Effect of a Fungal Cu/Zn Superoxide Dismutase on the Cell-Mediated Immune Response in Graffi Tumor Bearing Hamsters

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The antibody-dependent cell cytotoxicity (ADCC) of spleen lymphocytes, isolated from hamsters with progressing myeloid Graffi tumor, was studied. The effect of the application of Cu/Zn superoxide dismutase, isolated from the fungal strain Humicola lutea (HL SOD), before and during tumor transplantation on the lymphocyte ADCC was examined. Myeloid Graffi tumor cells as target cells were used. Antibodies from a rabbit hyper-immune anti-tumor Graffi cells serum, or from tumor-bearing hamsters serum were used in the test. The leukocyte adherence inhibition (LAI) in the presence of tumor antigen was examined also during tumor progression. ADCC of the spleen lymphocytes, determined by both, rabbit and hamster anti-tumor antibodies, decreased during tumor progression. The optimum treatment of the animals by HL SOD induced a 20–30% increase of lymphocyte cytotoxicity against myeloid Graffi tumor cells. Cytotoxicity in presence of tumor bearing hamsters serum was twofold lower as compared to that one determined in the presence of rabbit hyper-immune anti-myeloid Graffi tumor cells serum. Leukocyte adherence inhibition (LAI) index in the presence of tumor antigen increased during tumor development in the groups of treated and untreated animals. The LAI indices of HL SOD-treated tumor-bearing hamsters were lower than that of untreated animals with tumors, what can be explained by a higher adherence ability of leukocytes induced by HL SOD treatment (in formula for calculation of LAI index the adherence value is in the denominator). The results show the beneficial effect of HL SOD on the cell-mediated immune response of myeloid Graffi tumor bearing hamsters, what is probably due to the participation of the enzyme in the host’s oxidant-antioxidant balance.

Key words: Tumors, Superoxide Dismutase, Antibody-Dependent Cell Cytotoxicity

Introduction

Antibody-dependent cell cytotoxicity (ADCC) is one of the important mechanisms of the anti-tumor immune response. Several immune-competent cells realize this kind of immune response: macrophages, PMNs, cytotoxic T-lymphocytes, NK-cells, K-cells.

ADCC against tumor cells develop as follows: 1) Binding of specific antibodies to the cell surfaces of tumor cells, 2) interaction of cytotoxic cells with specific antibodies, preliminary binded to the target cell and 3) killing of the tumor cells (Benjamini and Leskowitz, 1994).

Several lymphocyte populations are able to realize ADCC of tumor cells. Specific cytotoxic T-lymphocytes (CTL) are important participators in this activity. T-helper lymphocytes participate in the induction and regulation of T- and B-cells. CD8+ cytotoxic T-lymphocytes (CTL), specific for the respective tumor antigen, are responsible for killing the tumor cells.

The natural killer cells (NK) are another type of lymphoid cells which destroy different cells including tumor cells. NK cells are part of the large granular lymphocyte (LGL) population. These cells have an important role in the host defense at the beginning of the tumor development, before the proliferation of killer cells and T-cell-mediated activated macrophages (Benjamini and Leskowitz, 1994).

It was found that ADCC, mediated by peripheral blood lymphocytes in hamsters with transplanted melanoma, depends on the changes of the
surface glycoprotein antigen on the target tumor cells. A decrease in susceptibility to ADCC against melanin-less melanoma cell lines in comparison to the native cell line of the tumor was established (Kozlowska et al., 1988).

Investigations of Dorothee et al. (2001) showed that cytotoxic T-lymphocyte clone (CTL) reacts specifically with the human autologous lung carcinoma cell line IGR-Heu. The tumor cell line was found to lack its Fas-receptor expression. The specific CTLs use mainly a granule exocytosis-dependent pathway to destroy the autologous target cells. These cells are able to circumvent alteration of Fas-triggering intracellular pathway via activation of a caspase-independent mechanism of cell death.

Our recent investigations showed that the Cu/Zn SOD, isolated from the fungal strain Humicola lutea 103 (HL SOD), produced a protective effect on the survivability and immune status of hamsters with transplanted myeloid Graffi tumor. Elongation of the mean survival time, elongation of the latent time of tumor appearance and suppression of the tumor growth, enhanced phagocyte indices of macrophages and PMNs and increase of the in vitro proliferating abilities of the spleen B-lymphocytes were observed (Toshkova et al., 2000). Enhancement of the spontaneous superoxide production by phagocyte cells (macrophages and PMNs) in tumor-bearing hamsters (TBH) and normalization of the values under the action of HL SOD treatment was found (Dimitrova et al., 2000). A progressive decrease of the liver antioxidant enzymes SOD and catalase during tumor development and a beneficial effect of HL SOD treatment were observed (Ivanova et al., 2002).

The aim of the present investigation was to study the ADCC of spleen lymphocytes and the leukocyte adherent activity in presence of tumor antigens in hamsters with transplanted myeloid Graffi tumors, treated with a fungal Cu/Zn superoxide dismutase.

**Material and Methods**

**Experimental animals**

“Golden Siberian” hamsters, two months of age, weighing 80–100 g, of both sexes were used for the experiments. The animals were grown up at the animal house of the Institute of Microbiology, Bulgarian Academy of Sciences, at standard conditions, accepted from the Bulgarian Veterinary Health Control Service. The animals were distributed in 4 experimental groups (30 in one group):

- Group 1 – hamsters with transplanted tumors, treated with *Humicola lutea 103* Cu/Zn SOD (SOD+T),
- Group 2 – hamsters with transplanted myeloid tumor (T),
- Group 3 – healthy hamsters, treated with HL SOD (SOD), and
- Group 4 – control group of healthy hamsters (Control).

**Tumor and transplantation**

The myeloid Graffi tumors were induced by Graffi virus in mice and adapted to hamsters by repeated subcutaneous inoculations of $1 \times 10^5$ viable tumor cells (Jakimov et al., 1979).

In the present experiments, 100% transplantability of tumors was achieved by s.c. injection of $2 \times 10^4$ viable tumor cells into the inter-scapular field of the animals. By this quantity of cells, 100% obligatory mortality in our previous studies was established (Toshkova, 1995).

Myeloid tumor cells were isolated from solid tumor mass 15 days after tumor appearance. The non-necrotic tumor tissue was cut into small pieces, suspended in RPMI 1640 (Fluka) medium (pH 7.2) and subsequently centrifuged at $200 \times g$ for 10 min at 4 °C. The cell pellets were re-suspended at the necessary concentrations for transplantation or the ADCC test with spleen lymphocytes. The trypan blue-tested viability of tumor cells was 90–95%.

**Cu/Zn superoxide dismutase from Humicola lutea 103 (HL SOD)**

The fungal strain *Humicola lutea 103* was used as a source of the Cu/Zn superoxide dismutase. The methods for cultivation of the strain, extraction and purification of the enzyme, determinations of the amino acid composition and electrophoretic properties were described earlier (Angelova et al., 2001). SOD activity was measured by the nitroblue tetrazolium (NBT) reduction method of Beauchamp and Fridovich (1971). One unit of SOD activity was defined as the amount of SOD required for a 50% inhibition of NBT and is expressed as units per mg protein (U/mg protein).
Cu/Zn HL SOD – doses and way of application

HL SOD was injected into hamsters i. p. 7 days before and 14 days after tumor transplantation in a single dose of 65 U per animal, with a frequency of two times a week. This dose and scheme of application of HL SOD proved to be the optimal according to our previous experiments (Toshkova et al., 2000).

Specific immune sera against myeloid Graffi tumor cells

Rabbit immune anti-serum against myeloid Graffi tumor-associated antigen, used for the ADCC test, was obtained previously (Toshkova et al., 1992). Briefly, anti-sera of rabbits were obtained after 6 injections of the animals with myeloid Graffi tumor cells according to the scheme: Day 1: s. c. injection of 3 ¥ 10^7 tumor cells/1 ml PBS, well mixed with 0.5 ml complete Freund’s adjuvant (CFA) (Difco Lab., Detroit, Michigan, USA); day 9: i. m. injection of 5 ¥ 10^7 tumor cells/1 ml PBS, day 16: i.m. injection of 5 ¥ 10^7 tumor cells/1 ml PBS, day 46: i. v. booster injection of 5 ¥ 10^7 tumor cells/1 ml PBS and day 52: i. v. booster injection of 5 ¥ 10^7 tumor cells/1 ml PBS.

Rabbit antiserum was collected 1 week after the last injection.

Absorptions of the serum were then performed with suspensions of hamster embryo, liver, kidney, spleen, bone marrow and lung cells also and with hamster serum and plasma as well as sheep erythrocytes. Specificity of the absorbed antiserum against the tumor-associated antigen was proven by immune-precipitation according to Ouchterlony, immune-fluorescence microscopy and immune-electron microscopy (Toshkova et al., 1992).

Tumor-specific anti-sera, obtained from experimental groups of tumor-bearing hamsters, were collected at days 14, 25 and 30 after tumor transplantation and comparatively used in the ADCC tests with spleen lymphocytes. Normal serum from the healthy control hamsters was used as a control in the ADCC test.

Spleen lymphocytes

Spleens were aseptically removed and homogenised. A single cell suspension was prepared. The spleen cells were diluted in 3 ml phosphate-buffered saline (PBS) in a ratio of 1:2 and were layered on 3 ml Ficoll-Paque (Pharmacia, Uppsala, Sweden). The gradient was achieved by centrifugation at 600 ¥ g for 40 min at 20 °C. The lymphocytes were collected from the inter-phase and washed three times with RPMI-1640 medium, supplemented with L-glutamine, sodium pyruvate and antibiotics. The viability of lymphocytes, tested by trypan blue exclusion, was about 95%. Cell suspensions with concentrations of 2 ¥ 10^7 cells/ml were adjusted and subsequently used for the ADCC test.

Antibody-dependent cell cytotoxicity (ADCC) of spleen lymphocytes

ADCC of spleen lymphocytes of hamsters was assessed at days 14, 25 and 30 of the tumor progression according to the method of Pearson (1978). Briefly, myeloid Graffi tumor cells, obtained from non-necrotic tumor tissue were used as target cells. Aliquots of 0.1 ml myeloid tumor cells (concentration 5 ¥ 10^4) in complete RPMI-1640 medium containing 10% Difco fetal calf serum (FCS) were distributed in plates of 96-wells. The cells were pre-incubated with 0.1 ml of serum at dilutions 1:10 or 1:50 at 37 °C for 30 min. Subsequently, 0.1 ml of 2 ¥ 10^7 cells/ml 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% CO2 incubator for 20 h. At the end of interaction, the cells were centrifuged and 0.05 ml of a 0.02 trypan blue solution was added to each well. The percentage of killed tumor cells was determined. The ADCC of the spleen lymphocytes was calculated as follows:

\[
\text{Cyt.index} = \frac{\% \text{ surviving tumor cells after incubation with control serum} - \% \text{ surviving tumor cells after incubation with anti-sera}}{\% \text{ surviving tumor cells after incubation with control serum}} \times 100
\]  
(1)

All experiments were done in triplicate.
Tumor antigen

Tumor antigen from myeloid Graffi tumor cells was obtained according to a method described previously (Toshkova et al., 1995). Briefly, tumor cell suspensions in saline were obtained as stated above. The cells were treated with an ultrasonic desintegrator (MSE, England) at amplitude 12 (peak to peak), for 3 min. After centrifugation, the cell pellets were re-suspended in a buffer, containing 0.5 mM sucrose, 10 mM Tris(2-amino,2-(hydroxymethyl)-1,3 propanediol)-HCl buffer (pH 7.5), 1 mM MgCl₂ and 1 mM PMSF (phenylmethane-sulfonyl-fluoride). A crude membrane fraction of tumor cells was obtained after sedimentation through a layer of 1 mM sucrose solution at 4000 g for 30 min. The protein concentration of the membrane fraction of the tumor cells was measured by the method of Bradford (1976).

Separation of polymorphous nuclear leukocytes (PMNs)

Blood samples from hamsters were collected in glass tubes and immediately diluted 1:3 with a 2% sodium citrate solution. PMNs were separated from other leukocytes by gradient centrifugation on Histopaque (Sigma, Grünwald, Germany) according to the method of Boyum (1968). Briefly, citrate blood samples were over-layered subsequently with two gradients (Histopaque 1107 and Histopaque 1119) with a ratio between gradients and blood of = 1:3 and centrifuged at 300 g for 30 min at room temperature. The PMNs from the inter-phase between the two layers were collected and washed by Hanks balanced salt solution (HBSS), and contaminating erythrocytes were destroyed by short lysis in distilled water.

Leukocyte adherence inhibition (LAI) technique

LAI assay was performed by the tube method, described by Grosser and Thomson (1975). The test is based on the finding that leukocytes from sensitized patients show a reduced adherence on glass, when mixed in vitro with relevant antigen, in contrast to the leukocytes from non-sensitized patients. LAI test response has been supposed to be caused by lymphokine, released from T-lymphocytes.

Briefly, 0.1 ml of 0.5 x 10⁶ PMNs and 0.1 ml tumor antigen (0.5 mg/ml protein content) were supplemented by 0.2 ml RPMI-1640 medium and very well mixed in glass tubes (16x150 mm). The tubes were horizontally situated, so that the contents covered about ¾ of the lower areas of the tubes. After a 2-h incubation at 37 °C and a 5% CO₂ atmosphere in a thermostat, the tubes were placed vertically and agitated. The numbers of the non-adherent cells/ml were counted with a Burker's chamber.

The LAI index was calculated according to the formula:

\[
\text{LAI index} = \frac{A - B}{B} \times 100
\]

A: percentage of the adherent cells in absence of antigen

B: percentage of the adherent cells in presence of antigen

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

It was established that ADCC of the spleen lymphocytes from tumor bearing hamsters (TBH) against myeloid Graffi tumor cells, determined in presence of specific rabbit antibodies decreased during the tumor progression. Cytotoxic indices (CI) of 40.6%, 22% and 15.6% at days 14, 25 and 30 after tumor transplantation of hamsters in the experimental group 2 (TBH without treatment) were found; the percentage of control lymphocytes from healthy hamsters was 7.3%. A strong enhancement of the lymphocyte CI (8 fold) at the time of tumor appearance (14th day) was observed. The treatment of hamsters by HL SOD enhanced the lymphocyte cytotoxicity by 20–30%, but the tendency concerning the decrease of ADCC remains during tumor progression (CI = 54.6%, 45.7% and 26.2%, determined at days 14, 25 and 30 of the investigation). Treatment with HL SOD did not cause any stimulation of the ADCC of lymphocytes in healthy hamsters (Fig. 1-A).
ADCC, determined in the presence of serum from tumor-bearing hamsters was lower compared to that estimated in presence of immune rabbit serum. A tendency of decrease of the anti-tumor lymphocyte cytotoxicity during the tumor progression of group 2 (TBH without treatment) was established (CI = 22.3%, 16.3% and 6.6%, determined at days 14, 25 and 30, control value for healthy animals being 7.8%). Treatment with HL SOD enhanced the cytotoxicity in presence of serum from TBH by 20–50% (CI = 35.3%, 24.6% and 15.7%, determined at the days of investigation, respectively). HL SOD did not influence significantly the ADCC of lymphocytes from healthy hamsters, determined in the presence of serum from TBH (Fig. 1-B).

The index of inhibition of leukocyte adherence on glass surface in presence of tumor antigen (I-LAI) was enhanced in TBH from both experimental groups, compared to the control values of healthy animals. The LAI-indexes of PNNs, isolated from hamsters without treatment (group 2) were higher than that of HL SOD-treated TBH. A gradual increase during the tumor development was found (LAI index = 61.5%, 77.7% and 88%). In gr. 1 were found lower LAI indexes (LAI index = 47.9%, 55% and 75%, respectively at days 14, 25 and 30 of the investigation, control healthy LAI index = 2.8% ± 0.8%) (Fig. 1-C).

Discussion

A progressive decrease of the values of the CI, determined in presence of rabbit serum or in serum from TBH, was observed (Figs. 1-A and B). The cytotoxic activity of lymphocytes from healthy animals treated and non-treated by HL SOD against tumor cells were low, demonstrating the importance of specific antibodies in the anti-tumor cytotoxic activity of lymphocytes. The suppression
of ADCC of lymphocytes from TBH could be due to the lower cytokine production, responsible for T-cell cytotoxicity (e.g. tumor-necrosis factor or gamma-interferon). On the other hand, this suppression of ADCC could be due to the decrease of cytotoxic T-lymphocyte subsets. Such a decrease of CD8 (T-suppressor) cytotoxic subsets of peripheral blood lymphocyte sub-populations was found for lung cancer patients (Mazzoccoli et al., 1999). Byrnes et al. (1998) reported on low levels of NK and cytotoxic/suppressor T-cells in HIV (positiv) black women, connected with risk for cervical cancer. Lower levels of T-helper lymphocytes and of eosinophils were found in blood of patients with cerebral tumors (gliomas). Post-operation infections occurred exclusively in patients with severe depression of helper T-cells, (Dauch et al., 1994).

Another factor responsible for different levels of the lymphocyte CI is the origin of antibodies used in the ADCC in vitro tests. The values of CI for lymphocytes from of TBH of both experimental groups, determined in presence of serum from TBH were 1.5–2.0-fold lower, compared to the corresponding values, determined in presence of rabbit antibodies (Figs. 1-A and B). The lower cytotoxic activity in presence of sera from TBH could be due to immuno-suppressive factors which are characteristic for the sera of tumor-bearing hosts. Different factors are responsible for the immuno-suppression of tumor-bearing organisms. In the case of the ADCC in vitro test for the most probable suppressing factor on the cytotoxic activity of host lymphocytes could be the circulating antigen-antibody complexes and tumor antigens in the sera of animals, as mentioned in our previous investigations (Toshkova, 1995).

The results of the adherence activities of leukocytes from TBH showed indirectly that the anti-tumor immune response increases during tumor progression in both groups of animals with tumors, as estimated according to the LAI-test of Grosser and Thompson (1975). The lower LAI indexes of leukocytes of HL SOD-treated TBH is due to the higher adherence activity of leukocytes (in the formula for calculation of LAI index the adherence value is in the denominator). We can conclude that HL SOD could produce a beneficial effect on the cytotoxic activity of PMNs, which is known to have a tumorocidal effect (Benjamini and Leskowitz, 1992; Ivanova et al., 1996).

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