

The effects of hemocyanin on T cells cultured *in vitro*

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Abstract. As a broad-spectrum antibiotic, gentamicin is used extensively in T cell culturing *in vitro*, but preliminary studies have identified that T cell activity is significantly affected by gentamicin. In the present study, the hemocyanin from *Litopenaeus vannamei* (*L. vannamei*) was selected as an additive for T cell cultures *in vitro*. Compared with those in the control group, the cell quantity exhibited no significant difference, and the formation rate of cell colony increased gradually with increases in the hemocyanin concentration. Additionally, flow cytometry assays identified that cluster of differentiation (CD)3⁺CD4⁺ and CD4⁺CD25⁺ T cells in the 0.2 µg/ml hemocyanin (Hem 3) group were all significantly increased. Furthermore, cell cycle analysis demonstrated that T cells in the G0/G1 phase were significantly decreased in the Hem 3 group compared with in the control, 0.05 µg/ml (Hem 1) and 0.1 µg/ml (Hem 2) groups, and cells in the S phase were significantly elevated in the Hem 3 group compared with in the control and Hem 1 groups. In addition, MTT analysis indicated that the cytotoxicity of T cells towards HepG2 cells was significantly increased in the Hem 3 group compared with in the control, Hem 1 and Hem 2 groups. Taken together, the present study identified that hemocyanin may improve the proliferation and cytotoxicity of T cells, and the results supported the use of hemocyanin in T cell adoptive immunotherapy.

Introduction

T cell adoptive immunotherapy is widely used in clinical settings, and gentamicin is used extensively in T cell cultures *in vitro* as a broad-spectrum antibiotic (1-3). However, our

previous study identified that T cell activity was inhibited by gentamicin (4). Hemocyanin was selected as an additive for T cell culture *in vitro* in order to maintain the antibacterial environment and enhance the activity of T cells.

Hemocyanin was identified in mollusks and arthropods as a respiratory protein (5). In addition to this primary function, hemocyanin performs multiple roles in immune defense, functioning similarly to a phenoloxidase-like enzyme (6), an antiviral agent (7), an antimicrobial protein (8) and agglutinin (9). Notably, Min *et al* (10) identified that 2 hemocyanin fractions from *Litopenaeus vannamei* (*L. vannamei*) exhibited hemolytic activity, which was likely associated with the diversity in amino acid sequence and glycosylation of the IgG fractions. Zheng *et al* (11) demonstrated that the hemocyanin from *L. vannamei* exhibited antiproliferative properties against HeLa cells *in vitro* through mediating the apoptosis mechanism via the mitochondria triggered pathway. Gesheva *et al* (12) noted that the hemocyanin from *Rapana thomasi* and *Helix pomatia* presented marked anticancer and antiproliferative effects in a murine colon carcinoma model. Although hemocyanin had been identified as a multifunctional protein with antimicrobial and anticancer activities, the effects of hemocyanin from *L. vannamei* on human T cells is unclear.

In the present study, hemocyanin, as an antimicrobial protein, was incorporated into the culturing process of human T cells; the T cells in all groups were analyzed by optical microscopy and flow cytometry, and the results indicated that the application of hemocyanin to T cells cultured *in vitro* has a beneficial effect.

Materials and methods

The peripheral blood was donated by healthy male volunteers (age, 25-35 years) who provided of verbal informed consent, and the present study was approved by the Animal Welfare and Research Ethics Committee of the University of South China (Henan, China). The hemocyanin, which was purified by gel filtration chromatography on a Sepharose 4B column from *L. vannamei* and stored in PBS buffer (0.01 mol/l, pH 7.4), was donated by Professor Yueling Zhang from Shantou University (Shantou, China).

T cells cultured *in vitro* and microscopic examination. T cells were cultured with 80 U/ml gentamicin (Yichang Humanwell Pharmaceutical Co., Ltd., Yichang, China), and were defined as the control group. T cells cultured with 0.05, 0.1 and

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0.2 $\mu\text{g/ml}$ hemocyanin were defined as Hem 1, 2 and 3 groups, respectively.

Lymphocytes were separated and cultured as described previously (13), with certain modifications: Peripheral blood with heparin sodium anticoagulant was centrifuged at 450 \times g at room temperature for 5 min. The plasma was collected and maintained at 4°C subsequent to inactivation at 56°C for 30 min, then centrifuged at 648 \times g at room temperature for 10 min. The supernatant was collected and stored at 4°C as autologous plasma. The peripheral blood cell layer was mixed 1:1 (v/v) with 0.9% saline and used for Ficoll® density gradient separation (LymphoPrep; PAA Laboratories GmbH, Cölbe, Germany). Following centrifugation at 648 \times g at room temperature for 20 min, the leukocyte layer was collected in new tubes. Then, the cells were washed twice with 0.9% saline and centrifuged at 450 \times g at room temperature for 7 min. Subsequently, the lymphocytes were cultured in GT-T551 medium (Takara Biotechnology Co., Ltd., Dalian, China) with 1,000 U/ml γ -interferon (Beijing Biocoen Biotechnology Co., Ltd., Beijing, China) and 10% autologous plasma was added. Following culture for 24 h at 37°C with 5% CO_2 , 50 $\mu\text{g/ml}$ cluster of differentiation 3 (CD3) monoclonal antibody (Skoda Biotechnology Co., Ltd., Beijing, China) and 100 U/ml interleukin-1 α (PeproTech China, Suzhou, China) were added on the first day, and 1,000 U/ml recombinant human interleukin-2 (SL Pharmaceutical Co., Ltd., Beijing, China) and 2% autologous plasma were included in the medium. The cells were cultured at 37°C and 5% CO_2 for 9 days, and the T cells were analyzed by pocH-100i (Sysmex, Kobe, Japan) and an optical microscope (magnification, $\times 50$) (Olympus Corporation, Tokyo, Japan).

T cell phenotype analysis. A total of 1 ml cell suspension was collected from each experimental group. Subsequent to centrifugation at 200 \times g at room temperature for 10 min, the precipitate was resuspended in 0.9% saline and centrifuged again at 200 \times g at room temperature for 10 min. Then, the precipitate was resuspended in 150 μl 0.9% saline and divided into two groups. The first group was an isotype control, and 5 μl allophycocyanin (APC) mouse IgG1 (1:10; cat. no. 555751), 5 μl fluorescein isothiocyanate (FITC) mouse IgG2 α (1:10; cat. no. 555573), 5 μl phycoerythrin (PE) mouse IgG1 (1:10; cat. no. 555749) and 1 μl PerCP-CyTM5.5 mouse IgG1 (1:50; cat. no. 550795; all from BD Biosciences, Franklin Lakes, NJ, USA) were added. The second group was the experimental group, and 5 μl FITC mouse anti-human CD3 (1:10; cat. no. 555339), 5 μl PE mouse anti-human CD4 (1:10; cat. no. 555347), 1 μl PerCP-CyTM5.5 mouse anti-human CD8 (1:50; cat. no. 560662) and 5 μl APC mouse anti-human CD25 (1:10; cat. no. 555434; all from BD Biosciences) were added. All groups were incubated for 15 min at room temperature, resuspended in 1 ml 0.9% saline and then centrifuged again at 200 \times g at room temperature for 10 min. Finally, the precipitate was resuspended in 200 μl 0.9% saline and prepared for detection with a BD Biosciences Accuri™ C6 Plus flow cytometer (BD Biosciences). The experiment was repeated in triplicate.

T cell cycle analysis. A total of 1 ml cell suspension was collected from each group and centrifuged at 200 \times g at room temperature for 10 min, and the precipitate was resuspended in 0.9% saline. Following centrifugation at 200 \times g at room

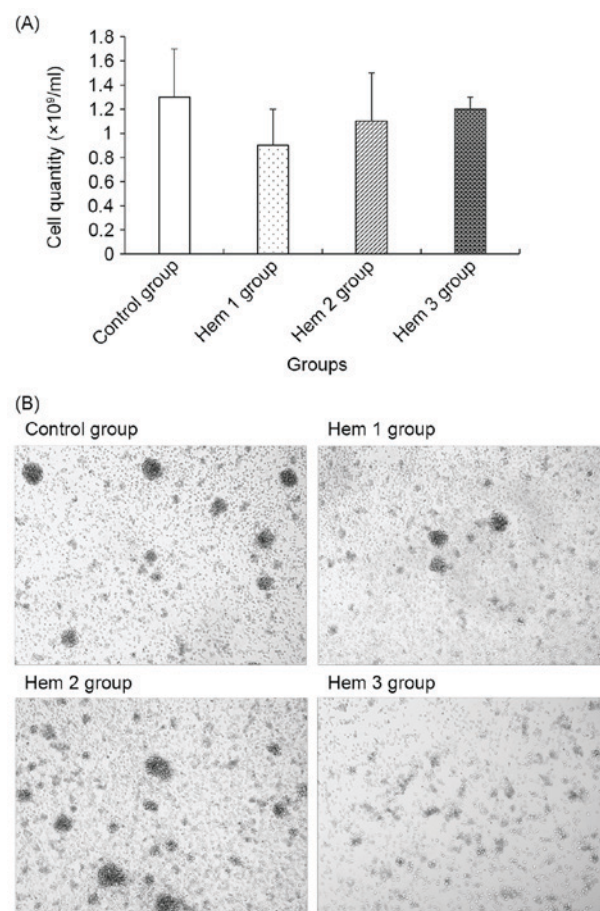


Figure 1. Cell quantity and morphology assay. (A) Quantity of T cells analyzed by cell counting. (B) T cell morphology analysis via optical microscopy (magnification, $\times 50$). Bars represent the mean \pm standard deviation ($n=3$). Hem, hemocyanin.

temperature for 10 min, the precipitate was resuspended with 75% frozen ethanol and maintained at -20°C for 1 h. Then, samples were centrifuged at 200 \times g at room temperature for 10 min. Then, the precipitate was resuspended with 200 μl 0.9% saline, and incubated with 10 μl propidium iodide (PI, 1 mg/ml; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at room temperature for 30 min. Finally, the cell suspension was detected using a BD Accuri™ C6 Plus flow cytometer (BD Biosciences) and analyzed by FlowJo v.7.6.2 software (Tree Star, Inc., Ashland, OR, USA).

T cell cytotoxicity analysis. HepG2 (American Type Culture Collection, Manassas, VA, USA) were collected at logarithmic growth phase for use as target cells, and the concentration of cells was adjusted to 1×10^5 cells/ml. T cells for use as effector cells were cultured at 37°C and 5% CO_2 for 9 days were resuspended with GT-T551 medium containing 2% autologous plasma and diluted to 5×10^6 cells/ml. These cells were divided into three groups: The effector-target group was 100 μl effector cells and 100 μl target T cells; the effector cell group comprised 100 μl effector cells and 100 μl GT-T551 medium; the target T cells group comprised 100 μl target T cells and 100 μl GT-T551 culture medium. All groups had 5 identical tubes, and were cultured at 37°C and 5% CO_2 for 24 h. A total of 10 μl thiazolyl blue tetrazolium blue (5 mg/ml;

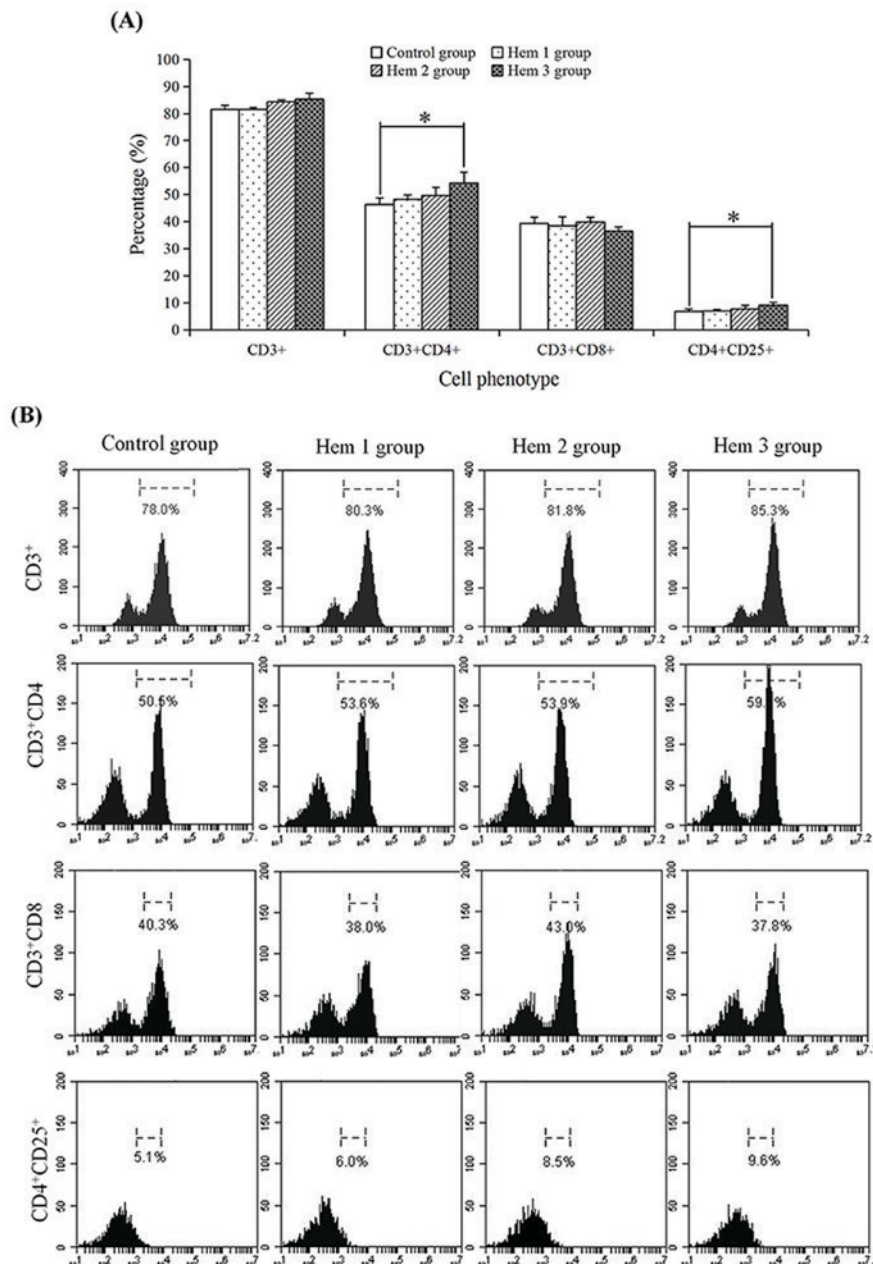


Figure 2. T cell phenotype assay by flow cytometry. (A) T cell phenotype distribution. * $P < 0.05$. (B) The flow cytometry results of the CD3⁺, CD3⁺CD4⁺, CD3⁺CD8⁺ and CD4⁺CD25⁺ phenotypes. Bars represent the mean \pm standard deviation ($n=3$). CD, cluster of differentiation; Hem, hemocyanin.

Sigma-Aldrich; Merck KGaA) was added and cultured at 37°C and 5% CO₂ for 4 h. Following centrifugation at 800 x g at room temperature for 5 min, the precipitate was dissolved in 100 μ l dimethyl sulfoxide (Sigma-Aldrich; Merck KGaA), agitated for 15 min, and the optical density (OD) was detected at 490 nm. The killing rate was calculated as follows:

$$\text{The killing rate (\%)} = [1 - (\text{OD}_{\text{effector-target T cells well}} - \text{OD}_{\text{effector cell well}}) / \text{OD}_{\text{target T cells well}}] \times 100\%.$$

Statistical analysis. The data are presented as the mean \pm standard deviation. Statistical analyses were performed using SPSS v.17.0 (SPSS, Inc., Chicago, IL, USA). Multiple comparisons between the groups were performed using one-way analysis of variance and the Bonferroni method as a post-hoc

test. $P < 0.05$ or $P < 0.01$ were considered to indicate statistically significant differences. All experiments were repeated ≥ 3 times.

Results

Cell quantity and morphology assay. As demonstrated in Fig. 1, the cell quantity in the Hem 1, 2 and 3 groups gradually increased, though all were decreased compared with the control group. No significant difference between the control group and the Hem 1, 2 or 3 groups was observed.

Effect of hemocyanin on the T cell phenotype. To determine the effect of hemocyanin on T cells subsets, the proportions of CD3⁺, CD3⁺CD4⁺, CD3⁺CD8⁺ and CD4⁺CD25⁺ T cells were detected by flow cytometry (Fig. 2). In the control, Hem 1, 2 and 3 groups, the proportion of CD3⁺

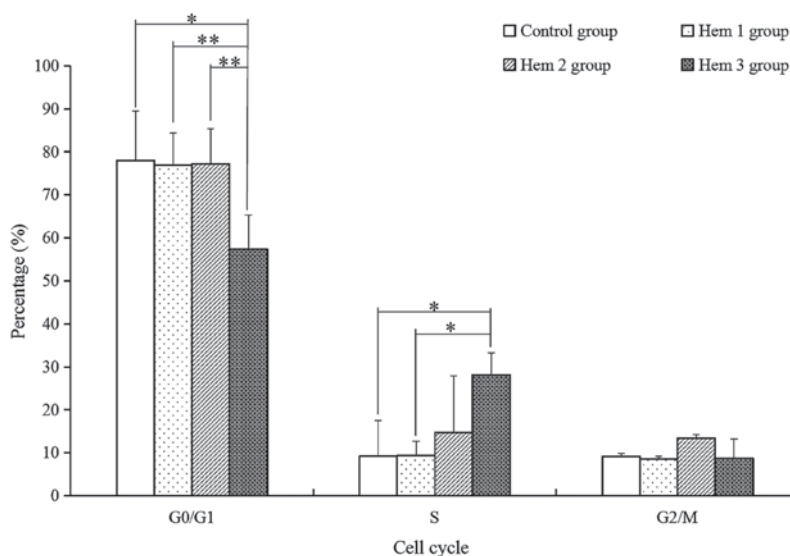


Figure 3. Cell cycle assay using flow cytometry. Bars represent the mean \pm standard deviation (n=3). *P<0.05 and **P<0.01. Hem, hemocyanin.

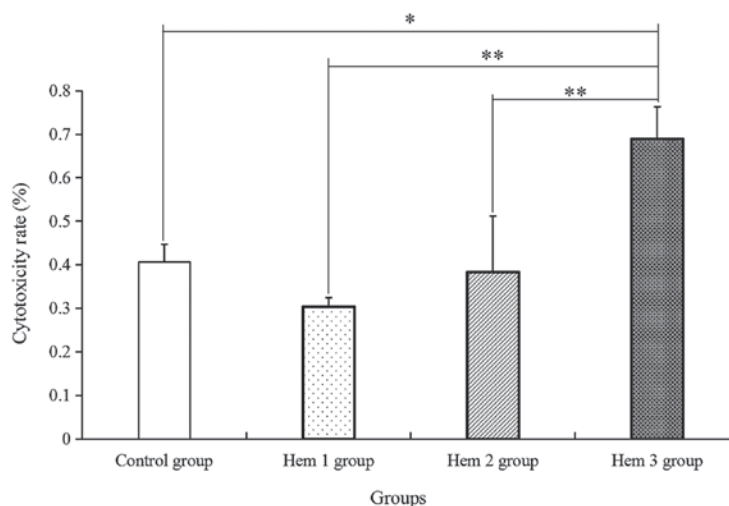


Figure 4. T cell cytotoxicity analysis via an MTT assay. Bars represent the mean \pm standard deviation (n=3). *P<0.05 and **P<0.01. Hem, hemocyanin.

(81.47, 81.53, 84.23 and 85.30%, respectively), CD3⁺CD4⁺ (46.40, 48.23, 49.73 and 54.33%, respectively) and CD4⁺CD25⁺ (6.77, 6.97, 7.63 and 9.10%) T cells were all increased with an increasing hemocyanin concentration, but no change was observed in the proportion of CD3⁺CD8⁺ T cells (39.37, 38.50, 39.83 and 36.47%). In addition, the proportions of CD3⁺CD4⁺ and CD4⁺CD25⁺ T cells in the Hem 3 group were respectively significantly increased 1.17-(P=0.02) and 1.34-fold (P=0.03) compared with the control group.

Effect of hemocyanin on T cell cycle distribution. For the subsequent analysis of the variety of T cells activity with hemocyanin treatment, the G0/G1, S and G2/M phase of T cells were analyzed with PI dye and a flow cytometry assay. As demonstrated in Fig. 3, the proportion of G0/G1 phase cells in the Hem 3 group were significantly decreased by 1.36-(P=0.012), 1.34-(P=0.002) and 1.34-(P=0.007) fold compared with the control, Hem 1 and 2 groups, respectively. The S phase cells of the Hem 3 group were significantly increased by 3.03-(P=0.027)

and 3.01-(P=0.028) fold compared with the control and Hem 1 groups respectively, and increased by 1.91-(P=0.143) fold compared with Hem 2 group. No significant change in the G2/M phase proportion was observed among all groups.

Effect of hemocyanin on T cell cytotoxicity. Furthermore, the T cell cytotoxicity in HepG2 cells was verified. As indicated in Fig. 4, the T cell cytotoxicity in the Hem 1, 2 and 3 groups was gradually enhanced along with increases in the hemocyanin concentration, as compared with that in the control group. Among the four groups, T cell cytotoxicity in the Hem 3 group was significantly increased by 1.70-, 2.26- and 1.80-fold, compared with in the control, Hem 1 and 2 groups, respectively.

Discussion

Hemocyanin is a metallo-glycoprotein oxygen transporter, previously indicated to potentially have multiple functionalities (14). Hemocyanin has exhibited excellent

antimicrobial (15-17), anti-tumor (18) and immune enhancement effects (19). In the present study, hemocyanin was used as an additive in T cell culture *in vitro*. The results indicated that the T cells normally proliferated during culture without gentamicin treatment, and the cell quantity between the control and experimental groups exhibited no significant changes (Fig. 1).

Furthermore, the CD3⁺, CD3⁺CD4⁺, CD3⁺CD8⁺ and CD4⁺CD25⁺ phenotypes, which all represent different T cell subsets, were analyzed by flow cytometry. CD3⁺ is the characteristic marker of T cells (20). The CD3⁺CD4⁺ phenotype is characteristic of T helper cells, which secrete anti-tumor agents for cytotoxic T lymphocytes (21). T cells with the CD3⁺CD8⁺ phenotype are critical factors in the immune response to viral infection and cancer (22). CD4⁺CD25⁺ T cells are considered to be regulatory, and to serve a central role in the prevention of autoimmunity and in the control of immune responses (23). In the present study, the quantity of CD3⁺CD4⁺ and CD4⁺CD25⁺ T cells in the Hem 3 group were significantly increased compared with in the control group (Fig. 2). Therefore, 0.2 µg/ml hemocyanin may be an effective concentration for use in T cell culture *in vitro*.

In addition, the cell cycle distributions of the T cells were assayed using flow cytometry. Among the four groups (Fig. 3), the number of G0/G1 phase cells in the Hem 3 group was significantly decreased compared with in the control, Hem 1 and 2 groups. However, the number of S phase cells in the Hem 3 group was significantly elevated compared with in the control and Hem 1 groups. Chen *et al* (24) demonstrated that the cell proliferative activity corresponded with an increase in the number of S phase cells, therefore, 0.2 µg/ml hemocyanin may be beneficial for T cells proliferative activity.

T cell cytotoxicity in HepG2 was analyzed. In the present study, HepG2 cells were used for T cell cytotoxicity analysis. Although the HepG2 cell line has been demonstrated to be misidentified and be derived from hepatoblastoma instead of hepatocellular carcinoma (25), the use of HepG2 cells as a model of target cells derived from tumor tissues is suitable for cytotoxicity analysis of immune cells cultured *in vitro* (26-28). Among the four groups (Fig. 4), the T cell cytotoxicity in the Hem 1, 2 and 3 groups was gradually increased with the increased of hemocyanin concentration, and the T cell cytotoxicity of the Hem 3 group was significantly increased compared with the control, Hem 1 and 2 groups. Previous studies have indicated that this killing activity is a major characteristic of immune cells (29-31). Consequently, the results of the present study suggest that the concentration of hemocyanin used in the Hem 3 group may positively contribute to the anti-tumor activity of T cells.

In conclusion, the present study explored the possibility that hemocyanin may be used as an additive in T cell culture *in vitro*. The results verified that 0.2 µg/ml hemocyanin may significantly improve T cell proliferative activity and cytotoxicity, and have potential value in the application of T cell adoptive immunotherapy.

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