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THE ROLE OF SUPEROXIDE ANIONS IN THE ESTABLISHMENT OF AN INTERFERON-α-MEDIATED ANTIVIRAL STATE

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It has been suggested that CuZn-superoxide dismutase (CuZnSOD) is required for the establishment of an interferon (IFN)-mediated antiviral state. To investigate this possibility further, a panel of 6 stably transfected HeLa clones, expressing CuZnSOD activity from 1.6 to 7.3 times the normal level, were treated with different concentrations of recombinant human interferon alpha A (rHuIFN-αA) followed by challenge with vesicular stomatitis virus (VSV). A biphasic response curve was generated ($r = 0.87$, $p < 0.025$). Clones with up to 3-fold basal level CuZnSOD activity exhibited an *inverse* relationship between their ability to generate an IFN-α-mediated antiviral state and CuZnSOD activity: the *higher* the CuZnSOD activity, the *lower* the sensitivity to IFN-α and the more IFN-α required for antiviral defense. Clones with between 4 to 7.3 times higher CuZnSOD activity than the non-transfected HeLa control showed a *direct* relationship between the CuZnSOD activity and the sensitivity to IFN-α. Furthermore, in agreement with the results obtained with the *SOD1*-transfected HeLa cells with up to 3 times the basal SOD activity, fetal fibroblasts derived from *SOD1*-transgenic mouse strains, TgHS-229 and TgHS-218, which also express 3 times the basal CuZnSOD activity, required higher IFN-α to achieve 50% protection. These results suggest a possible role for superoxide anion in the establishment of IFN-mediated antiviral effect, especially in the dose-response region in which the *inverse* relationship between the generation of the IFN-α-mediated antiviral state and CuZnSOD activity was observed. To assess this possibility, allopurinol was used as a xanthine oxidase inhibitor and hydroxyl radical scavenger in the IFN-α-mediated antiviral assay. Addition of 3 mM allopurinol diminished the IFN-mediated antiviral effect by between 40 and 50% ($p < 0.01$), and there was a reduction in superoxide generation ($p < 0.05$). The degree of reduction caused by allopurinol treatment was higher at an IFN-α concentration of 10 U/ml than at 100 U/ml, and there was no correlation between CuZnSOD activity and the degree of reduction. To establish further the role of superoxide as an antiviral agent, paraquat was used as a superoxide generator in the absence of IFN-α in the antiviral assay. Although paraquat at high concentrations is toxic to the cells, it actually showed a protective effect against VSV infection, and an inverse relationship ($r = 0.79$, $r < 0.025$) between cell survival and CuZnSOD activity was observed with 150 mM paraquat treatment. Treatment with paraquat plus IFN-α had a greater effect than treatment with either agent alone, and the degree of the antiviral response was inversely proportional to CuZnSOD activity over the entire range analyzed. Taken together, these results strongly support a role for superoxide and CuZnSOD in the generation and regulation of the IFN-α mediated antiviral state.

KEY WORDS: CuZn superoxide dismutase, HeLa cells, allopurinol, paraquat, transgenic mice

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INTRODUCTION

The interferons (IFNs) comprise a family of regulatory proteins that have antiviral and antiproliferative effects when bound to target cells.[1] Following binding to specific cell surface receptors, signals are transduced to the nucleus where transcriptional stimulation of a set of responsive genes takes place. Identification and characterization of interferon responsive genes and their products have led to an understanding of some of the mechanisms underlying IFN-mediated antiviral and antiproliferative effects.[1,2] Cellular responses to interferon treatment include the activation and induction of over 10 different polypeptides.[1,2] Interestingly, among the interferon-inducible proteins are two involved in oxygen metabolism, xanthine oxidase and 2,3-dioxygenase,[2] implying a possible role of superoxide in the interferon-mediated antiviral and antiproliferative states.

Superoxide radicals are generated in all oxygen-utilizing organisms during normal metabolic reactions. In polymorphonuclear neutrophils (PMNs) and macrophages, a large quantity of superoxide is produced in the "respiratory burst" and serves as a potent antimicrobial agent. In addition, the generation of superoxide can be induced by a number of chemicals and proteins, such as quinones,[3] interferons and tumor necrosis factors.[4,5] It is now well established that superoxide radicals and their products, hydrogen peroxide and hydroxyl radicals, may lead to DNA and RNA single-strand breaks, lipid peroxidation, and protein crosslinks, which may eventually lead to cell death.[6–8] To protect cells from these deleterious effects, superoxide is either physically separated from other cellular components, as in the phagosomes of PMNs and macrophages, or is immediately removed by one of the superoxide dismutases.

The genes encoding the IFN-α receptor (*IFNAR*), the IFN-α and the IFN-γ receptor accessory or transducing factors, and CuZn-superoxide dismutase (*SOD1*) are alllocated on human chromosome 21 and mouse chromosome 16,[9–12]an evolutionarily conserved physical linkage that might imply some functional relatedness of the gene products. The relationship between the IFN-mediated antiviral effect and CuZnSOD activity was examined previously[13] and revealed a possible requirement for CuZnSOD in the establishment of an IFN-α-mediated antiviral state. However, this relationship was not conclusively established, and the role of superoxide in the cellular antiviral defense system was not explored. Therefore, we have used a panel of 6 stably transfected HeLa clones expressing up to 7.3 times normal levels of CuZnSOD[14] and two lines of mouse *SOD1*-transgenic fetal fibroblasts expressing 3 times normal CuZnSOD activity[15] to investigate the possible function of superoxide radicals and to explore further the role of CuZnSOD in the establishment of the IFN-α-mediated antiviral state.

MATERIALS AND METHODS

Tissue Culture Media and Reagents

Dulbecco's modified Eagle's medium (DMEM), HEPES buffer (N-[2-hydroxyethyl]-piperazine-N′-[2-ethanesulfonic acid]), Hank's balanced salt solution (HBSS), and F12 nutrient mix were prepared by the UCSF Tissue Culture Facility. Fetal calf serum (FCS) was from Hyclone Laboratories (Logan, UT), and the serum substitute Ultroser G was from IBF Biotechnics (France). Neomycin sulfate (G418, Geneticin) was purchased from GIBCO Laboratories (Grand Island, NY). Recombinant human

IFN-α (rHuIFN-αA) was provided by Hoffmann-LaRoche Inc. (Nutley, NJ) and the recombinant mouse IFN-α (rMuIFN-α) was purchased from Holland Biotechnology (Holland). All other chemicals and reagents were purchased from Sigma (St. Louis, MO).

Primary Cultures of Embryonic Fibroblasts

Fetal fibroblasts were derived from 14 to 15-day-old mouse embryos of *SOD1*-transgenic mouse strains TgHS-218 and TgHS-229[15] and from non-transgenic littermates and were cultivated in serum-free medium composed of 50% DMEM in 15 mM HEPES/50% F12 medium supplemented with 2% Ultroser G as previously described.[14]

SOD1-Transfected HeLa Cells

HeLa cells were transfected with the plasmid pGSOD-SVneo,[16] containing human genomic *SOD1*, by liposome transfection,[17] The stably transfected clones were selected with G418 at 800 μg/ml. Six clones expressing progressively higher levels of CuZnSOD activity, ranging from 1.6–7.3 times basal level, were obtained for further studies.[14] The established lines, designated SOD1-1, SOD1-2, SOD1-14, SOD1-17, SOD1-22, and SOD1-29, were routinely maintained in DMEM with 10% FCS and G418 at 500 μg/ml.

Enzyme Assay

CuZnSOD activity in cell lysates, expressed in U/mg protein, was measured by procedures described previously.[18,19] Ferricytochrome c was used as the electron acceptor and xanthine/xanthine oxidase as the superoxide generating system in a Na-bicarbonate buffer, pH 10.0. Human CuZnSOD was used to generate a standard curve in each assay.

Antiviral Assay

The microtiter antiviral assay was based on the method described by Beresini *et al.*[20] with some modifications. The transfected and control HeLa cells were seeded at 1×10^4 per 100 μl per well in flat-bottom 96-well microtiter plates and incubated at 37°C overnight. The culture medium was then removed by gentle suction, and different combinations of IFN-α, allopurinol and paraquat were added at 100 μl per well to a row of 6 wells in a 96-well microtiter plate. The plates were incubated at 37°C for 24 h, the cells challenged with vesicular stomatitis virus (VSV), and the incubation continued for another 24 h. The culture medium was then removed by gentle suction, and the cells were washed 3 times with 160 μl per well of Ca^{2+}, Mg^{2+}-free PBS. Cell lysis was achieved by adding 160 μl H_2O and 40 μl concentrated Bio-Rad protein reagent to each well, and absorbance at 600 nm was determined with a Titertek microtiter plate reader. Mean ± SEM cell survivals were calculated for each treatment from the optical density (OD) readings of the 6 replicate wells, and the degree of protection (in percent) resulting from each treatment was calculated as the mean OD_{600} of the VSV-infected cells divided by that of the mock infected controls × 100. The antiviral assay of mouse fetal fibroblasts followed the same procedure except that the fetal fibroblasts were plated at 2×10^4/100 μl/well and treated with rMuIFN-α only.

Measurement of Superoxide (O_2^-)

O_2^- generation from the IFN-αA-treated cells were measured according to Johnston *et al.*[21] with slight modifications. Confluent monolayers of HeLa cells in 6-well tissue culture plates were washed three times with prewarmed HBSS without phenol red. One milliliter ferricytochrome *c* (80 μM in HBSS without phenol red), either with IFN-αA alone (10 or 100 U/ml), or with IFN-αA plus allopurinol (3 mM), was then added to triplicate wells. The plates incubated at 37°C in 10% CO_2/90% air for 6 h, and the ferricytochrome *c* solution was then collected and measured for OD changes at 550 nm. Purified human CuZnSOD (Sigma) at 100 U/ml was added to some wells along with the IFN-αA to confirm that the reduction of cytochrome *c* was caused by O_2^-. The molar extinction coefficient for ferricytochrome *c* at 550 nm is 2.1×10^4/mol/cm.

Statistical Analysis

Linear regressions of cell survival *vs* IFN-α concentration were generated with Cricket Graph Version 1.0 (Cricket Software, Philadelphia, PA), and the 50% cell survival point was used to calculate the ED_{50}. The statistics program, StatView SE$^+$ Graphics (Abacus Concepts, Inc., CA), was used to perform paired *t*-tests.

RESULTS

Biphasic Relationship Between CuZnSOD Activity and the IFN-α ED_{50}

The CuZn SOD activities of the *SOD1*-transfected HeLa clones are shown in Table I. An *inverse* relationship between the sensitivity of the clones to rHuIFN-αA in the establishment of IFN-mediated antiviral state and CuZnSOD activity was observed in the clones (SOD1-22, SOD1-29, and SOD1-14) with up to a 3-fold increase in CuZnSOD activity. On the other hand, a *direct* relationship between the sensitivity to IFN-αA and CuZnSOD activity was observed for clones (SOD1-1, SOD1-2, and SOD1-17) with between a 3 and 7.3-fold increase in CuZnSOD activity (Figure 1). Because of the variations among the ED_{50} obtained from each experiment, all ED_{50}s were normalized to the ED_{50} of the non-transfected control HeLa clone (Table I). The increase in the IFN-α ED_{50} in clone SOD1-29 is significantly different ($p < 0.05$) from the non-transfected HeLa cells and clones SOD1-1, SOD1-2 and SOD1-22 in all 4 experiments (Table I).

Higher ED_{50} for IFN-α in SOD1-Transgenic Fetal Fibroblasts

Both strains of mouse fetal fibroblasts derived from *SOD1*-transgenic mouse strains TgHS-218 and TgHS-229, with 3.5- and 3.3-fold increases in CuZnSOD activity, respectively, required more than control amounts of IFN-α to achieve 50% protection (Table I and Figure 2). Although the differences between the IFN ED_{50}s of the transgenic fibroblasts and their non-transgenic controls are not as large as was observed between the *SOD1*-transfected and the non-transfected HeLa cells, the differences are statistically significant at $p = 0.05$. More importantly, the results are in accordance with those obtained with the transfected HeLa cell clones with up to a 3-fold increase in CuZnSOD activity.

TABLE I
The IFN-α effective dose 50% (ED_{50}) of cell lines studied[a]

	HeLa	SOD1-22	SOD1-29	SOD1-14	SOD1-1	SOD1-2	SOD1-17	Tg-218	Tg-229
	CuZnSOD ACTIVITY (U/mg PROT.)[b]								
	4.7 ± 0.3	7.4 ± 0.1	12.1 ± 0.9	12.8 ± 0.5	27.7 ± 2.9	31.3 ± 1.3	34.5 ± 1.3	38.1 ± 2.4	35.8 ± 2.7
	IFN-αA ED_{50} (U/ml)[c]								
EXP. 1	8.1	7.9	11.5[g]	10.7	7.9	N.D.	20.9	18.6[e]	3.85[f]
	(1.00)	(0.98)	(1.42)	(1.32)	(0.98)		(2.58)	(1.47)	(1.42)
EXP. 2	1.9	1.2	8.5[g]	5.3	1.1	0.9	3.5		
	(1.00)	(0.63)	(4.47)	(2.79)	(0.58)	(0.47)	(1.84)		
EXP. 3	2.4	0.74	8.3[g]	6.0	3.6	1.5	9.0		
	(1.00)	(0.31)	(3.46)	(2.50)	(1.50)	(0.63)	(3.75)		
EXP. 4	14.0	7.5	107.8[g]	133.1	13.4	16.7	14.8		
	(1.00)	(0.54)	(7.70)	(9.51)	(0.96)	(1.19)	(1.06)		
Mean of ratios[d]	1.00 ± 0.00	0.62 ± 0.14	4.26 ± 1.31	4.03 ± 1.86	1.00 ± 0.19	0.76 ± 0.19	2.31 ± 0.57	1.47	1.42

[a]*SOD1*-transferred HeLa clones and mouse fetal fibroblasts derived from the *SOD1*-transgenic mice were used in this study. [b]CuZnSOD activities are presented as mean ± SEM, $n = 4$; the SOD activity for the non-transgenic mouse fetal fibroblasts is 11.0 ± 0.8 U/mg. [c]Numbers in parentheses represent the ratio of IFN-α ED_{50} of *SOD1*-transfected HeLa clones to that of the non-transfected HeLa control. [d]Mean ± SEM. [e]The IFN-α ED_{50} of the non-transgenic littermate control for Tg-218 is 12.7 U/ml. [f]The IFN-α ED_{50} of the non-transgenic littermate control for Tg-229 is 2.72 U/ml. [g]The ED_{50} of SOD1-29 is significantly different ($p < 0.05$) from that of the other HeLa clones.

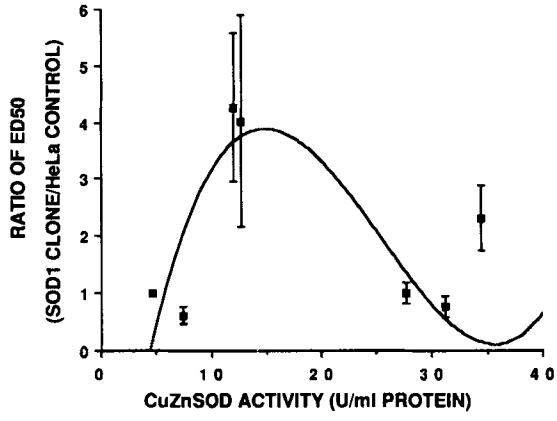

FIGURE 1 Relationship of the ED_{50} of interferon-αA (rHuIFN-αA) to CuZnSOD activity in *SOD1*-transfected HeLa clones. $r = 0.87$, $p < 0.025$. $n = 4$. Error bars represent ± SEM.

Allopurinol Causes a Significant Reduction in the IFN-α-Mediated Antiviral Effect

The addition of a 3 mM concentration of allopurinol [4-hydroxypyrazolo(3,4-d)pyrimidine], an inhibitor of xanthine oxidase[22] and a scavenger of hydroxyl radicals,[23] to the culture medium 24 hours prior to challenge with VSV caused a dramatic reduction ($p < 0.01$) in cell survival in the IFN-α-mediated antiviral assay. In all the HeLa clones tested, there were 48–69% and 31–58% reductions in cell survival with 10 U/ml and 100 U/ml IFN-α, respectively (Table II). No correlation was observed between the degree of reduction in protection and the CuZnSOD activity. The production of superoxide by the HeLa clones treated with IFN-α was significantly reduced ($p < 0.05$) when the cells were treated with 3 mM allopurinol (Figure 3).

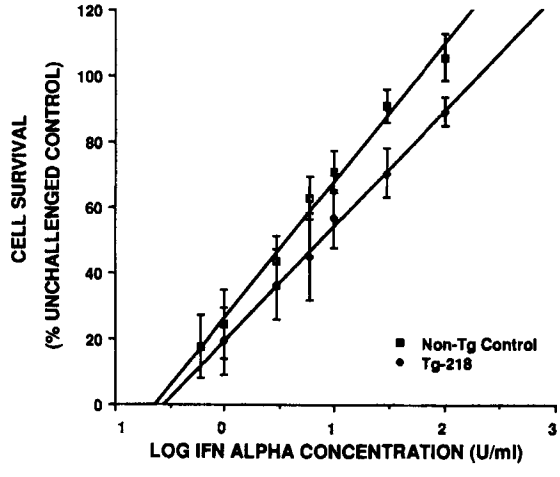

FIGURE 2 Survival of *SOD1*-transgenic cells at different concentrations of mouse interferon-α (rMuIFN-α) 48 h after challenge with VSV. Results from a representative experiment with heterozygous *SOD1*-transgenic TgHS-218 fetal fibroblasts are presented. $n = 3$. Error bars represent ± SEM.

TABLE II
Reduction of IFN-α-mediated antiviral effect by allopurinol[a]

Cell lines	% Reduction[b]	
	10 U/ml IFN-αA	100 U/ml IFN-αA
HeLa	59.6 ± 10.0	55.0 ± 5.3
SOD1-22	56.1 ± 9.4	38.9 ± 4.7
SOD1-29	62.0 ± 7.0	57.7 ± 5.5
SOD1-14	47.9 ± 9.7	31.2 ± 2.7
SOD1-1	58.9 ± 11.5	40.5 ± 6.1
SOD1-2	54.5 ± 12.2	38.3 ± 6.4
SOD1-17	68.8 ± 0.8	50.2 ± 5.7

[a] 3 mM allopurinol was added to the culture medium as a xanthine oxidase inhibitor and hydroxyl radical scavanger. [b]Results were obtained from 5 independent experiments and are expressed as mean ± SEM; the results were significantly different from that of the IFN-α-treated control at $p < 0.01$.

Paraquat Increases Survival of Virus-infected Cells

Exposure of HeLa cells to paraquat, a superoxide generator, produced a protective effect against viral infection (Figure 4), even without the addition of interferon. An inverse relationship was observed between cell survival and CuZnSOD activity in the *SOD1*-transfected HeLa clones ($r = 0.79$, $p < 0.025$) when the cells were treated with 150 mM paraquat prior to challenge with VSV (Figure 5). Moreover, an additive effect was observed when IFN-α was added simultaneously with paraquat in the

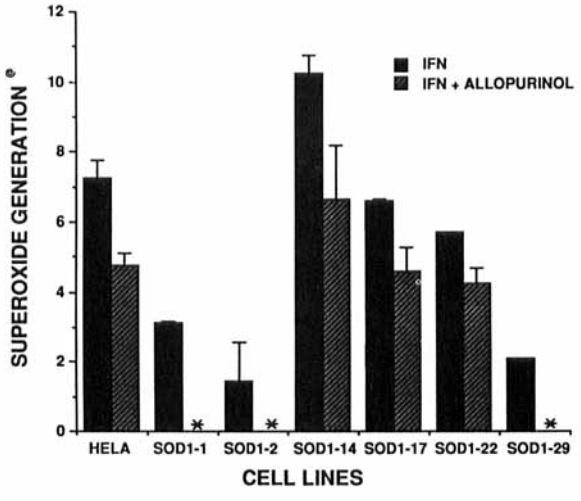

FIGURE 3 Effect of allopurinol on the production of superoxide in the *SOD1*-transfected HeLa cells treated with 100 U/ml of rHuIFN-αA. Allopurinol was used at a concentration of 3 mM. O_2^- production by IFN-αA was reduced by 82 ± 7% in the wells treated with 100 U/ml purified human CuZnSOD. [a] The superoxide generation is presented as nmol oxidized cytochrome c/mg protein. *Superoxide generation in clones SOD1-1, SOD1-2, and SOD1-29 after allopurinol treatment was not detectable. Error bars represent ± SEM.

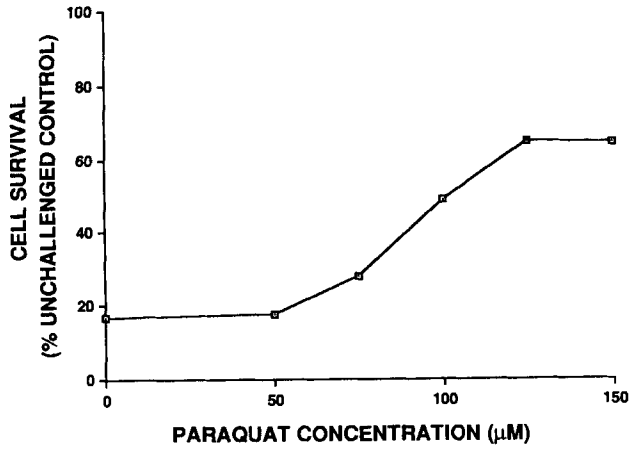

FIGURE 4 Cell survival at different concentrations of paraquat after challenge with VSV. Results from a representative experiment with non-transfected HeLa cells are presented.

antiviral assay. However, the relationship between CuZnSOD activity and cell survival was not changed (Figure 6).

DISCUSSION

The report of Pottathil *et al.*[13] suggested a possible requirement for CuZnSOD, a cytoplasmic enzyme[19,24,25] that catalyzes the dismutation of superoxide anions to hydrogen peroxide and oxygen, in the establishment of the IFN-mediated antiviral state. They used the copper chelator, diethyldithiocarbamate (DDC), to lower the

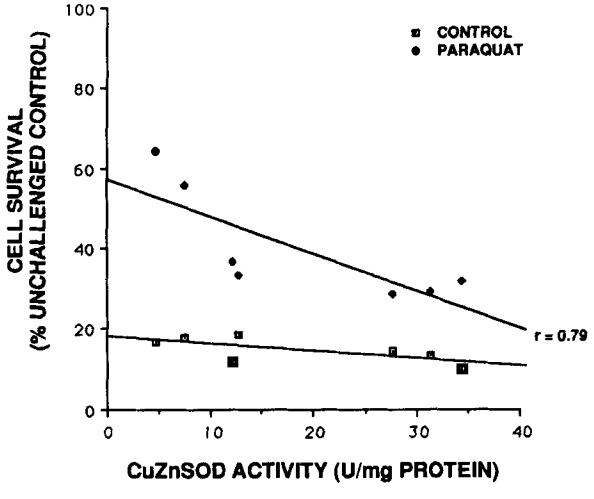

FIGURE 5 Relationship between cell survival and CuZnSOD activity in *SOD1*-transfected HeLa clones treated with 150 mM paraquat and challenged with VSV. $r = 0.79$, $p < 0.025$.

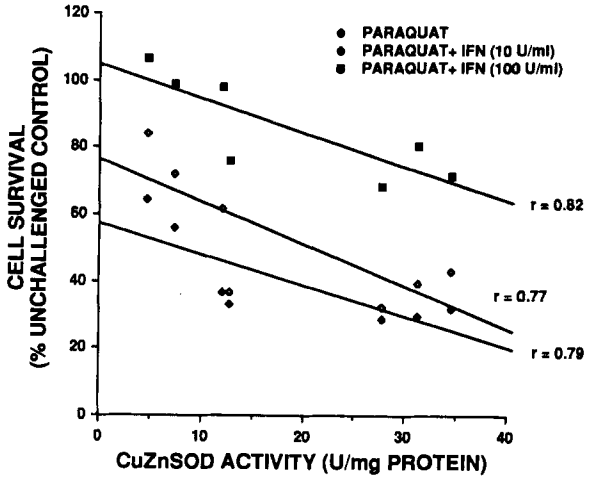

FIGURE 6 Relationship between cell survival and CuZnSOD activity in *SOD1*-transfected HeLa clones treated with paraquat alone, paraquat plus 10 U/ml rHuIFN-αA, or paraquat plus 100 U/ml rHuIFN-αA, and challenged with VSV. The respective correlation coefficients are 0.79, 0.77, and 0.82, with $p < 0.025$ for each line.

CuZnSOD activity of cells treated with IFN-α and observed a reduced antiviral effect of IFN-α. There appeared to be, therefore, a *direct* relationship between CuZnSOD activity and the degree of antiviral effect established by treatment with IFN-α, and this is what we expected to find in our studies. However, this is not what we found when we tested our panel of *SOD1*-transfected HeLa cell clones with a broad range of CuZnSOD activities. Rather, we observed a biphasic response ($r = 0.87, p < 0.025$) in which the two clones with intermediate CuZnSOD activities (2.6–2.7-times the control level) required a greater amount of IFN-α than did clones with higher or lower activities to achieve an equivalent response (Figure 1 and Table I). Thus at low levels of CuZnSOD the effectiveness of IFN-α in establishing an antiviral response was *inversely* related to enzyme activity. At higher levels of CuZnSOD, the response was closer to what the earlier work would have predicted.

In earlier studies of the same HeLa cell panel we demonstrated a direct relationship between resistance to paraquat, a superoxide generator, and CuZnSOD activity.[14] These results indicate that the biphasic nature of the response of IFN-α is not the result of a biphasic reaction in the handling of superoxide radicals by CuZnSOD and suggest that there may be more than one mechanism by which an interaction of superoxide and the IFN-α response system are involved in establishing an antiviral state. We postulated, therefore, that at low levels of CuZnSOD activity superoxide participates in a positive way in the IFN-α mediated resistance to VSV infection. To determine whether the same relationship would hold for another cell type, we examined the behavior of two *SOD1*-transgenic strains of mouse fibroblasts which had 3-times the control level of CuZnSOD activity and had been shown to be more resistant to the growth-inhibiting effect of paraquat.[14] The agreement between the responses to IFN-α observed in these fibroblasts (Figure 2 and Table I) and in the transfected HeLa clones which made up the ascending limb of the biphasic response curve, both groups having roughly equivalent increases in CuZnSOD activity, suggests

strongly that CuZnSOD does play a role, either directly or indirectly, in the establishment of IFN-mediated antiviral state and that the inverse relationship between enzyme activity and the sensitivity to IFN-α is not restricted to one specific cell type.

The observations just cited led to the prediction that if increased CuZnSOD activity should lead to a lower intracellular concentration of superoxide, then reduction in superoxide generation by some other means should make the cells less responsive to IFN-α. Allopurinol, a purine analogue which has been shown to serve as substrate for and inhibitor of xanthine oxidase,[22] was chosen for this purpose. Xanthine oxidase, acting on its substrate, xanthine or hypoxanthine, leads to the generation of superoxide, and a marked increase in xanthine oxidase activity has been reported in mice treated with interferon-α/β and interferon inducers.[26,27] Increased oxygen consumption has also been observed in human polymorphonuclear leukocytes treated with recombinant human IFN-α and IFN-γ.[4] Furthermore, at the concentrations used in our studies, allopurinol also serves as a hydroxyl radical scavenger[23] and/or an electron transfer agent[28] and prevents oxidative damage by superoxide radicals.

Following treatment with allopurinol, a dramatic reduction of the IFN-mediated antiviral effect, as compared to that produced by treatment with IFN-α alone, was observed. Interestingly, allopurinol treatment caused more reduction (Table II) in the IFN-mediated antiviral effect at low IFN concentration (10 U/ml). The results suggest that superoxide generation may play a more important role against viruses at low interferon concentration, while mechanisms involving other IFN-inducible proteins may play a more important role at high interferon concentration. Furthermore, the reduction of antiviral effect caused by allopurinol coincides with a reduction in measured superoxide generation (Figure 3), which further implicates an involvement of superoxide in generating the antiviral effect.

Based on the inhibitory effect of allopurinol on the action of IFN-α, it was predicted that paraquat itself, as a superoxide generator, might have an antiviral activity of its own, and this is what was observed. Although high concentrations of paraquat were toxic, proportional cell survival was significantly higher ($p < 0.01$) in VSV-infected cells treated with paraquat than in control infected cells (Figure 4). Furthermore, there was an inverse relationship between cell survival and CuZnSOD activity after paraquat treatment (Figure 5), further supporting the hypothesis that superoxide radicals have an antiviral effect.

When paraquat and IFN-α were used together, the antiviral effect produced by treatment with the two agents was greater than that produced from either treatment alone (Figure 6). The degree of protection was inversely proportional to the CuZnSOD activity across the entire range, and 100 U/ml of IFN-α had a greater effect than did 10 U/ml (Figure 6). Thus, the direct relationship between the antiviral effect produced by IFN-α alone and CuZnSOD activity, which characterizes the second half of the biphasic curve, is masked by the presence of paraquat in the system. This suggests, in turn, that the biphasic curve observed with IFN-α treatment alone does not reflect just the effect of IFN-α induced superoxide generation. A more complicated mechanism or mechanisms involving the delicate balance of intracellular redox state and/or of some redox-sensitive proteins is likely to be involved in the inverse relationship between IFN-α ED_{50} and CuZnSOD activity in the HeLa clones expressing over 3-fold basal levels of CuZnSOD.

Oxygen free radicals are well known to lead to DNA and RNA single strand breaks and protein denaturation.[6-8] Furthermore, DNA damage has been implicated as the key factor in the lethality of paraquat to *E. coli*,[27] and the antiviral effect of the

antiviral drug, avarol, has been proposed to be due to the generation of superoxide.[30] These observations lend further support to our inference from the results presented above that superoxide can act as an antiviral agent, possibly through its ability to lead to DNA and RNA strand breaks by the subsequent formation of hydroxyl radicals or the activation of nucleases.[8]

Another mechanism that could be operative in the relationship between IFN-α action and CuZnSOD activity involves the signal transduction pathway for IFN-α. The activation of specific gene expression by IFN-α requires the binding of interferon to specific cell surface receptors,[1,2] and activation of protein kinase C (PKC) has been shown to play a crucial role in signal transduction.[31,32] Increased lipid peroxidation has been observed in cells overexpressing CuZnSOD,[11] and this could, by its effects on the plasma membrane, change the affinity of ligand/receptor binding. Furthermore, an enhanced PKC activity has been produced by redox-cycling quinones through a reduction-sensitive modification of the thiol/disulphide status of PKC.[33] Therefore, changes in the intracellular redox state resulting from changes of CuZnSOD activity could affect the maximum reactivity of PKC and thus the degree of gene expression induced by IFN-α and the ultimate establishment of the IFN-mediated antiviral effect.

With the argument that altered CuZnSOD level is responsible for the changes in the establishment of the IFN-α-mediated antiviral state, one must consider the possible involvement of the other intracellular superoxide dismutase, MnSOD, and the other two antitoxidant enzymes, catalase and glutathione peroxidase, in the process. The levels of MnSOD mRNA expression, as quantitated from the intensities of hybridization signals from RNA slot blots, varied slightly among the different *SOD1*-transfected HeLa cell clones (data not shown). However, the MnSOD levels are only about 1/10 of the activity of CuZnSOD. Therefore, the distribution of the total SOD levels is not changed among the HeLa clones. Furthermore, since it has been reported that MnSOD is not induced by IFN-α[20,34] in fibroblasts, the MnSOD level should not have affected the outcome of this study. We have previously reported slight variations of catalase and glutathione peroxidase activity among the *SOD1*-transfected HeLa clones.[14] However, no correlation was observed between the generation of the IFN-α-mediated antiviral state and the activities of catalase and glutathione peroxidase.

As was noted earlier, our results are, in part, contrary to those published previously by Pottathil *et al.*[13] in which CuZnSOD activity was inhibited by DDC. However, the cell lines and the ranges of CuZnSOD activity used differed between the two studies. In addition, DDC reduces not only the CuZnSOD activity, but also the activities of other enzymes, such as cytochrome c oxidase, which used copper as the cofactor. The latter complication is not present in our study, since CuZnSOD activity was stably altered by genetic means. Similar contradictory conclusions have been drawn in different experimental systems looking at the biological consequences of having elevated levels of CuZnSOD activity. While the protective effects of CuZnSOD have been reported in cultured human and mouse cells,[14] transgenic mice,[35–37] and transgenic *Drosophila*,[38] deleterious effects of increased CuZnSOD activity have also been reported in the same systems when different cell strains or transgenes were used.[39–45] Moreover, increases in CuZnSOD activity have been reported in some,[46,47] but not all,[14] experimental systems to be accompanied by an increase in other antioxidant enzymes with which it works in concert to maintain the intracellular redox state. Thus, each experimental system is unique in its own way and should be analyzed individually.

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