Immunoprotective Effect of Cu/Zn Superoxide Dismutase on Myeloid Graffi Tumor-Bearing Hamsters

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Investigation on the immunoprotective activity of Cu/Zn superoxide dismutase from *Humicola lutea* 103 AL (HLSOD) in hamsters with transplanted myeloid tumor was performed. Survivability, tumor growth and tumor transplantability were followed. The immune status of tumor-bearing animals, injected with the optimal protective HLSOD dose, was examined during 27 days after tumor transplantation by the following parameters: (i) the number, migration and phagocytic activity of peritoneal macrophages, (ii) the phagocytic activity of blood polymorphonuclear leukocytes (PMNs), (iii) the responsibility *in vitro* of spleen lymphocytes to T and B cell mitogens.

It was established that intraperitoneal inoculation of HLSOD produced a protective effect on the development of tumors. Elongation of the latent time for tumor appearance and inhibition of the tumor growth were observed. The decreased percentage of mortality in early stage of tumor progression was established. Immunological studies on tumor-bearing hamsters (TBH) induced a temporary immunorestoring effect on the suppressed phagocytic activities of peritoneal macrophages and blood PMNs during the first 14 days of tumor development. Moreover, HLSOD showed an expressed stimulating effect on proliferative activity *in vitro* of spleen B lymphocytes from healthy and TBH as well. The immunorestoring and protective effect of the enzyme was probably due to improve of the oxidant-antioxidant balance in peritoneal phagocytes. The temporary character of the effect can be explained with the interference of immunosuppressing factors produced by tumor tissue as well as by the presence of tumor antigens, tumor cells and antigen-antibody complexes in the circulation.

Introduction

There has been a considerable interest in the use of SOD in medicine over the last years arising from its ability to reduce deleterious effects of O_2^- in the cells. These radicals as well as H_2O_2 and the hydroxyl radicals are continuously generated in all aerobic organisms. They can cause widespread damage to the cell affecting nucleic acids, proteins and lipids (Sies, 1993). In addition to their generation, reactive oxygen radical (ROS) formation appears to be markedly increased during stress conditions. Oxidative stress is strongly implicated in more than 50 human diseases including cancer (Okada *et al.,* 1999; Seidman *et al.,* 1999; Weber and Bruch, 1999). During the last years, clinical investigations have indicated that oxidant-antioxidant balance is changeable during the oncogenic process. Several authors have demonstrated that antioxidant levels in more human and animal cancers are low (Oberley and Oberley, 1997; Janssen *et al.*, 1999; Ambrosone *et al.*, 1999; Bittinger *et al.*, 1998; Grammatico *et al.*, 1998). Zhong *et al.* (1997) found a suppression of the malignant phenotype of human glyoma cells by overexpression of MnSOD. Kiningham *et al.* (1997) supposed that MnSOD is a new type of a suppressor of tumor genes, and overexpression of this enzyme selec-

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tively modulates the activity of some transcription factors in fibrosarcoma cells. However, in some cancer types elevated levels of antioxidant enzymes have been found, particularly SOD (Arivazhagan *et al.*, 1997; Jung *et al.*, 1997; Kahlos *et al.*, 1999). Animal experiments have supported the concept that SOD has a protective effect during the promotional phases of cancer development. Recently, Li *et al.* (1998) established a protective action of several antioxidants on the tumor transformation, both, *in vivo* and *in vitro*. Inoculation of Cu/ZnSOD was shown to prolong the survival of experimental animals with Ehrlich ascites and Sarcoma 180 tumors (Oberley and Buetner, 1979).

Materials and Methods

Tumor

The myeloid tumor, induced by Graffi virus, was adapted to hamsters (Jakimov *et al.*, 1979). The tumor was initiated in the Institute of Experimental Pathology and Parasitology, Sofia by subcutaneous inoculations of 1×10^5 viable tumor cells. In the present experiments, 2×10^4 viable tumor cells were inoculated s.c. in the interscapular field of hamsters. Such quantity of cells induced 100% transplantability and 100% mortality of hamsters according to our previous investigations (Toshkova, 1995).

Experimental animals

Two months old "Golden Siberian" hamsters from both sexes, weighing 80–100 g were used for the experiments. The animals were obtained from the animal house of the Institute of Microbiology, Bulgarian Academy of Sciences, Sofia. They were bred and grown under the standard conditions, accepted from the Bulgarian Veterinary Health Control Service.

Superoxide dismutase

The fungal strain *H. lutea* 103 from the Mycological Collection of the Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, was used for the production of Cu/ZnSOD. Cultivation was performed in a 31 bioreactor ABR-09, developed and realized by the Central Laboratory for Bioinstrumentation and Automatisation (CLBA) of the Bulgarian Academy of Sciences, Sofia. The bioreactor was equipped with a pH-monitoring system and an automatic monitoring and controlling dissolved oxygen concentration (DO) system. The fermentation parameters were the same as described earlier (Pashova *et al.*, 1999). The purified HLSOD with specific activity 3100 U/mg protein was prepared according to Dolashka-Angelova *et al.* (1999). It is a water-soluble substance, a glycoprotein homodimer with a molecular mass of approximately 30700 Da (unpubl data). Purity control of the enzyme was performed on 7.5% polyacrylamide gels, as described by Davis (1964), and the gel stained for protein detection was compared with a duplicate gel, stained for superoxide dismutase activity, as described by Misra and Fridovich (1977). The 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 inhibition of the reduction of NBT by 50% and was expressed as units per mg protein (U/mg protein). Sodium cyanide (2 mM) was used to distinguish between the cyanide-sensitive isoenzyme Cu/ZnSOD and the cyanide-resistant MnSOD. The Cu/ZnSOD activity was obtained as total activity minus that in the presence of 2 mM sodium cyanide. The protein contents was estimated after Lowry *et al.* (1951), using crystalline bovine serum albumin as standard.

Doses and way of application

For the examination of the optimal protective effect of HLSOD, 30 animals were separated in several experimental groups, and were injected intraperitoneally (i.p.) with different doses before and after tumor transplantation (Fig. 1) as follows:

- *1st group:* Hamsters, treated before transplantation of tumors, two times with 65 U HLSOD per animal and 5 times after tumor transplantation, 2 times a week.
- *2nd group:* Hamsters were injected 5 times with 65 U HLSOD per animal, starting simultaneously with tumor transplantation, 2 times a week.
- *3rd group:* Hamsters were injected 5 times with 125 U HLSOD per animal, starting simultaneously with tumor transplantation, 2 times a week.

- *4th group:* Tumor-bearing hamsters were investigated without HLSOD treatment.
- *Control groups:* Healthy hamsters and HLSOD-treated healthy hamsters were used for the investigations.

Biometric parameters

Tumor transplantability (TT). Tumor transplantability (%) was determined for each experimental group at days 10, 12 and 15 as ratios between the number of tumor-bearing hamsters to the number of all hamsters in the group.

Inhibition of tumor growth (ITG). The tumor size in mm (mean arithmetical value of two tumor diameters) was determined for each animal at days 10, 15, 20, 25 and 30 after tumor transplantation. Inhibition of tumor growth (ITG) was calculated according to the formula (Toshkova, 1995):

$$ITG = [(A-B)/A]x100,$$

A = mean arithmetical value of diameters (mm) in TBH without HLSOD treatment and
B = mean arithmetical value of diameters (mm) in HLSOD-treated TBH.

Mortality (M). Mortality (%) was followed at days 30, 35, 40 and 45 after tumor transplantation.

Immune status

The immune status of the experimental animal injected by the optimal protective dose of 65 U HLSOD per animal, 2 times before and/or 5 times after tumor transplantation was examined by the following parameters:

Number, migration ability and phagocytic indices of peritoneal macrophages were examined at days 7, 14 and 27 after tumor transplantation. The peritoneal cells were collected by washing of the peritoneal cavity with 20 ml cold Hank's balanced salt solution (pH 7.2). After two washings with RPMI 1640 medium (Sigma; Deisenhofen, Germany) without supplements, the cells were adjusted to the necessary concentrations.

In vitro phagocytosis by peritoneal macrophages. Macrophages in concentrations of $1x10^7$ cells/ml in RPMI 1640 without supplements were allowed to adhere on glass lamellae for 2 h at 37 °C in humidified 5% CO_2 atmosphere. After washing with phosphate-buffered saline (PBS), a suspension of killed *Staphylococcus aureus* Smith cells was added to a macrophage monolayer (the ratio between macrophages to bacteria was 1:20). The cells were allowed to interact 1 h under the same conditions, washed and stained according to Pappenheim (1911). The phagocytic index (PI) was calculated according to the formula proposed by Ossada *et al.* (1982):

PI(%)=(number of macrophages, containing bacterial cells per 200 counted macrophages) x100.

Migration of peritoneal macrophages. Migration of macrophages in RPMI 1640 without the presence of antigen was examined by the method described by Leu *et al.* (1972). Briefly, $6x10^7$/ml macrophages in RPMI 1640 were introduced in hematocrit-siliconized glass capillaries (75/1 mm; for clinical laboratory use), one side stopped by bee vax. The capillaries were centrifuged at 4 °C for 5 min at 500 rpm, then they were cut at the level of the visible cell pools and fixed with silicon at the bottom of plastic petri dishes, containing RPMI 1640, supplemented by 10% fetal calf serum (Sigma; Diesenhofen, Germany), L-glutamine and antibiotics. The migration fields were estimated after 24 h incubation at 37 °C in humidified 5% CO_2 atmosphere, using a projection apparatus and planimeter.

In vitro responsibility of spleen lymphocytes to mitogens was estimated according to the method described by Masson and Guanzura (1990). Briefly, the splenocytes were aseptically disintegrated in RPMI 1640 without supplements. The obtained cell suspensions were separated by gradient centrifugation on Ficoll-Paque (Pharmacia; Uppsala, Sweden). The cells from the interphase were collected, then washed 3 times and adjusted to $1x10^6$/ml RPMI 1640, supplemented with L-glutamine, antibiotics and 20% fetal calf serum. The 100 ml/well lymphocyte suspensions were distributed in 96 plaque wells. Then, 50 ml phytohaemagglutinine from *Phaseolus vulgaris* (PHA; 25 mg/ml; Sigma; Germany) or 50 ml lipopolysaccharide from *E.coli* (LPS; 25 mg/ml; Sigma, Germany) were added per well. After incubation for 48 h at 37 °C in 5% CO_2 atmosphere, the cells were pulsed with 1 mCi ^{3}H-thymidine per well and then reincubated for 18 h under the same conditions. The cells from each well were collected on nitrocellulose filters using a semiautomatic collector and distributed in scintillation bottles. The isotope uptake was determined with a scintillation counter

(Intertechnique, France). Stimulation indices of lymphocytes (SI) were calculated according to the formula:

SI=number of Cpm of lymphocytes incubated with mitogen to Cpm of lymphocytes without mitogen.

Phagocytic indices of PMNs in the blood were estimated at days 7, 14 and 27 after tumor transplantation. They were determined according to the method of Vulchanov (1954). Briefly, 0.10 ml peripheral blood and 0.05 ml of a 2% sodium citrate solution and 0.05 ml 2×10^6 of a suspension of killed *Staphylococcus aureus* cells were allowed to interact in glass tubes for 1 h at 37 °C. Smears from each sample were prepared and stained by Pappenheim (1911). The phagocytic indices (PI) were calculated according to the formula (Ossada *et al.*, 1982):

PI(%) = (number of PMNs, containing bacterial cells per 200 counted PMNs) × 100

Statistical methods

Differences between the results of experimental groups were analyzed by the Student's T test. Data are presented as mean arithmetical values ±SD. $P<0.05$ was accepted statistically significant.

Results

According to the results, presented in Fig. 1 and 2, it could be established that a single dose of 65 U HLSOD per hamster tended to be the most effective one during the treatment period (experimental groups 1 and 2, see Fig. 1). For groups 1 and 2, treated by a single 65 U HLSOD dose per animal, on day 10 the tumor transplantability was only 20%, while animals treated by a single 125 U HLSOD dose per animal, was 75%, and for untreated TBH, 100% tumor transplantabilities were observed. On day 12, the experimental group 3 (125 U HLSOD/animal) showed already 100% tumor transplantability, while the experimental groups 1 and 2 (65 U HLSOD/animal) reached values of only 80%.

Furthermore, a decreased mortality (%) at the early stage of tumor progression in hamsters treated by HLSOD was registered (Fig. 3). At day 30 after tumor transplantation, the mortality for groups 1 and 2 (65 U HLSOD/animal) was 20% and for group 3 (125 U HLSOD/animal) 0%. For the control group it was 33%, and the latter increased to 100% within 5 days, while for groups 1 and 2 (65 U HLSOD/animal) a mortality value of a only 40% was observed.

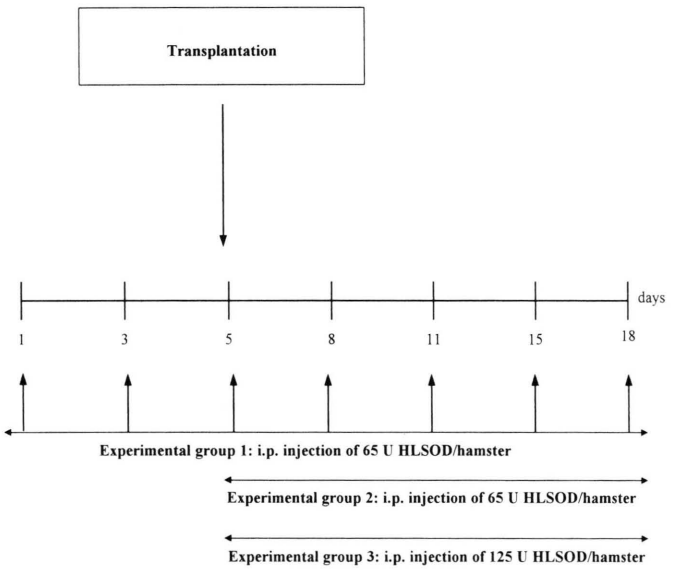

Fig. 1. Schedules for tumor transplantation and doses of HLSOD injections.

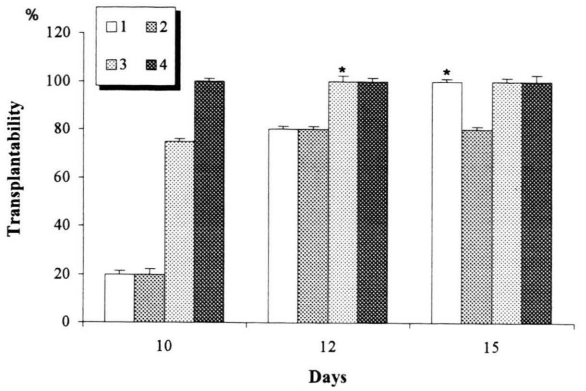

Fig. 2. Transplantability (%) in hamsters, injected subcutaneously with 1×10^5 viable Graffi tumor cells and treated by Zn/CuSOD. **Experimental groups**: 1. Tumor-bearing hamsters (TBH), treated i.p. by a 65 U single HLSOD dose per animal *prior* and after tumor transplantation; 2. TBH, treated i.p. by a 65 U single HLSOD dose per animal, starting at the day of tumor transplantation; 3. TBH, i.p.-treated by a 125 U single HLSOD dose per animal, starting at the day of tumor transplantation; 4. Hamsters with transplanted tumors without any treatment. Results are expressed as mean statistical values ± SD. *Note*: * $p<0.05$ as compared to the control group.

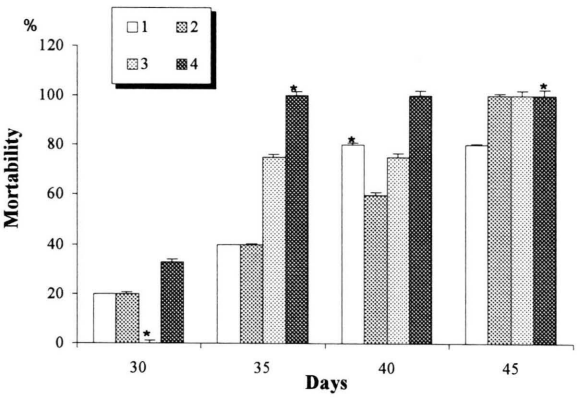

Fig. 3. Mortality (%) in hamsters with progressing Graffi tumors, treated i.p.with HLSOD in different doses. Definition of experimental groups: see legend to Fig. 2. *Note*: *$p<0.05$ as compared to the control group.

As demonstrated by Fig. 4, an inhibition of tumor growth in the groups of HLSOD- treated tumor-bearing hamsters at the early stages of tumorgenesis (ITG=50% at day 15) was observed.

The results observed from the studies on mortality, on tumor transplantability and inhibition of tumor growth demonstrate throughout that the application of 65 U HLSOD/hamster has an optimal

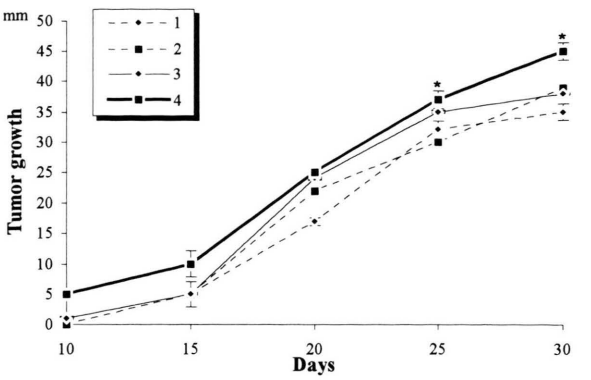

Fig. 4. Inhibition of tumor growth in hamsters, treated i.p. with different doses HLSOD. Definition of experimental groups: see legend to Fig. 2. *Note*: *$p<0.05$ as compared to the control group.

protective effect for tumor-bearing hamsters. As next, observations of the effect of HLSOD at its optimal dose on the immune status of tumor-bearing hamsters were performed.

I.p. application of a single 65 U dose of HLSOD per animal induced a 2–4-fold increase of the peritoneal macrophage number in healthy animals (group 1) and a 1–3-fold increase in TBH during the whole tested period (27 days) with a maximum at day 7 (10.31×10^6 and 7.51×10^6, respectively; number of macrophages of control group: 2.21×10^6; Table I). In the group of TBH without treatment, the number of macrophage was similar to that of the control group, with the exception around day 7, when a lower value (1.33×10^6) was observed.

Futhermore, a suppression of the migration ability of peritoneal macrophages during tumor progression was registered (migration=1.60 mm^2, 1.00 mm^2 and 0.93 mm^2; control group: 2.27 mm^2 at days 7, 14 and 27, respectively; Table I). HLSOD-treatment induced an increase of the macrophage migration ability of TBH, compared to the untreated animals with transplanted tumors (migration=1.91 mm^2, 1.80 mm^2 and 1.71 mm^2, determined at the same days of observation; HLSOD+T). HLSOD-treatement produced also a significant increase of macrophage migration in healthy control animals with a maximum at day 7 (migration=3.26 mm^2, 3.12 mm^2 and 2.25 mm^2 at days 7, 14 and 27, respectively; Table I, HLSOD).

Results of examinations of phagocytic abilities of peritoneal macrophages are presented in Ta-

Table I. Immunological parameters of hamsters with progressing myeloid tumor, treated by a 65 U HLSOD single dose per animal. **Experimental groups**: 1. Tumor-bearing hamsters, treated with a 65 U HLSOD single dose per animal (HLSOD+T); 2. Tumor-bearing hamsters (T); 3. Hamsters, treated only with a 65 U HLSOD single dose per animal (HLSOD); 4. Healthy control animals (control). Results are expressed as mean statistical values ± SD. *Note:* *$p<0.05$ as compared to the control group.

Experimental groups	Number of macrophages ($\times 10^6$)			Migration of macrophages (mm^2)			Phagocytic index of macrophages (%)			Phagocytic index of PMNs (%)			Stimulation index to PHA			Stimulation index to LPS		
Days	*7*	*14*	*27*	*7*	*14*	*27*	*7*	*14*	*27*	*7*	*14*	*27*	*7*	*14*	*27*	*7*	*14*	*27*
1. HLSOD+T	7.51 ±0.40	5.00 ±0.17	3.34 ±0.25	1.91 ±0.12	1.80 ±0.20	1.71 ±0.29	53.53* ±2.12	43.56 ±0.71	54.00* ±2.83	54.51* ±0.71	41.51 ±0.71	64.00 ±1.42	1.22 ±0.08	1.13 ±0.01	0.98* ±0.11	3.17 ±0.07	2.15 ±0.05	0.52 ±0.05
2. T	1.33 ±0.15	2.21 ±0.25	2.52 ±0.40	1.60* ±0.21	1.00 ±0.22	0.93 ±0.01	31.51 ±2.12	28.25 ±1.41	26.56 ±2.12	38.50 ±4.95	24.53* ±0.71	20.00 ±2.12	1.02 ±0.01	1.09 ±0.05	1.45* ±0.06	1.70 ±0.01	1.74 ±0.05	0.73 ±0.07
3. HLSOD	10.31* ±2.56	5.53* ±0.10	4.33* ±1.52	3.26* ±0.38	3.12 ±0.02	2.25 ±0.04	71.21 ±2.83	60.51 ±0.71	44.22 ±2.83	71.55 ±0.71	54.52 ±2.12	38.00 ±4.24	1.04 ±0.01	1.07 ±0.05	1.26 ±0.01	2.51 ±0.01	3.39* ±0.05	1.15* ±0.02
4. Control	2.22 ±0.40	2.21 ±0.40	2.20 ±0.40	2.17 ±0.02	2.18 ±0.02	2.17 ±0.02	45.50 ±0.71	45.50 ±0.71	45.50 ±0.81	45.51 ±2.83	45.50 ±0.71	45.50 ±1.41	1.24 ±0.05	1.24 ±0.05	1.24 ±0.01	1.43 ±0.04	1.43 ±0.04	1.43 ±0.02

ble I. Tumor progression induced a suppression of the macrophage phagocytic indices (PI= 38.51%, 28.25% and 26.56%, established at days 7, 14 and 27, control value 45.50%). HLSOD-treatment produced a stimulating effect on the macrophage phagocytic ability in TBH and in healthy animals at the 7th day (PI=53.53% and 71.21%, respectively). Later, the PI values of both groups gradually decreased, but after passing day 14, the HLSOD-treated TBH showed increasing PI values again (T and HLSOD+T).

Phagocytic indices of PMNs in peripheral blood of tumor-bearing hamsters were decreased during the whole period of the observation (PI= 38.50%, 24.53% and 20.00% at days 7, 14 and 27, respectively, control value: 45.50%; Table I: T and Control). In the group of HLSOD-treated TBH, the phagocytic ability of PMNs was higher compared to the untreated animals with tumors (PI=54.51%, 41.51% and 64.00% at days 7, 14 and 27, respectively, HLSOD+T). The highest phagocytic index (71.55%) was found for the group of HLSOD-treated healthy animals at day 7 of observation (HLSOD).

Results of the investigations on the *in vitro* responsibility of spleen lymphocytes to mitogens are presented on Table I. It demonstrated that tumor progression and/or HLSOD application did not produce any significant changes in lymphocyte stimulating indices (SI) in the presence of phytohaemagglutinine (PHA). The SI of proliferative responses of spleen lymphocytes to *E. coli* LPS in the group of TBH were insignificantly impaired till day 14 compared to the controls (SI=1.70 and 1.74, at days 7 and 14 respectively, values for control animals: 1.43; Table I; T and Control), but followed by a 100% decrease at day 27. HLSOD treatment induced in TBH a marked increase of B lymphocyte mitogen reactivity to LPS with a maximum at day 7 of the investigation (SI=3.17 and 2.15 at days 7 and 14, respectively; HLSOD+T), followed by a 3-fold decrease at day 27 compared to the control. HLSOD increased also significantly B lymphocyte mitogen reactivity in healthy animals (SI=2.51 and 3.39 at days 7 and 14; HLSOD).

Discussion

The obtained experimental data demonstrate the protective effect of *H. lutea* SOD on the survivability of hamsters with transplanted myeloid Graffi tumors, as expressed by (i) decreased tumor transplantability, (ii) inhibition of tumor growth and (iii) reduced mortality percentage. Similarly Oberley and Buettner (1979) established elongation of survival time of experimental animals with Ehrlich ascites and Sarcoma 180 tumor after intravenous and intramuscular injection of Cu/ZnSOD.

This protective effect can be partly explained by the temporary immunostimulating and immunorestoring action of the preparation on the phagocytic activity of macrophages and PMNs at the beginning stage of tumor progression (7–14th day) as well as by the stimulating effect on the proliferating ability of B lymphocytes.

The transitory character of immunorestorating and protective effects of the preparation can be explained by the intervention of the tumor immunosuppression during tumor progression (Finke *et al.*, 1999; Alexandroff *et al.*, 1998). Reactive oxygen species (ROS) and antioxidants were shown to influence the development of malignant diseases. Kogawa *et al.* (1999) reported that ROS enhanced inhibition of tumor cell metastasis of mouse Meth A sarcoma and Lewis lung carcinoma after treatment with Cu/ZnSOD and adriamycin.

Increased expression of MnSOD has been detected in several classes of human and experimental tumors and appears to correlate with poorer prognosis in human neuroepithelial, ovarian and cervical tumors. These observations suggest that MnSOD provides a mechanism for counteracting the intracellular oxidative processes that impairs cell growth and viability in the context of growth factor withdrawal which may promote tumor-cell survival *in vivo* under conditions unfavourable to cell growth (Palazzotti *et al.*, 1999). Siemankowski *et al.* (1999) found a selective increase of MnSOD in MGF-7 tumor cells after TNF treatment. The expression of Cu/ZnSOD, catalase or thiozedoxin were not altered.

Data about the levels of SOD in tumor tissues are contradictory depending on the kind of tumor tissues, cell lines and stages of development of the diseases. Bittinger *et al.* (1998) found that melanoma cells without stimulants produced large amounts of superoxide anions at an increasing rate in relation with time. Liaw *et al.* (1997) found lower Cu/ZnSOD in human hepatocellular carcinoma compared to normal surrounding and cirrhotic tissue. Tanaka *et al.* (1997) reported about a suppression of intracellular Cu/ZnSOD as a result of enhanced motility and metastasis of Meth A sarcoma cells. Lu *et al.* (1997) established enhanced skin carcinogenesis in transgenic mice with high expression of glutathione peroxidase or, both, glutathione peroxidase and SOD.

In conclusion, i.p. application of Cu/ZnSOD from *Humicola lutea* induces a protective and immunorestoring effect in hamsters with progressing myeloid Graffi tumor. Three possible mechanisms for this action can be hypothesized:

- Cu/ZnSOD from *Humicola lutea* improves the oxidant-antioxidant balance in peritoneal macrophages and PMNs resulting in decreased toxic changes and increased phagocytic abilities of these cells.
- Cu/ZnSOD from *Humicola lutea* increases the levels of H_2O_2 from radicals which supposedly can not be detoxified by most cancer cell types (Oberley and Oberley, 1997).
- The presence of polysaccharide chains in the HLSOD molecule probably contribute to the immunostimulating action of the preparation.

Acknowledgments

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