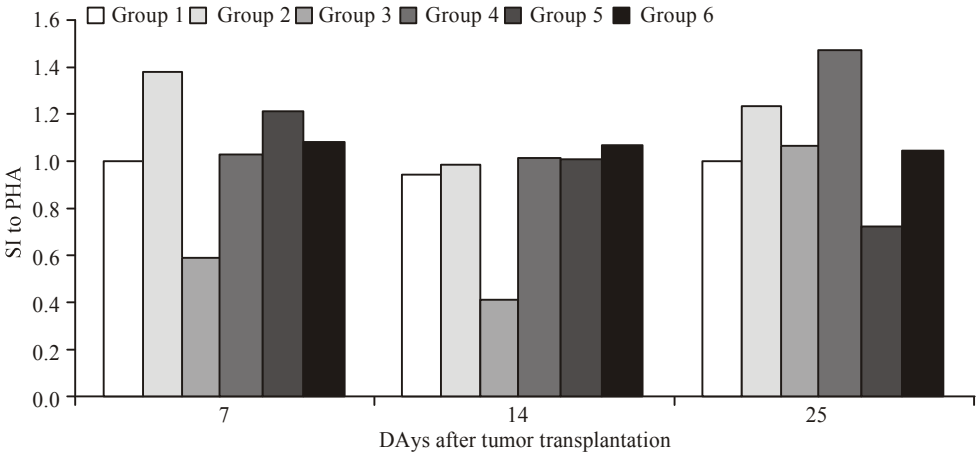

Fig. 4: Stimulation indices (SI) of spleen lymphocytes to PHA in Graffi TBH treated with molluscan Hcs from *Rapana venosa* (RVH) and keyhole limpet (KLH). For experimental groups see legend to Fig. 1

The effect of *Rapana venosa* hemocyanin on Antibody-dependent Cell Cytotoxicity (ADCC) of lymphocytes from hamsters with progressing myeloid tumors was studied in comparison to keyhole limpet *Megathura crenulata* Hc which is known to posses remarkable immunostimulatory properties. Treatment of TBH by KLH (group 2) induced a similar enhance of lymphocyte ADCC compared to RvH during the total observation time (on days 7, 14 and 25) (Fig. 1-3). Only about a 3% higher cytotoxicity was found for TBH treated with Rapana Hc (group 1) in comparison to those treated with keyhole limpet Hc (Fig. 1-3; group 2).

Figsures 1, 2 and 3 show that both, Rapana Hc and KLH, do not influence significantly the ADCC of healthy hamsters (groups 4, 5) and the cytotoxic indices are near to the control group.

The results of PHA-mitogen reactivity of spleen lymphocytes are presented in Fig. 4. It was found that the stimulation indices (SI) of spleen lymphocytes from tumor bearing hamsters (TBH, group 3) after PHA incubation decreased during early stages of tumor progression (days 7 and 14; SI values 0.58 and 0.41, respectively), while the SI values were found to be 1.07 for control healthy hamsters (group 6). Treatment of TBH with RvH (group 1) restored the mitogen reactivity of lymphocytes to againts PHA and the SI values are near to those of the control during the observation (25 days).

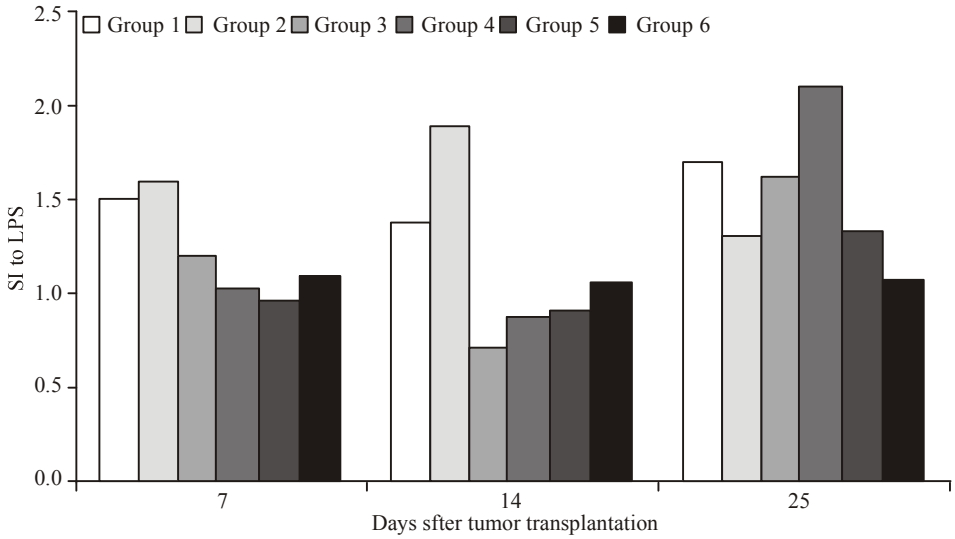

Fig. 5: Stimulation Indices (SI) of spleen lymphocytes to lypopolysaccharide (LPS) in Graffi TBH treated with molluscan Hcs *Rapana venosa* (RVH) and keyhole limpet (KLH). For experimental groups see legend to Fig. 1

Treatment of TBH with molluscan keyhole limpet hemocyanin (group 2) induces an increase of SI after PHA treatment (SI 1.38, 0.97 and 1.23 at days 7, 14 and 25, respectively) compared to the SI of TBH (Fig. 4, groups 2, 3).

It was found that RvH stimulated the responsibility of lymphocytes to PHA of healthy animals (group 4) at day 25 after treatment (SI = 1.47). Using for the treatment KLH (group 5) instead of RvH, only a minor increase of this parameter was observed at day 7 (SI=1.21, control group SI=1.07) (Fig. 4).

The effects of molluscan *Rapana* and keyhole limpet Hcs on mitogen responsibilities of spleen B-lymphocytes to lypopolysaccharide (LPS) are presented in Fig. 5. The change of stimulation indices of lymphocytes from TBH were found to be 1.2, 0.73 and 1.63 at days 7, 14 and 25 respectively, compared to 1.07 for the healthy untreated hamsters (Fig. 5, groups 3, 6).

Both, *Rapana* and keyhole limpet Hc enhanced the responsibility to LPS of spleen lymphocytes of TBH during tumor progression. Stimulation indices for Hcs-treated TBH (groups 1, 2) are higher after 7,14 and 25 days of treatment (SI=1.5,1.39 and 1.69 for RvH and 1.59, 1.89 and 1.31 for KLH) in comparison to the control group (SI = 1.07) (Fig. 5).

A marked stimulation (stimulation index 2.11) was produced by RvH (group 4) on spleen B-lymphocyte population in healthy animals at day 25 of the investigation. During the first 14 days similar SI values compared to these of the controls were found. Compared to RvH, treatment of healthy hamsters with keyhole limpet hemocyanin (group 5) induced a lower than of the B-lymphocyte mitogen responsibility (SI = 1.34) at day 25 (Fig. 5).

DISCUSSION

The results of the investigations demonstrate a progressive decrease of ADCC of the spleen lymphocytes during the progression of myeloid tumors transplanted in hamsters (Fig. 1-3). Lower values of CI of the spleen lymphocytes from TBH in presence of sera from TBH could be explained by the presence of immuno-suppressive factors characteristic for tumor growth. Renk *et al*. [19] reported on an immuno-regulator isolated from different tumors (melanoma, pulmonal carcinoma, sarcoma) which inhibits mitogen response of lymphocytes to PHA, Con A etc. Later, it was found that mouse melanoma K-1735 cells produce a factor inhibiting proliferation of splenocytes, protein synthesis and blastogenesis in mixed leukocyte population. Probably myeloid Graffi tumor produce similar suppressive factor, which suppress the ADCC activity of the spleen lymphocytes of TBH.

In this study we observed that the treatment of TBH with RvH or KLH produced a 2, 0-3, 0-fold increase of the spleen lymphocyte ADCC during the observation. KLH was applied to compare the results obtained for RvH, as KLH is a subject of many clinical trials and animal experiments during the last years and is used to synthetic antitumor vaccines against relevant tumor antigens [20]. It is clinically applied as a participator in human dendritic

anti-tumor vaccines, as a factor accelerating the maturation of dendritic cells and stimulating the Th1-lymphocyte population resulting in an enhancement of T-cytotoxic lymphocyte population [9]. It was shown that KLH directly inhibits the growth of human breast and pancreatic cancer cells *in vitro* by apoptotic and non-apoptotic (cytokine production) mechanisms [11]. A similar effect of KLH on bladder carcinoma cells was also found [21].

These mechanisms could be responsible for the stimulation effect of RvH on lymphocytes' ADCC in TBH: i/hemocyanins stimulate maturatiion of dendritic cells, proliferation of Th1 and specific T-cytotoxic lymphocyte populations; ii/RVH acts directly on tumor cells *via* apoptosis; iii/RVH stimulates production of cytokines.

Furthermore, we found that RvH stimulates the responsibility to PHA of lymphocytes from healthy animals (group 4) at day 25 after treatment which is clearly more pronounced using KLH (Fig. 4, group 4).

Both, *Rapana* and keyhole limpet Hcs enhance the responsibility of spleen lymphocytes of TBH to LPS during tumor progression. The stimulating effect of Hcs on the B-lymphocyte responsibility to LPS, in tumor bearing hamsters as well as in healthy animals, is probably due to their unspecific lymphoproliferative action. KLH is known to act as a low-level non-specific blastogenic/lymphoproliferative factor similar to PHA, *Corynebacterium parvum* and bacterial LPS [22].

In conclusion, the results of these investigations demonstrate that RvH and to a minor exent KLH stimulate the ADCC and mitogen responsibility of spleen lymphocytes from hamsters with progressing myeloid tumors. We suggest that this action is caused by stimulation of the Th-1 and T-cytotoxic lymphocyte population as well as unspecific lymphoproliferative properties of Hc preparations.

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