COMBINED PROTECTIVE EFFECT OF A FUNGAL CU/ZN-CONTAINING SUPEROXIDE DISMUTASE AND RIMANTADINE HYDROCHLORIDE IN EXPERIMENTAL MURINE INFLUENZA A VIRUS INFECTION

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Summary. – The combined protective effect of a novel naturally glycosylated Cu/Zn-containing superoxide dismutase, produced by the fungus *Humicula lutea* (HL-SOD) strain 103, and the selective anti-influenza drug rimantadine hydrochloride (Rim) was evaluated in experimental virus infection in mice, induced with influenza virus A/Aichi/2/68 (H3N2). A combined application of HL-SOD and Rim in doses, which by themselves did not protect significantly mice against the infection, resulted in a synergistically increased protection, determined on the basis of protective indices. Lung virus titers, lung weights and consolidation and mortality rates were all decreased significantly, while survival times were prolonged.

Key words: fungus Humicula lutea; superoxide dismutase, rimantadine hydrochloride, combined protective effect, murine influenza virus infection

Introduction

Influenza continues to be a major cause of high morbidity and significant mortality both in humans and domestic animals. Parallel to the search for novel potent anti-influenza drugs, the strategy of combined antiviral therapy with available viral inhibitors has proved its usefulness. It has been found that a novel naturally glycosylated Cu/Zn-containing superoxide dismutase, produced by the fungus *Humicula lutea* (HL-SOD) strain 103, applied four times from day 4 to 7 post infection (p.i.), induced with influenza virus A/Aichi/2/68 (H3N2) in a dose of 500 U/mouse/day intravenously (i.v.), increased the survival rate by 66% and prolonged the survival time to 5.2 days (Angelova *et al*., 2001). Rim, an analogue of amantadine hydrochloride, has a well documented prophylactic (Dolin *et al*., 1982) and therapeutic (Van Voris *et al*., 1981) effect on uncomplicated influenza A virus infection after oral administration. However, development of viral resistance to Rim has been identified as a problem in the use of this drug (Hayden and Couch, 1992). In general, a combined use of antiviral agents is likely to enable potentiation of viral inhibition, reduction of toxicity and prevention of antiviral resistance.

The aim of the present study was to investigate the combined effect of HL-SOD and Rim on a murine model of experimental influenza virus A infection with regard to reduction of morbidity and mortality due to infection.

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Abbreviations: CEF = chick embryo fibroblasts; ELISA = enzyme-linked immunosorbent assay; FCS = fetal calf serum; HA = hemagglutinin; HL-SOD = Cu/Zn-containing superoxide dismutase produced by *Humicula lutea* 103; i.n. = intranasal; i.v = intravenously; i.p. = intraperitoneally; MDCK = Madin-Darby canine kidney; MAb = monoclonal antibody; MST = mean survival time; NBT = nitro-blue tetrazolium; Rim = rimantadine hydrochloride; p.i. = post infection; PI = protection index; p.o. = orally; PR = protection ratio

Materials and Methods

Compounds. Rimantadine hydrochloride (Rim) was obtained from Hoffman-La Roche Inc., Nutley, NJ, USA.

Microorganism, cultivation and equipment. The fungus *H. lutea* strain 103 from the Institute of Microbiology, Bulgarian Academy of Sciences, at 4°C on beer agar pH 6.3. Cultivation was performed in a 3 l bioreactor ABR-09, developed by CLBA, Bulgarian Academy of Sciences, Sofia, in a culture medium described earlier (Angelova *et al*., 1996).

Analysis, purification and characterization of HL-SOD. The cell-free extract was prepared as described earlier (Angelova *et al*., 1996). The SOD activity was measured by the nitro-blue tetrazolium (NBT) reduction method (Beauchamp and Fridovich, 1971). One unit of SOD activity was defined as the amount of SOD required for inhibition of the reduction of 16 µmol/l NBT by 50% (A_{560}), and the SOD activity was expressed as the number of units per mg of protein (U/mg). The purification and characterization of HL-SOD was done according to Angelova *et al*. (2001).

Cell cultures. Primary cultures of chick embryo fibroblasts (CEF) were prepared in a standard way and were maintained in the 199 medium (50%) with Hanks solution (40%) lactalbumine hydrolysate (5%), calf serum (5%) and100 IU/ml benzylpenicillin and 100 µg/ml streptomycin. Madin-Darby canine kidney (MDCK) cells were passaged in Dulbecco's modification of Eagle's medium (DMEM, Gibco BRL, Scotland), supplemented with 5% of fetal calf serum (FCS) and antibiotics. Cell cultures were kept at 37°C in the presence of 5% CO_2 until confluent monolayers were formed. In the experiments the medium contained 0.5% of FCS and 2 µg/ml trypsin.

Viruses. Avian influenza virus A/chicken/Germany/34 strain Rostock (H7N1) (A/Rostock), grown in CEF and human influenza virus A/Aichi/2/68 (H3N2) (A/Aichi), adapted to mouse lungs were maintained by passaging in mice lungs and fertile hen's eggs. The virus stocks were kept at -70°C. The viruses originated from the collection of viruses of the Institute of Microbiology, Bulgarian Academy of Sciences, Sofia.

Enzyme-linked immunosorbent assay (ELISA) was carried out according to Belshe *et al*. (1988). The monoclonal antibody (MAb) to viral hemagglutinin (HA) HC58 was kindly provided by Dr. A. Douglas, the World Collaborative Centre of Influenza, Mill Hill, London, UK.

Mice. Male and female inbred ICR mice of body weight of 16-18 g were obtained from the Experimental Animal Station, Bulgarian Academy of Sciences, Slivnitza, Bulgaria. They were maintained on a standard laboratory chow and tap water *ad libitum.* The animals were bred under standard conditions accepted by the Bulgarian Veterinary Health Service. A specialized personnel took care of the welfare of the animals.

Virus infection was induced under light ether anesthesia by intranasal (i.n.) inoculation of the A/Aichi virus. To cause lethal infection, mice were infected with 10 LD_{50} of the virus in 0.05 ml of saline per mice.

Experimental design. HL-SOD was applied i.v. in the dose of 125–1000 U/mice/day on days 4–7 p.i. Rim was applied orally (p.o.) 24 and 2 hrs before and 24, 48, and 72 hrs p.i in the dose of 10–40 mg/kg per application. The experimental groups consisted of 10 animals each. The mice were observed for death daily for 21 days p.i. After the end of the experiments the surviving mice were sacrificed by cervical dislocation. Additional groups of 3 animals each from each group were killed on day 4 and 7, their lungs were weighed and the lung consolidation was scored as described earlier (Serkedjieva and Ivanova, 1997). Infectious virus in mouse lungs was titrated in MDCK cells on the basis of CPE (Serkedjieva and Ivanova, 1997). Virus titers were calculated and expressed as log $TCID_{50}$/0.2 ml in a standard way. Virus-infected placebo-treated animals were used as virus controls. Toxicity controls for each combination under study were run on 5 mice in parallel.

Protective effects of HL-SOD, Rim and their combinations were estimated by reduction of infectious virus titer in the lungs, lung weight and consolidation and mortality rate; determination of the increase of survival rate and the indices of protection and prolongation of mean survival time (MST) was estimated as described earlier (Serkedjieva and Ivanova, 1997). The protective index (PI) was determined from the equation

$$PI = (PR–1)/PR \times 100$$

where PR (protection ratio)) is $M_{control}/M_{experiment}$ and M is mortality. The effect of the combination ($E_{1,2}$) was evaluated according to Webb (1966):

$$E_{1,2} = PI_{1,2}/100$$
$$E_{1,2} = E_1+E_2–(E_1xE_2)$$

where the effects of the individual substances E_1 and E_2 are defined as follows:

$$E_1 = PI_1/100$$
$$E_2 = PI_2/100$$

There are, in general, three possibilities for the effect of a combination of two different substances (1 and 2):

a synergisitic effect if $E_{1,2} > E_1+E_2–(E_1xE_2)$
an additive effect if $E_{1,2} = E_1+E_2–(E_1xE_2)$
an antagonistic effect if $E_{1,2} < E_1+E_2–(E_1xE_2)$

Results and Discussion

Intranasal (i.n.) inoculation of the A/Aichi (H3N2) virus to mice produced a damaging infection of the lungs which, depending on the dose of the viral inoculum, was highly lethal to the animals. HL-SOD did not protect significantly mice under the conditions of a severe viral infection (100% mortality in the virus control group); further experiments were carried out under the conditions of 70–80% mortality in the virus control induced by 5–10 LD_{50}. HL-SOD, applied four times i.v. on days 4 to 7 p.i. in a dose of 500 U/mouse/day, increased the survival rate by 66% and prolonged the survival time by 5.2 days. The effect was dependent on the dose (Fig. 1A), duration of treatment (Fig. 1B) and route of inoculation (Fig. 1C). It is known that exogenous SOD is rapidly excreted (Nimrod *et al*., 1994); presumably, the i.v. inoculation provides faster delivery to the target organ of infection. In further experiments HL-SOD was applied by the i.v. route.

Table 1. Combined protective effect of HL-SOD and Rim on experimental murine influenza infection

Treatment group	Dosage	Lung weight (g)	Lung consolidation[a]	Lung virus titer[b]	Mortality (%)	Protective index (%)	Effect	MST (days)
Control		0.237	4.0	6.0	75.0			14.2
HL-SOD	500[d]	0.126	1.7	5.5[c]	10.3	86.1		19.7
HL-SOD1	250[d]	0.164	2.3	5.5[c]	30.2	59.5		18.2
HL-SOD2	125[d]	0.232	4.0	6.0	65.0[c]	12.4[c]		14.3[c]
Rim	40[e]	0.109	1.0	2.3	10.6	85.5		14.0
Rim1	20[e]	0.148	2.0	4.0	41.6	44.1		17.8
Rim2	10[e]	0.172	3.3	5.0	65.0[c]	12.3[c]		19.4[c]
HL-SOD1 +Rim1	250+20	0.077	0.0	0.0	0.0	100.0	S	21.0
HL-SOD1 +Rim2	250+10	0.134	2.3	2.5	16.7	77.7	S	18.0
HL-SOD2 +Rim1	125+20	0.106	1.0	1.7	10.0	86.7	S	18.8
HL-SOD2 +Rim2	125+10	0.132	1.7	3.0	35.0	53.0	S	14.4

[a]Scores 0–4, assigned to % of visible consolidation. Samples taken on day 7 p.i.
[b]Log $TCID_{50}$/0.2 ml. Samples taken on day 7 p.i.
[c]The difference was not significant.
[d]U/mouse/day, administered i.v. on days 4–7 p.i.
[e]mg/kg, administered p.o. 24 and 2 hrs before and 24, 48, and 72 hrs p.i.
S = synergistic.

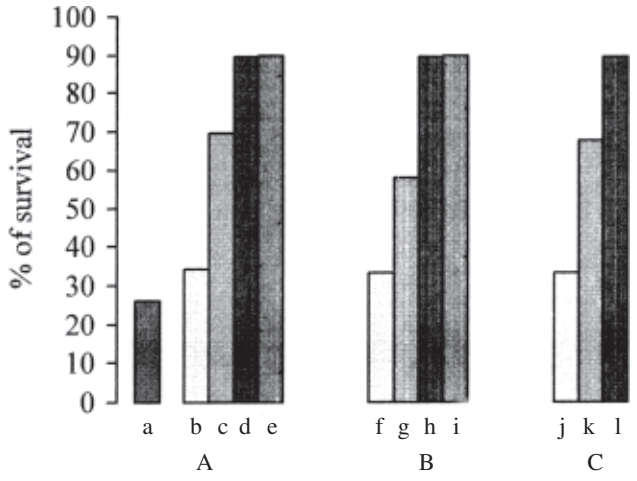

Fig. 1
Protective effect of HL-SOD on the experimental murine influenza infection

a: virus control.
A: dose dependence (number of units/mouse/day applied i.v. on days 4–7 p.i.; 125 U (b), 250 U (c), 500 U (d) and 1000 U (e).
B: dependence on the duration of i. v. treatment with 500 U/mouse/day; 2-fold (f), 3-fold (g), 4-fold (h) and 5-fold treatment.
C: dependence on the route of application of 500 U/mouse/day on days 4–7 p.i.; p.o. (j), i.p. (k) and i.v. (l).

To evaluate a possible *in vitro* selective virus-inhibitory effect, the preparation was tested on the growth of the A/Rostock virus in CEF. If applied in a concentration of 500 U/ml HL-SOD did not reduce the expression of viral HA on the infected cell surface as a measure of virus growth by ELISA with a MAb to HA. As no specific virus-inhibitory activity was found the protective effect of HL-SOD was most probably mediated by its intrinsic oxygen radical-scavenging properties. As a result of HL-SOD treatment the oxidative damage to the lungs of infected animals was apparently reduced and the prooxidant-antioxidant balance was restored. It should be noted that HL-SOD is a naturally glycosylated enzyme (Angelova *et al*., 2001), which could be isolated in few cases only. This feature of the enzyme is very important with regard to its *in vivo* pharmacological activity, e.g. in all probability its half-life in plasma and blood is prolonged.

Rim is a highly effective drug in the prophylaxis and treatment of influenza A virus infection. With many influenza virus strains the inhibition occurs at an early stage of virus reproduction, preventing virus uncoating (Bukrinskaya *et al*., 1982). For certain inhibition of influenza H7 infection takes place at a later stage during replication and prevents virus release by a specific interaction with the viral M2 protein (Hay, 1989).

The combined application of HL-SOD and Rim in doses, which by themselves were either ineffective or of a low potency, led to a marked increase in survival and resulted in a higher protective effect, determined on the basis of protective indices. The calculated combination effect was of the synergistic type. HL-SOD did not reduce significantly virus titers in the lungs, presumably, its protective effect was mediated by a reduction of virus toxicity in the target organ (Akaike *at al*., 1996). The lung weight, lung consolidation and lung virus titer, evaluated on day 7 p.i. and the mortality rate of infected animals were all significantly reduced and MST markedly increased (Table 1). The drug combinations were well tolerated by

the experimental animals and the improved protection was not associated with an increased toxicity.

Recently it has been found that reactive oxygen species play an important role in influenza infection pathogenesis (Akaike *et al*., 1996). It has been suggested that the main cause of mortality from influenza virus-induced pneumonia is the cytotoxicity, which in its turn is determined by the substantially increased levels of O_2^- rather than by the viral replication per se in the bronchial epithelial cells (Akaike *et al*., 1996). Thus the use of exogenous superoxide dismutase could be a new approach for the control of the disease. Oda *et al*. (1989) reported a dramatical reduction of mortality rates in experimental animals by application of Cu/Zn SOD conjugated with pyran polymer. Significant improvement of survival in lethal influenza virus infection has been achieved also by the application of recombinant Mn SOD (Sidwell *et al*., 1996) and Cu/Zn SOD from human erythrocytes (Sharonov *et al*., 1991). The protective effect of a novel naturally glycosylated Cu/Zn-containing SOD, produced from the fungal strain *H. lutea* 103 (HL-SOD) was evaluated in experimental influenza virus infection in mice, induced with virus A/Aichi/2/68 (Angelova *et al*., 2001). The experiments were done in parallel with SOD from bovine erythrocytes, the selective antiviral drug ribavirin and a plant polyphenol extract with established anti-influenza activity. HL-SOD proved to be the most effective in protecting the animals from mortality.

The presented results on the combined protective effect of HL-SOD and Rim indicate a beneficial role of combined use of viral inhibitors with diverse mechanisms of action for the treatment of experimental influenza virus infection in mice. This could be explained with an effective integration of different functions in the control of the infection.

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