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Recombinant superoxide dismutase (SOD) administered by aerosol inhibits respiratory syncytial virus infection in cotton rats

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Abstract

Recombinant (r) human (hu) manganese (Mn) and copper-zinc (CuZn) superoxide dismutase (SOD) were evaluated for their cytotoxicity and antiviral activity against respiratory syncytial virus (RSV) in tissue culture and in cotton rats. No apparent cytotoxicity or inhibition of RSV was observed in the tissue culture studies (both compounds had IC_{50} and EC_{50} values $\geqslant 1000$ μg/ml and a selective index = 1). However, significant reductions in mean pulmonary RSV titers (ranging between 0.5 and 1.9 $\log_{10}$/g of lung compared with the mean pulmonary viral titers detected in similarly inoculated, placebo-treated control animals) were seen in most of the experiments, in which experimentally infected cotton rats were exposed to continuous small-particle aerosols (reservoir concentrations $\geqslant 20$ mg/ml) containing either rhuMnSOD or rhuCuZnSOD. This protective effect was dose dependent and not observed when either rSOD compound was administered parenterally (intraperitoneally) or intranasally. No toxic effects were noted in any of the cotton rats exposed to aerosols of either rhuMn or CuZnSOD; nor was any evidence of drug-induced histopathology observed in sections of lung prepared from these animals.

Keywords: Respiratory syncytial virus; Respiratory syncytial virus, RSV; Antiviral; SOD; MnSOD; CuZnSOD; Cotton rats

1. Introduction

During serious respiratory virus infections, reactive oxygen species (e.g. O_2^-, OH* and H_2O_2) can be produced intracellularly by lung parenchymal cells and extracellularly by responding polymorphonuclear neutrophils (PMN) and macrophages (Freeman and Crapo, 1981; Fox et al., 1981; Shasby et al., 1982; Oda et al., 1989). Although these reactive oxygen species may benefit the host by contributing to the oxidative

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killing of the invading pathogens by activated phagocytes, they may also have detrimental effects, for example by increasing local inflammation and adding to the pulmonary tissue injury, morbidity and mortality induced by the virus (Blake, 1983; Tate and Repine, 1983; Oda et al., 1989).

Superoxide dismutase (SOD) enzymes can catalyze the dismutation of O_2^- to H_2O_2, thereby reducing tissue concentrations of O_2^- and diminishing the potential for tissue injury by the reactive oxygen species induced during infection (reviewed in Tsan, 1993). In eukaryotes, there are at least three forms of SOD, each encoded by a different gene, which are distinguishable by their tissue distribution and associated metal component. These isomers include two intracellular enzymes, manganese (Mn) SOD and copper–zinc (CuZn) SOD (Keller et al., 1991), and one extracellular form which also contains Cu and Zn as coenzymes (Fridovich, 1976; Marklund, 1984). The dominant SOD isoenzyme found extracellularly in the serum and on endothelial cells contains Cu and Zn (Marklund, 1984).

The mouse-influenza model has been used to study the role of oxygen free radicals in the pathogenicity of respiratory infections (Oda et al., 1989; Akaike et al., 1989; Maeda and Akaike, 1991; Buffinton et al., 1992). In this model, inoculation of mice with mouse-adapted influenza virus usually leads to marked infiltration of lymphocytes, PMN and macrophages around bronchi, bronchioles and interstitially, to extensive fibrosis of the lungs, to pulmonary hemorrhaging and widespread destruction of pulmonary tissues, and frequently (the extent depending on the dose of virus administered) to death of the inoculated animals (Loosli, 1949; Loosli et al., 1971; Wyde et al., 1977). Convincing evidence that oxygen free radicals contribute significantly to this pathology was published in 1989 by Oda et al., and by Akaike et al. In these studies, influenza virus-infected mice administered SOD conjugated to pyran intraveneously (i.v.), or allopurinol (an inhibitor of xanthine oxidase) orally, manifested significantly reduced inflammation, morbidity and mortality than matched animals given placebo. The protection observed from lethal infection did not correlate with reductions in pulmonary influenza virus titers (these were similar in both treated and placebo control groups), but did correspond to significant reductions in the levels of O_2^- and xanthine oxidase activity in the lungs of infected SOD-treated mice. It was concluded that oxygen radicals are important in the pathogenesis of influenza virus infection, and that SOD-containing compounds have therapeutic potential for this and other diseases associated with free radicals.

Oxygen free radicals may play a significant role in the pathogenesis of respiratory syncytial virus (RSV), a leading cause of serious lower respiratory tract infection in infants and children under two years of age (Parrott et al., 1973 and Glezen et al., 1982). No vaccines are currently available for prevention of RSV disease, and only one antiviral, ribavirin, is approved to treat infections caused by this virus. This paucity of available prophylactic and therapeutic agents to prevent or treat RSV disease, as well as the encouraging results seen in the mouse-influenza SOD studies, prompted us to evaluate genetically engineered recombinant human (rhu) Mn and CuZnSOD for their cytotoxicity and therapeutic potential against RSV in tissue culture and in cotton rats. The results obtained in these studies are summarized below.

2. Materials and methods

2.1. Animals

The cotton rats (*Sigmoden hispidus*) used in these studies were all descendants of six pairs of animals obtained in 1984 from the Small Animal Section of the Veterinary Research Branch, Division of Research Services, National Institutes of Health. All were housed in the Baylor College of Medicine (BCM) vivarium in cages covered with barrier filters, and all were given water and food ad libitum. Animals of either sex and weighing between 50 and 100 g were used in all experiments.

2.2. Tissue culture

Seed stocks of Hep-2 (human epithelial carcinoma; ATCC CCL 23), A549 (human lung carcinoma; ATCC CCL 185) and Vero (African green monkey kidney; ATCC CCL 81) tissue culture cells were obtained from the American Type Culture Collection (ATCC), Rockville, MD. Cultures of these cell lines were grown in monolayers using Eagle's minimal essential medium (MEM; BioWhittaker, Inc.), supplemented with 10% fetal calf serum (FCS; Sigma Chemical Co.), 100 units/ml penicillin (GIBCO Laboratories), 100 μg/ml streptomycin sulfate (GIBCO), 2 mM L-glutamine (GIBCO), and 0.2% sodium bicarbonate (Sigma Chemical Co.) as a growth medium. This same medium containing only 2% FCS was used to maintain cell cultures and as a diluent for all assays.

2.3. Viruses

The two RSV A subtypes utilized in these studies, RSVA2 (ATCC VR1302) and RSV Long (ATCC VR26), were obtained from the ATCC. The only RSV B subtype tested, RSV 18537, was obtained from Dr. Tony Piedra, Department of Microbiology and Immunology, Baylor College of Medicine. Working stocks of each of these viruses were prepared as described in detail previously (Wyde et al., 1993).

2.4. Compounds

Lyophilized rhuMnSOD (50% compound:50% sucrose) and CuZnSOD (no added sucrose) were provided by BioTechnology General, Kiryat Weizmann, Israel. The expression of both human enzymes in *Escherichia coli*, their specific activity and their purification to apparent homogeneity have been described previously (Hartman et al., 1986; Beck et al., 1988). The level of endotoxin in all preparations was < 0.4 units/mg protein.

Just prior to each experiment, the lyophilized rhuMn (40 mg/ml) or CuZnSOD (20 mg/ml) was suspended in distilled water (Baxter Healthcare Corp., Deerfield, IL) to make stock solutions containing 20 mg rhuSOD/ml. These solutions were sterilized by passing them through 0.2 mμ filters (Costar). Sterile distilled water (Baxter) was used as a placebo in experiments testing the rCuZnSOD or MnSOD lacking sucrose. Sucrose solution (20 mg sucrose in sterile, distilled water) was used as the placebo in experiments testing the rMnSOD containing 50% sucrose.

Concentrations ranging from 10 to 40 mg/ml (dose range: 10–80 mg/kg per day) were used in cotton rat experiments in which the SOD compounds were administered intraperitoneally (i.p.) or intranasally (i.n.). In all experiments in which the recombinant enzymes were delivered by aerosol, stock rhuMn or rhuCuZnSOD was added to the aerosol generating unit reservoirs (i.e. 20 mg rSOD/ml). In these experiments, the amount of rSOD administered to the animals was controlled by varying the interval of time during which the cotton rats were exposed to the aerosols.

Ribavirin was used in all in vitro and many in vivo experiments as a positive control. This compound was obtained from Viratek, Inc., Covina, CA, in powdered form. Just prior to the start of each experiment, sufficient ribavirin was dissolved in distilled water to make a solution containing 90 mg ribavirin/ml. This solution was then filter sterilized, and if used in in vitro experiments, diluted in MEM supplemented with 2% FCS to a make a new stock containing 4 mg ribavirin/ml. In in vivo experiments, the test animals were inoculated i.p. or i.n. with 0.10-ml volumes of the 90-mg ribavirin/ml stock solution, or exposed to aerosols generated from reservoirs containing 60 mg ribavirin/ml.

2.5. Aerosol delivery

Collison aerosol generators were used to produce the continuous small-particle aerosols described in this report. A detailed description of these nebulizers and their use to generate aerosols has been described in detail previously (May, 1973; Wilson et al., 1980; Knight and Gilbert, 1988). The concentration of SOD generated in the aerosols and their particle size distribution were determined by using an all-glass impinger and an

Andersen sampler, respectively (see Andersen, 1958 and Knight and Gilbert, 1988 for a more complete description). Samples from the all-glass impingers were analyzed directly, while those collected by the Andersen sampler were eluted from the collecting filters by first soaking the filters in 10 ml water for 1 h prior to analysis. The SOD eluted was then quantitated spectrophotometrically (absorbance at 210 nm) using a standard curve (range 0–50 μg/ml). Fig. 1 shows the particle size distribution and mass medium aerodynamic diameter (MMAD) obtained in these studies for rhuCuZnSOD. The particle size distribution and MMAD obtained for rhuMnSOD aerosols were almost identical (data not shown).

Cotton rats were exposed to the test aerosols by placing them in sealed plastic cages connected to the aerosol generating units by corrugated tubing as previously described (Gilbert and Wyde, 1988). The estimated doses of drug retained by the test animals were calculated as reported previously (Knight and Gilbert, 1988; Wyde et al., 1985), and were based on the amount of drug in the aerosol (determined using an all-glass impinger), the minute volume of the cotton rats (Phalen, 1984), an estimated retention factor for these animals (Hatch and Gross, 1964), and the duration of treatment. In several experiments, the actual concentrations of rhuMn or CuZnSOD in the lungs of cotton rats exposed to aerosols of these

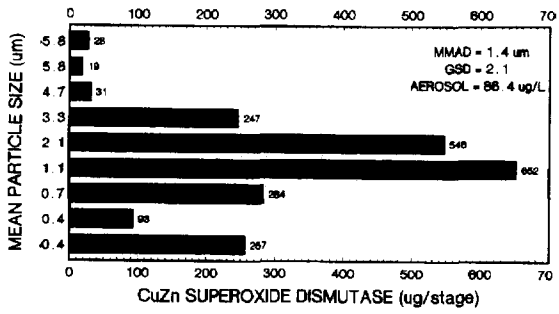

Fig. 1. Particle size distribution of recombinant human copper/zinc superoxide dismutase (CuZnSOD) generated by the Collison nebulizers utilized in the experiments presented in this report. Sizes were determined using an Anderson sampler. The mass median aerodynamic diameter (MMAD) and geometric standard deviation (G.S.D.) obtained for this particle distribution are also shown.

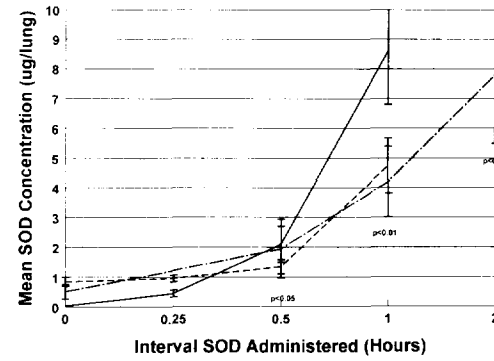

Fig. 2. Comparison of levels of recombinant human manganese superoxide dismutase (——) or copper/zinc superoxide dismutase (two experiments: Experiment 1, – – –, Experiment 2, – · – ·) in lungs of cotton rats exposed for different time intervals to continuous small-particle aerosols of these compounds. *P*-values were obtained by comparing the mean SOD concentrations obtained at each time interval with the mean background SOD concentration (indicated at time zero) in each experiment, using the nonparametric Kruskal-Wallis analysis of variance; number of animals per group = 4.

compounds were calculated after measuring the levels of rSOD in the broncho-alveolar lavage (BAL) fluids obtained from these animals using a radioimmunoassay (RIA; described below) specific for SOD (see Fig. 2 for examples).

2.6. *In vitro cytotoxicity assays*

In vitro cytotoxicity assays were performed in quadruplicate in sterile 96-well microtiter plates (Falcon 3072) as described previously (Wyde et al., 1993), with two exceptions: (1) XTT (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolium-5-carboxanilide; Sigma Chemical Co.) was used instead of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma) to measure mitochondrial respiration (and indirectly cell viability); and (2) the two compounds were tested in A549 and Vero, as well as Hep-2, tissue culture cells. All monolayers were observed microscopically prior to the addition of the XTT for inhibition of cellular proliferation and for evidence of drug-induced cytopathology.

The maximum concentration of each compound tested in these assays was 4 mg/ml (1 mg/ml final concentration). Test monolayers were

approximately 30% confluent at the start of each assay, and the optical density (OD) in each test well was ascertained using a 96-well plate reader (Molecular Devices UVMax spectrophotometer) set at a wave length of 490 nM. The median inhibitory concentration (IC_{50}) for each compound was calculated after determining the minimum concentration (μg/ml) of test compound that caused $\geq 50\%$ reduction in OD, compared to the mean OD obtained for the cell control wells, in half of the replicate wells.

2.7. In vitro antiviral assays

The assay used to assess and compare the antiviral activity of rhuMnSOD, rhuCuZnSOD and ribavirin in tissue culture has also been described previously (Wyde et al., 1993). In these studies, the two recombinant SOD enzymes were always tested in quadruplicate using a maximum final concentration of 4 mg/ml (1 mg/ml final concentration). All assay plates were incubated at 36°C in a (5%) CO_2 incubator until the virus control wells exhibited 70–100% cytopathic effects (CPE) including syncytia. All wells were then observed and scored for virus-induced CPE. The EC_{50} (median efficacious concentration) for each compound was calculated after determining the lowest concentration of antiviral that completely inhibited CPE in half of the replicate wells. If interpolation was required to help calculate either the IC_{50} or EC_{50}, the method of Karber (Rhodes and Van Rooyen, 1953) was used. At the conclusion of these assays, the IC_{50} obtained for rhuMnSOD, for rhuCuZnSOD and for ribavirin were divided by their respective EC_{50} values to obtain a selective index (SI) for each compound.

2.8. Collection of samples

Cotton rats were sacrificed using CO_2. The lungs from these animals were removed, rinsed in sterile phosphate-buffered saline (PBS; pH 7.2) and weighed. Each set of lungs was then transpleurally lavaged using 3 ml of 2% FCS-MEM media as reported previously (Wilson et al., 1980). The resulting BAL fluids were kept on ice until assayed for RSV (usually within 2 h of collecting the lungs). They were then frozen until tested for SOD levels in the RIA assay.

2.9. Virus quantification

Quantitative viral assays to determine the median tissue culture infectious doses ($TCID_{50}$/ml) present in viral pools and lungs were performed in quadruplicate or duplicate in 96-well tissue culture plates as described previously (Wyde et al., 1993; Gilbert et al., 1993). Median cotton rat infectious doses ($CRID_{50}$) in virus pools were determined by inoculating groups of cotton rats i.n. with serial 10-fold dilutions of RSV. The animals in each group were sacrificed 4 days later and their lungs were removed and assessed for the presence of RSV. The maximal dilution of the virus pool that infected half of the inoculated animals was then determined. The minimum amount of virus that was detectable was 1.3 $\log_{10}$/g lung.

2.10. Antiviral testing in vivo

Cotton rats were lightly anesthetized with Metofane (methoxyfurane, Pitman-Moore, Mundelein, IL) on day 0. While unconcious, each animal was weighed and then inoculated i.n. with approximately 100 $CRID_{50}$ of RSV in 0.1 ml. On days 1 through 3, the animals were either injected i.p. or i.n. with placebo, ribavirin or graded doses of rhuMn or CuZnSOD, or exposed for different periods of time on each of these days to continuous small-particle aerosols containing these enzymes. Each day the animals were observed for morbidity, death, diarrhea, lethargy or other untoward behavior. On day 4 after virus inoculation, the day of maximum RSV pulmonary infection in untreated cotton rats, all of the test animals were killed and weighed. The lungs of these animals were removed, weighed and transpleurally lavaged (Wilson et al., 1980). Each lavage fluid was then assessed for pulmonary RSV titers as described above, or for SOD levels as related below.

2.11. Quantitation of SOD in lungs

Levels of rhuMn and CuZnSOD in BAL fluids were determined using an RIA. This assay was performed in 96-well multiwell plates and utilized antibodies specific for rhuMn or CuZnSOD, ^{125}I-CuZnSOD labeled by the Bolton-Hunter reagent, and a diluent consisting of sodium chloride buffer (0.14 M NaCl, pH 7.4) containing 0.01 M sodium phosphate and 2 mg/ml of bovine serum albumin.

At the start of each assay, the wells of each plate contained approximately 5 ng ^{125}I-CuZnSOD and an appropriate amount of monoclonal antibody to give about 50% binding of SOD. BAL fluid samples or standard SOD solutions were added in 50 μl volume to these wells. After an overnight incubation at 4°C, immunocomplexes were separated from the unbound label by addition of normal mouse serum and a rabbit anti-mouse antibody. Under these conditions, the log displacement curve was linear between 2 and 20 ng/well of SOD. In SOD-exposed cotton rats, serum antibody levels were too low and could not be detected by this method.

2.12. Histologic studies

Lungs designated for histologic studies were collected as described above and placed in buffered formalin. After 24 h, each set of lungs was embedded in low-melting-point paraffin, and sectioned to 5-μm thickness. The resulting sections were deparaffinized, stained with hematoxylin and eosin and evaluated in a blinded fashion for evidence of histopathology.

2.13. Statistics

The Newman-Keuls multiple comparison test and the Kruskal-Wallis analysis of variance (ANOVA) were used to compare geometric mean RSV titers obtained for each cotton rat group. BAL samples with undetectable virus titers were assigned a value of 0.8. These same tests were also used to compare mean rhuSOD levels in the lung lavage fluids obtained from the different groups of test animals. Student's t-test was used to compare mean body weights. These tests and the determination of all descriptive statisitics (i.e. geometric mean titers and standard deviations) were performed using True Epistat, a statistical program designed by T.L. Gustafson of Epistat Services, Richardson, Texas, for IBM-compatible computers.

3. Results

3.1. Cytotoxicity of rhuMn and CuZnSOD in vitro

No evidence of cytotoxicity for either rhuMn or CuZnSOD was seen in the in vitro cell proliferation assays performed during the course of these studies. Thus, no significant inhibition of cell growth or cytopathology was observed in any of the replicate wells containing these recombinant human enzymes, in any of these assays. Nor were any significant reductions in mean OD readings measured in these wells following the addition of XTT. This was true regardless of the tissue culture cell line (i.e. Hep-2, A549 or Vero) or concentration of rhuMnSOD or rhuCuZnSOD tested (1 mg/ml being the maximum final concentration of both compounds tested). In contrast, the highest dose of ribavirin tested (1 mg/ml final concentration) did inhibit tissue culture cell growth, cause significant rounding up of test cells, and reduce mean OD readings by approximately 50% in the XTT assay (data not shown).

3.2. Antiviral activity of rhuMn and CuZnSOD in vitro

The rhuMn and CuZnSOD also did not exhibit any apparent antiviral activity in tissue culture assays. Accordingly, no significant inhibition of RSV-induced CPE was observed even at the maximum concentrations of rhuMn or CuZnSOD tested (i.e. 1 mg/ml final concentration; data not shown). In contrast, RSV-induced CPE was totally inhibited in all test wells containing 15.6 μg ribavirin/ml, leading to a calculated (using interpolation) EC_{50} for this compound of 11.7 μg/ml (data not shown). Based on the results obtained in

Table 1
Mean RSV titers obtained in three representative experiments testing recombinant human MnSOD delivered by continuous small-particle aerosol to cotton rats experimentally inoculated with RSV[a]

Expt. no.	Treatment	Reservoir conc. ribavirin or SOD (mg/ml) [b]	Pulmonary RSV (GMT $\log_{10}$/g lung) ($\pm$ S.D.)	$\log_{10}$ reduction pulmonary virus	P-value [c]
1	Placebo 2 × 1 h	0	5.3 ± 0.3		–
	MnSOD 2 × 1 h	20	3.8 ± 0.3	1.5	0.004
2	Placebo 2 × 1 h	0	4.9 ± 0.4		
	MnSOD 2 × 1 h	20	4.4 ± 0.1	0.5	0.04
3	Placebo 2 × 1 h	0	3.6 ± 0.4	–	
	MnSOD 2 × 1 h	20	3.1 ± 0.1	0.5	0.06

[a] In all three of these experiments, the cotton rats were inoculated intranasally with approximately 100 median cotton rat infectious doses of RSV A2 on day 0 and then exposed twice daily (from 08:00–09:00 and 15:00–16:00) on days 1 through 3 to continuous aerosols of placebo (20 mg/ml sucrose in sterile, distilled water) or MnSOD. All of the animals were sacrificed on day 4 when their lungs were removed and assessed for levels of RSV. The other seven experiments in this series of 10 had similar experimental designs, except that in some the animals were inoculated with RSV Long or RSV 18537, and sometimes the duration of treatment was varied.
[b] Abbreviations: conc. = concentration; expt. = experiment; GMT = geometric mean titer; S.D. = standard deviation; no. = number; h = hour.
[c] P-values were obtained by comparing the mean virus titers obtained in each experiment using the nonparametric Kruskal-Wallis analysis of variance; number of animals per group = 4 or 5.

the in vitro cytotoxicity and antiviral assays, rhuMnSOD, rhuCuZnSOD and ribavirin were assigned selective indices of 1 (> 1 mg/ml divided by > 1 mg/ml = 1), 1 and 85.5 (1 mg/ml divided by 0.0117 mg/ml = 85.5), respectively.

3.3. Antiviral activity of rhuMn and CuZnSOD in vivo

The rhuMn and CuZnSOD also failed to exhibit antiviral activity in cotton rats following i.p. or i.n. administration. With the exception of the control groups treated with ribavirin, in none of the six experiments performed in this series was a significant reduction in mean pulmonary RSV titer seen on day 4 in any test group, compared with the mean pulmonary virus titer seen in control animals comparably given placebo (data not shown). This was true of even the groups of cotton rats inoculated on days 1 through 3 with the maximum doses of rhuMn or rhuCuZnSOD tested (i.e. 80 mg of either rhuMn or CuZnSOD/kg per day).

In contrast to the results obtained when the rhuMn and CuZnSOD were administered i.p. or i.n., significantly reduced mean pulmonary RSV titers were observed in eight of 10 experiments assessing the antiviral activity of rhuMnSOD administered by aerosol, and in four of five experiments evaluating the antiviral activity of rhuCuZnSOD delivered by this route of administration. Data obtained from three representative experiments performed in the series of 10 experiments testing rhuMnSOD are shown in Table 1. Results obtained in the four successful experiments evaluating rhuCuZnSOD are presented in Tables 2 and 3.

As the data in Table 1 reflect, there was some variability in the decreases in mean pulmonary virus titer observed in the experiments testing rhuMnSOD by aerosol. Thus in three experiments, reductions in pulmonary RSV titers were ≥ 1.5 $\log_{10}$/g lung (e.g. Experiment 1 in Table 1); in five experiments, significant ($P \leq 0.05$), but lower reductions in mean pulmonary virus titers ranging from 0.5 to 0.9 $\log_{10}$/g were observed (e.g. Experiment 2 in Table 1); and in two experiments, no significant ($P > 0.05$) reductions in mean pulmonary virus titers were seen (e.g. Experiment 3 in Table 1). Less variability in mean virus titer reductions was observed in the experiments testing rhuCuZnSOD. Thus in all four experiments

Table 2
Comparison of the mean pulmonary RSV titers in cotton rats experimentally inoculated with this virus and treated with either ribavirin or recombinant human CuZnSOD delivered by continuous small-particle aerosol [a]

Expt.no.	Treatment	Reservoir conc. ribavirin or SOD (mg/ml) [b]	Pulmonary RSV (GMT $\log_{10}$/g lung) ($\pm$S.D.)	Log_{10} reduction pulmonary virus	P-value [c]
1	H_2O 2 × 1 h	0	4.4 ± 0.3	–	–
	Ribavirin 2 × 1 h	60	2.5 ± 1.2	1.9	0.01
	CuZnSOD 2 × 1 h	20	3.2 ± 0.6	1.2	0.05
2	H_2O 2 × 1 h	0	4.9 ± 0.3		–
	Ribavirin 2 × 1 h	60	2.9 ± 0.7	2.0	0.01
	CuZnSOD 2 × 1 h	20	3.4 ± 0.9	1.5	0.05
	CuZnSOD 2 × 0.5 h	20	3.8 ± 0.3	1.1	Not sig.

[a] In each experiment, the cotton rats were inoculated intranasally with approximately 100 median cotton rat infectious doses of RSV on day 0, and with the exception of the last group in Experiment 2, exposed twice daily for 1 h (from 08:00–09:00 and 15:00–16:00) on days 1–3 to continuous aerosols of water, ribavirin or CuZnSOD. The last group in Experiment 2 was exposed twice daily for 30 min (from 08:00–09:30 and 16:00–16:30). All of the animals were sacrificed on day 4, when their lungs were removed and assessed for levels of RSV.
[b] Abbreviations: conc. = concentrations; GMT = geometric mean titer; S.D. = standard deviation; h = hour; not sig. = not statistically significant.
[c] P-values were obtained by comparing the mean virus titers obtained in each experiment using the nonparametric Kruskal-Wallis analysis of variance; number of animals per group = 4.

involving this recombinant enzyme in which significant reductions in mean pulmonary RSV titers occurred, the maximum decreases in mean pulmonary virus titers was $\geqslant 1.1$ $\log_{10}$/g lung.

3.4. Determination of the minimal effective dose

The minimum dose of rSOD needed to inhibit growth of RSV in cotton rats was determined by exposing groups of these animals for different intervals of time to aerosols containing these enzymes (e.g. Experiment 3 in Table 2, and both experiments in Table 3), while keeping the starting concentrations of rSOD in the aerosol reservoirs constant (i.e. 20 mg rSOD/ml). In these experiments, statistically significant reductions in mean pulmonary RSV titer occurred only in groups of cotton rats exposed to aerosols for a minimum of 2 h/day (1 h each morning and 1 h each afternoon), and not in groups exposed to aerosols of the rSOD enzymes for $\leqslant 1$ h/day. Using this information and the equation for calculating the dosage of drug delivered to the animals by aerosol (see Knight and Gilbert, 1988 and Wyde et al., 1985), the estimated minimal effective dose for both rhuMnSOD and CuZnSOD was determined to be between 8.4 (the dose estimated to be delivered to animals exposed for 2 h) and 4.2 (the dose estimated to be delivered to animals exposed for 1 h) mg/kg per day.

3.5. Pulmonary rSOD levels and protection

The mean concentrations of rhuMn and CuZnSOD measured by RIA in the lungs of cotton rats exposed to aerosols of rhuSOD for different intervals of time in three different experiments are shown in Fig. 2. As the curves in this figure indicate, in each test, the levels of rhuMn (——) and CuZnSOD (– – – or – · –) measured in the BAL fluids initially rose slowly and did not reach statistically significant levels ($P < 0.05$) in any experiment (compared to the levels of SOD measured in control cotton rats not exposed to the aerosols [i.e. the values shown at time 0]), until 0.5 h after initiating aerosolization. Moreover, in all three experiments, a more rapid accumulation of SOD was observed after this time, resulting in highly statistically different (i.e. $P < 0.01$) differences at the 1 h interval. The marked increases in

Table 3
Comparison of the mean pulmonary RSV titers in lungs of RSV-inoculated cotton rats exposed to continuous small-particle aerosols of recombinant human CuZnSOD for different periods of time [a]

Expt. no.	Treatment	Reservoir conc. ribavirin or SOD (mg/ml) [b]	Pulmonary RSV (GMT $\log_{10}$/g lung) ($\pm$S.D.)	Log_{10} reduction pulmonary virus	P-value [c]
1	H_2O 2×1 h	0	4.4 ± 0.3		
	Ribavirin 2×1 h	60	3.7 ± 0.3	0.7	0.05
	CuZnSOD 2×0.25 h	20	4.2 ± 0.8	0.2	Not sig.
	CuZnSOD 2×0.5 h	20	3.9 ± 0.3	0.5	Not sig.
	CuZnSOD 2×1 h	60	2.5 ± 1.3	1.9	0.001
2	H_2O 2×2 h	0	4.0 ± 0.3		
	CuZnSOD 2×0.5 h	20	3.5 ± 0.3	0.5	Not sig.
	CuZnSOD 2×1 h	20	3.1 ± 0.3	0.9	Not sig.
	CuZnSOD 2×2 h	20	2.8 ± 0	1.2	0.01

[a] In each experiment, the cotton rats were inoculated intranasally with approximately 100 median cotton rat infectious doses of RSV on day 0, exposed to aerosols of water or CuZnSOD for different intervals of time on days 1–3, and killed on day 4, when their lungs were assessed for levels of RSV.
[b] Abbreviations: conc. = concentration; GMT = geometric mean titer; S.D. = standard deviation; h = hour(s); Not sig. = not significant.
[c] P-values were obtained by comparing the mean virus titers obtained in each experiment using the nonparametric Kruskal-Wallis analysis of variance; number of animals per group = 4 or 5.

rhuSOD seen in the lungs of test animals in these experiments after 1 h of aerosolization coincided with the minimal interval of aerosolization needed for protection (see Experiment 2, Table 2 and both experiments in Table 3 for examples).

3.6. Characterization of the aerosolized rSOD particles

The size distribution and MMAD of the particles of rhuCuZnSOD generated in the aerosol experiments performed in these studies are shown in Fig. 1. Virtually identical results were obtained in similar testing of rhuMnSOD aerosols. As the bars in Fig. 1 indicate, the distribution of the rhuCuZnSOD particles produced in these experiments was essentially normal. Moreover, the MMAD was determined to be 1.4 μm ($\pm$ a GSD 2.1).

3.7. Preliminary toxicity testing in cotton rats

No diarrhea, morbidity, death, weight loss or other untoward symptoms were evident in any of the cotton rats inoculated or exposed to aerosols of rhuMn or CuZnSOD in these studies. Moreover, no significant histopathologic findings (leukocyte infiltration, hyperplasia, edema) were evident in hematoxylin- and eosin-stained lung sections prepared from lungs obtained from uninfected cotton rats exposed to aerosols of placebo, rhuMnSOD or rhuCuZnSOD. A predominantly peribronchilar infiltration of leukocytes (PMN and mononuclear leukocytes) was evident in stained lung sections obtained from both RSV-infected cotton rats treated with placebo, and those exposed for different intervals to aerosols of either recombinant enzyme. However, there was no significant difference between the degree of infiltration or histopathology seen in the placebo- and rSOD-treated infected animals. Thus, in these studies, neither rSOD compound appeared to be cytotoxic.

4. Discussion

The studies described in this report were directed at evaluating the antiviral potential of genetically engineered rhuMn and CuZnSOD

against RSV. No evidence of antiviral activity was seen in in vitro testing of either compound. Nor was any significant inhibition of RSV observed when either of the rSOD enzymes was administered parenterally or i.n. to cotton rats. (Intravenous administration was not considered because the unusual architecture of cotton rat tails make the veins in this appendage virtually inaccessable for injection.) In contrast, when rhuMn or CuZnSOD was administered directly to the pulmonary tract by aerosol, significant reductions in pulmonary RSV titers were observed in infected treated cotton rats (see Table 1).

A total of 10 experiments administering rhuMnSOD by aerosol were performed. The reason for this repetition was three-fold: 1) the inhibition of pulmonary RSV replication did not correspond with what had been reported in the mouse-influenza model, nor with the results obtained in our own in vitro antiviral assays. Therefore, it was important to ascertain that the finding of inhibition of virus following aerosol delivery was accurate; 2) notwithstanding that in 8 of 10 of the experiments performed significant reductions in pulmonary RSV titers compared with placebo control animals were seen, variations in the magnitude of protection were observed from experiment to experiment (see Table 1 and Section 3 above); and 3) while the initial experiments in this series looked at a single dose of SOD and treatment interval (i.e. 20 mg rhuMnSOD/ml reservoir concentration administered twice daily for 1 h), later experiments looked at the effects of this compound administered for different intervals of time. At the end of this set of experiments it was concluded that rhuMnSOD administered by aerosol indeed inhibits RSV replication in the lungs of cotton rats.

All of the findings with rhuMnSOD were duplicated in the succeeding studies performed with rhuCuZnSOD. Thus, as with the rhuMnSOD, antiviral activity was not seen in in vitro testing or in cotton rats following i.p. or i.n. administration (data not shown), but was observed when the rhuCuZnSOD was delivered by aerosol (see Tables 2 and 3). In these experiments, the reductions in pulmonary RSV titers seen in animals exposed twice daily to 1-h treatments of rhuCuZnSOD ranged from 0.9 to 1.9 $\log_{10}$/g of lung, which was generally higher than the magnitude of reductions seen in infected cotton rats similarly exposed to rhuMnSOD (i.e. most of the reductions in pulmonary virus titers seen in the experiments testing rhuMnSOD ranged from 0.5 to 0.9 $\log_{10}$/g lung).

In no experiment performed during the course of these studies was a significant decrease in mean pulmonary virus titer seen in any group of animals exposed to aerosols of either rhuMn or CuZnSOD twice daily for less than 1 h/treatment. However, significant reductions in mean pulmonary virus titers were usually observed in cotton rats exposed two times/day for at least 1 h/treatment (see Tables 1–3). It thus appeared that the minimal effective dose for both rhuMnSOD and CuZnSOD was between 8.4 and 4.2 mg/kg per day.

It should be noted that in all of the assays measuring rSOD levels in the lungs of cotton rats exposed for different intervals to aerosols of either rhuMn or CuZnSOD, the pulmonary concentrations of these compounds appeared to still be rising at the time that the aerosolizations were stopped (i.e. after 1 or 2 h; see Fig. 2). This failure to see a plateauing of pulmonary drug levels in these studies suggest that most longer therapy intervals may have provided greater protection against virus infection than were seen. Although this supposition was testable, it was not due to the limited amount of rhuMn and CuZnSOD available to us.

The mechanism of action by which the rhuMn or CuZnSOD used in these studies inhibited the growth of RSV in cotton rats is not clear. The inability of even the highest doses of either rSOD compound to inhibit RSV replication in tissue culture suggests that the inhibition seen in cotton rats was not due to a direct antiviral activity, but more likely to an indirect mechanism (for example, by activating macrophages and/or natural killer cells). However, in preliminary experiments looking at the number and activation states of the cells obtained by lavage from lungs of RSV-infected cotton rats exposed to aerosols of placebo or rSOD cells (95% macrophages, lymphocytes and PMN), we have not been able to detect significant differences. Although uninformative,

these results are in agreement with our observation (stated above in the Section 3) that there was no significant difference in the number of inflammatory cells present in sections of lungs prepared from RSV-infected cotton rats treated with placebo or either rhuSOD in these studies.

It is possible that the antiviral activity of the rSOD enzymes was mediated by interferon. Very recently data has been published that indicates that MnSOD is involved in the establishment of the antiviral states induced in mice by alpha and gamma interferons (Raineri et al., 1996). Experiments are currently being designed to investigate whether a similar relationship is taking place in the cotton rat-RSV model.

It is also not known why virus-inhibition was observed in the present studies and not in those performed in the mouse-influenza model. However, comparisons are difficult because the different studies utilized different host animals (mice versus cotton rats), viruses (influenza versus RSV) and routes of drug administration (intravenous versus aerosol).

It has previously been established that aerosolized particles exceeding a MMAD of 5 μm deposit primarily in the upper respiratory tract where they are swallowed rapidly, whereas particles with an MMAD of 3 μm or less penetrate throughout the respiratory tract (Hatch and Gross, 1964). It was thus of interest to determine if the aerosol particles of SOD generated in the present studies were of the appropriate size to account for the antiviral activity seen. As indicated by the data shown in Fig. 1, the Collison nebulizers used in these studies generated rSOD containing particles with a MMAD of 1.4 μm ($\pm$2.1 geometric mean standard deviation). Thus, the particles were likely to be distributed throughout the lungs of the treated cotton rats. On the other hand, it is likely that neither compound was efficacious when given i.p., due to failure to get enough compound to the lungs, or when administered i.n., because of poor or uneven distribution of the compounds throughout the respiratory tract when given by this route of administration.

Although only preliminary toxicity testing was performed in these studies, no evidence of untoward activity was observed. It should be noted that rhuCuZnSOD enzyme has been used in human trials to treat reperfusion injuries following myocardial infraction and in kidney transplantations without any apparent adverse activity (personal communication, Biotechnology General). Based on the apparent safety of this material in these clinical trials, and on the toxicity and efficacy results presented in this report, further investigations of rhuMn and CuZnSOD enzymes to treat RSV infections in humans appear warranted.

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References

Akaike, T., Ando, M., Oda, T., Doi, T., Iijiri, S., Araki, S. and Maeda, H. (1989) Dependence on O_2 generation by xanthine oxidase of pathogenesis of influenza virus infection in mice. J. Clin. Invest. 85, 739–745.

Andersen, A.A. (1958) New sampler for the collection, sizing, and enumeration of viable airborne particles. J. Bacteriol. 76, 471–484.

Beck, Y., Bartfeld, D., Yavin, Z., Levanon, A., Gorecki, M. and Hartman, J.R. (1988) Efficient production of active human manganese superoxide dismutase in *Escherichia coli*. Biotechnology 6, 930–935.

Blake, D.R. (1983) Effect of a specific iron chelating agent on animal models of inflammation. Ann. Rheum. Dis. 42, 89–93.

Buffinton, G.D., Christen, S., Peterhans, E. and Stocker, R. (1992) Oxidative stress in lungs of mice infected with influenza A virus. Free Rad. Res. Commun. 16, 99–110.

Fox, R.B., Hoidal, J.R., Brown, D.M. and Repine, J.E. (1981) Pulmonary inflammation due to oxygen toxicity: involvement of chemotactic factor and polymorphonuclear leukocytes. Am. Rev. Respir. Dis. 123, 521–523.

Freeman, B.A. and Crapo, J.D. (1981) Hyperoxia increases oxygen radical production in rat lungs and lung mitochondria. J. Biol. Chem. 256, 10986–10992.

Fridovich, I. (1976) Oxygen radicals, hydrogen peroxide and oxygen toxicity. In: W.A. Pryor (Ed), Free Radicals in Biology, pp. 239–277. Academic Press, New York.

Gilbert, B.E. and Wyde, P.R. (1988) Pharmacokinetics of ribavirin aerosol in mice. Antimicrob. Agents Chemother. 32, 117–121.

Gilbert, B.E., Wyde, P.R., Wilson, S.Z. and Meyerson, L.R. (1993) SP-303 small-particle aerosol treatment of influenza A virus infection in mice and respiratory syncytial virus infection in cotton rats. Antiviral Res. 21, 37–45.

Glezen, W.P., Loda, F.A. and Denny, F.W. (1982) The parainfluenza viruses. In: A.S. Evans (Ed), Viral Infections of Humans: Epidemiology and Control, pp. 337–349. Plenum Press, New York.

Hartman, J.R., Geller, T., Yavin, Z., Bartfeld, D., Kanner, D., Aviv, H. and Gorecki, M. (1986) High-level expression of enzymatically active human Cu Zn superoxide dismutase in *Escherichia coli*. Proc. Natl. Acad. Sci. USA 83, 7142–7146.

Hatch, T.F. and Gross, P. (1964) Experimental studies on deposition of inhaled aerosols. In: T.F. Hatch and P. Gross (Eds), Pulmonary Deposition and Retention of Inhaled Aerosols, pp. 45–65. Academic Press, New York.

Keller, G.A., Warner, T.G., Steimer, K.S. and Hallewell, R.A. (1991) CuZn superoxide dismutase is a peroxisomal enzyme in human fibroblasts and hepatoma cells. Proc. Natl. Acad. Sci. USA 88, 7381–7385.

Knight, V. and Gilbert, B. (1988) Antiviral therapy with small particle aerosols. Eur. J. Clin. Microbiol. Infect. Dis. 71, 721–731.

Loosli, C.G. (1949) The pathogenesis and pathology of experimental air-borne influenza virus A infections in mice. J. Infect. Dis. 84, 153–168.

Loosli, C.G., Buckley, R.D., Hardy, J.D., Hertweck, M.S., Kow, S.H., Serebrin, R., Ryan, D.P. and Stinson, S.F. (1971) The pathogenesis of postinfluenzal collapse of the lungs of mice. Trans. Assoc. Am. Physicians 84, 182–189.

Marklund, S.L. (1984) Extracellular superoxide dismutase and other superoxide dismutase isozymes in tissues from nine mammalian species. Biochem. J. 222, 649–655.

Maeda, H. and Akaike, T. (1991) Oxygen free radicals as pathogenic molecules in viral diseases. Proc. Soc. Exp. Biol. Med. 198, 721–726.

May, K.R. (1973) The collision nebulizer: description, performance and application. Aerosol Sci. 4, 235–243.

Oda, T., Akaike, T., Hamamoto, T., Suzuki, F., Hirano, T. and Maeda, H. (1989) Oxygen radicals in influenza-induced pathogenesis and treatment with pyran polymer-conjugated SOD. Science 244, 974–976.

Parrot, R.H., Kim, H.W., Arrobio, J.O., Hodes, D.S., Murphy, B.R., Brandt, C.D., Camargo, E., and Chanock, R.M. (1973) Epidemiology of respiratory syncytial virus infection in Washington, D.C. Am. J. Epidemiol. 98: 289–300.

Phalen, R.F. (1984) Inhalation Studies: Foundations and Techniques, p. 222. CRC Press, Boca Raton, FL.

Raineri, I., Huang, T.T., Epstein, C.J. and Epstein, L.B. (1996) Antisense manganese superoxide dimutase mRNA inhibits the antiviral action of interferon-gamma and interferon-alpha. J. Interferon and Cytokine Res. 16, 61–68.

Rhodes, A.J. and Van Rooyen, C.E. (1953) Textbook of Virology (2nd edition), pp. 66–69. Williams and Wilkins Co., Baltimore, MD.

Shasby, D.M., Fox, R.B., Harada, R.N. and Repine, J.E. (1982) Reduction of the edema of acute hyperoxic injury by granulocyte depletion. J. Appl. Physiol. 52, 1237–1244.

Tate, R.M. and Repine, J.E. (1983) Neutrophils and the adult respiratory distress syndrome. Am. Rev. Respir. Dis. 128, 552–559.

Tsan, M.-F. (1993) Superoxide dismutase and pulmonary oxygen toxicity. Proc. Soc. Exp. Biol. Med. 203, 286–290.

Wilson, S.Z., Knight, V., Wyde, P.R., Drake, S. and Couch, R.B. (1980) Amantadine and ribavirin aerosol treatment of influenza A and B infection in mice. Antimicrob. Agents and Chemother. 17, 642–648.

Wyde, P.R., Couch, R.B., Mackler, B.R., Cate, T.R. and Levy, B. (1977) Effects of low and high passage influenza virus infection in normal and nude mice. Infect. Immun. 15, 221–229.

Wyde, P.R., Sun, C.-S., Wilson, S.Z. and Knight, V. (1985) Duration of effect of interferon aerosol prophylaxis of vesicular stomatitis virus infection in mice. Antimicrob. Agents Chemother. 27, 60–64.

Wyde, P.R., Meyerson, L.R. and Gilbert, B.E. (1993) An in vitro evaluation of the antiviral activity of SP-303, an Euphorbiaceae shrub extract, against a panel of respiratory viruses. Drug Dev. Res. 28, 467–472.