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# Effect of Expression of Manganese Superoxide Dismutase in Baculovirus-Infected Insect Cells

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## Abstract

It has previously been demonstrated that baculovirus infection of the *Spodoptera frugiperda* Sf-9 (Sf-9) and *Trichoplusia ni* BTI-Tn-5B1-4 (Tn-5B1-4) insect cell lines leads to oxidative stress as measured by protein and membrane lipid oxidation and that this oxidative damage contributes to cell death. As a result of these findings, it was hypothesized that baculovirus infection stimulates superoxide radical ($O_2^{\bullet-}$) synthesis in the mitochondria and that the resulting $O_2^{\bullet-}$ accumulation overwhelms the cells' antioxidant defenses. We investigated the ability of manganese superoxide dismutase (MnSOD) expression (which reduces $O_2^{\bullet-}$ to $H_2O_2$) to overcome the oxidative damage caused by baculovirus infection. It was found that MnSOD expression significantly reduced oxidative damage in baculovirus-infected Tn-5B1-4 cells but had no significant effect on oxidative damage in baculovirus-infected Sf-9 cells. The results are consistent with the hypothesis that $O_2^{\bullet-}$ accumulation in the mitochondria is at least partially responsible for the oxidative damage resulting from the baculovirus infection of insect cells.

**Index Entries:** *Spodoptera frugiperda* Sf-9; *Trichoplusia ni* BTI-Tn-5B1-4; baculovirus expression vector system; manganese superoxide dismutase; catalase; protein oxidation; lipid oxidation.

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## Introduction

The *Spodoptera frugiperda* Sf-9 (Sf-9) and *Trichoplusia ni* BTI-Tn-5B1-4 (Tn-5B1-4) insect cell lines are widely used with the baculovirus expression vector system to express recombinant eukaryotic proteins *(1)*. In addition, these cell lines can be used as hosts to produce baculovirus bioinsecticides *(2)*. Unfortunately, baculovirus infection kills the cells, thereby bringing recombinant protein and/or bioinsecticide synthesis to an end. Therefore, it follows that extending the life-span of baculovirus-infected cells could significantly improve productivity. Furthermore, corresponding information obtained regarding viral cytotoxicity, in general, could provide clues that are valuable in the development of antiviral drugs.

It has recently been demonstrated that baculovirus infection of Sf-9 and Tn-5B1-4 cells induces oxidative stress that leads to the oxidative damage of cellular proteins and membranes *(3)*. This oxidative damage corresponds to reduced cell viability; in other words, these results are consistent with the premise that oxidative damage contributes to cell death in baculovirus-infected cells. Because baculovirus infection of Sf-9 and Tn-5B1-4 cells has a minimal effect on the activities of most antioxidant enzymes, it was suggested that the oxidative stress resulted from increased levels of reactive oxygen species (ROS), such as, superoxide radical ($O_2^{\bullet -}$). Indeed, it is known that viral infection leads to increased intracellular $O_2^{\bullet -}$ levels in mammalian cells *(4–7)*.

It has been demonstrated that the overexpression of antioxidant enzymes in mammalian cells can increase cellular resistance to oxidative damage *(8–11)*. These antioxidant enzymes include manganese superoxide dismutase (MnSOD) and copper-zinc superoxide dismutase (CuZnSOD), which reduce $O_2^{\bullet -}$ to $H_2O_2$, and catalase (CAT) and glutathione peroxidase (GPX), which reduce $H_2O_2$ to $H_2O$. Although an increase in antioxidant enzyme activities generally provides protection against oxidative damage, some studies have shown that overexpression of SOD sensitizes cells to oxidative stress *(8,12–15)*. It is believed that this sensitization results from an imbalance between SOD and GPX or CAT, thereby leading to intracellular $H_2O_2$ accumulation, which can result in oxidative cell damage *(15)*. Therefore, overexpression of both SOD and GPX or CAT may be necessary in order to increase the resistance of some cell types to oxidative stress.

It has recently been demonstrated that the assemblages of antioxidant enzymes in the Sf-9 and Tn-5B1-4 insect cell lines differ from each other and from that typically found in mammalian cells *(16)*. Both cell lines contain MnSOD and CuZnSOD activities for reducing $O_2^{\bullet -}$ to $H_2O_2$, as is the case in typical mammalian cells. In addition, both the Sf-9 and Tn-5B1-4 cell lines contain ascorbate peroxidase (APOX), but not GPX, which is ubiquitous in mammalian cells, for reducing $H_2O_2$ to $H_2O$. APOX is commonly found in plants *(17)* and has been found in insect larvae *(18)*. The Tn-5B1-4 cell line, but not the Sf-9 cell line, also contains CAT for the reduction of $H_2O_2$ to $H_2O$ *(16)*. Consistent with this finding, it has been demonstrated that Sf-9 insect

cells do not contain peroxisomes (Tn-5B1-4 cells do contain peroxisomes), which are the intracellular location of CAT activity in mammalian cells *(16)*.

In the present study, the effect of overexpression of human MnSOD was investigated in baculovirus-infected Sf-9 and Tn-5B1-4 insect cell lines. Specifically, the effect on cell life-span, lipid oxidation, and protein oxidation were investigated.

## Materials and Methods

### *Chemicals*

All of the chemicals were purchased from Sigma (St. Louis, MO) unless indicated otherwise.

### *Cell Lines and Virus*

Sf-9 and Tn-5B1-4 insect cell lines were obtained from Gibco-BRL (Grand Island, NY) and Invitrogen (San Diego, CA), respectively. The Sf-9 and Tn-5B1-4 cell lines were grown in Sf-900 II and Express Five serum-free media (SFM) (both purchased from Gibco-BRL), respectively, and adapted to growth in SFM as described previously *(19)*.

Recombinant *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) expressing human secreted alkaline phosphatase (AcMNPV-SEAP) under control of the polyhedrin promoter was obtained from H. A. Wood (Mississippi State University, Mississippi State, MS).

The recombinant baculovirus used was isolated by standard homologous recombination and plaque purification procedures, as described previously *(20,21)*. Briefly, the baculovirus expressing human MnSOD cDNA *(22)* was generated as follows. The oligonucleotides pcDNAnot5'-GCATGC TCGAGCGGCCGCCAGT-3' (forward) and pcNDApme5'-AGCTTAGTT TAAACCCCTCGAGGTCGAC-3' (reverse) were synthesized. The *not* and *pme* primers were then used to amplify the MnSOD gene in pcDNA3/MnSOD by polymerase chain reaction. The resulting amplicon was next digested with *Not1* and *Pme1* and ligated into similarly digested pAc(+)IE1TV4 *(23)* to give plasmid pAc(+)1E1TV4/MnSOD. This positioned the MnSOD coding sequence under the transcriptional control of a baculovirus *ie1* promoter and *hr5* enhancer, which allows the foreign gene to be expressed immediately after recombinant baculovirus infection. The resulting transfer plasmid was mixed with Bsu36I-digested BakPAK6 viral DNA *(24)* and the mixture was used to cotransfect Sf-9 cells by a standard calcium phosphate precipitation method. The cell-free culture supernatant was harvested when viral occlusions were observed in the transfected cells, and viral progeny were resolved by standard plaque assays in the presence of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Recombinant viruses were identified in these plaque assays by their white-plaque, occlusion-positive phenotypes and subjected to two additional rounds of plaque purification. These clones were then amplified

and tested for the ability to express MnSOD by immunoblotting. Working stocks of four positive clones were amplified in Sf-9 cells and titered by the end-point dilution method. The MnSOD expression levels of these four clones were then tested in Sf-9 and Tn-5B1-4 cells. One clone designated AcP(+)IEMnSOD had the highest MnSOD expression level in both Sf-9 and Tn-5B1-4 cells and was used for the remainder of the study.

AcMNPV-SEAP and AcP(+)IEMnSOD stocks were produced in Sf-9 cells in Sf-900 II SFM with 10% fetal bovine serum (FBS) and titered in Sf-9 cells by the end-point dilution method *(20)*. FBS was added to increase the stability of the virus, which was stored at 4°C *(25)*.

## Cell Growth and Infection

Sf-9 and Tn-5B1-4 shake-flask cultures were routinely grown in 250-mL Erlenmeyer flasks with a working volume of 40 mL in a temperature-controlled (27°C) incubator shaker (Series 25; New Brunswick Scientific, Edison, NJ) at a rotational speed of 125 rpm. Cells in mid-exponential growth phase (~$2 \times 10^6$ cells/mL) were infected with AcMNPV-SEAP or AcP(+)IEMnSOD at a multiplicity of infection (MOI) of 10. At this MOI, essentially all of the cells are infected during the primary infection, thereby stopping cell replication *(20)*. Infected cultures were typically sampled at 24-h intervals for a period of 4 d post-infection. Samples were centrifuged at 1000*g* for 5 min to separate the supernatant and cells. The cells were washed with potassium phosphate buffer (pH 7.8) and centrifuged at 1000*g* for 5 min. The resulting cell pellets were stored at –80°C until assayed. Immediately following removal from the –80°C freezer, the cells were thawed and resuspended in potassium phosphate buffer (pH 7.8). The cells were then lysed twice for 20 s each in an ice-water bath using a Virsonic 300 sonicator (Virtis, Gardiner, NY) operated at approx 20% full power (400-W maximum power). Protein concentration was determined by the method of Lowry et al. *(26)* using defatted bovine serum albumin as the standard. The samples were then used for the assays discussed subsequently.

Cell counts were determined with a Coulter Counter Model ZM interfaced with a Coulter Channelyzer 256 (Coulter, Hialeah, FL). Cell viability was determined by the trypan blue dye exclusion method.

## Error Analysis

Confidence limits (95%) were calculated by the student's *t* method *(27)*.

## SOD Enzyme Assay

SOD activity was determined by a method developed previously *(28,29)* in which nitroblue tetrazolium (NBT) and SOD compete to react with $O_2^{\bullet -}$. The reaction of NBT with $O_2^{\bullet -}$ produces a product that absorbs at 560 nm. Thus, SOD activity in a sample can be determined from the level to which it inhibits the increase in absorbance at 560 nm. This assay was performed as described by Wang et al. *(16)*.

### *SOD Activity Gel*

The SOD activity gel assay was based on the inhibition of NBT reduction of $O_2^{\bullet -}$ by SOD *(30)*. CuZnSOD activity was inhibited by the addition of 0.75 m*M* NaCN, thereby allowing direct visualization of MnSOD activity. Briefly, proteins (400 µg each) were separated on 12.5% native acrylamide gels as described by Ornstein *(31)*. The gels were run for 1 h at 4°C in pre-electrophoresis buffer (pH 8.8). Total cell lysates were first run on these gels for 3 h in this buffer at 4°C. The buffer was then changed to electrophoresis buffer (pH 8.3), and the gels were run at 4°C for 2.5 to 3 h. SOD expression was visualized by first staining with 40 µg/mL of NBT for 20 min in phosphate-buffered saline (PBS) (pH 7.4). Superoxide radical was produced using TEMED/5-phospho-riboflavin in $H_2O$ for 15 min. Gels were washed with double-distilled $H_2O$, and SOD achromatic bands were viewed after exposure to fluorescent light.

### *Western Blot*

Protein preparations were performed on ice. Briefly, cells were washed twice with PBS (pH 7.0) and sonicated in 50 m*M* phosphate buffer (pH 7.8) on ice. Total protein concentrations were determined by the Bio-Rad (Hercules, CA) Bradford Protein Assay Kit using lyophilized bovine plasma gamma globulin as a standard. A total of 100 µg of denatured cell protein for each condition was separated by 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis, electrotransferred onto a nitrocellulose membrane, and then probed with antiserum to human MnSOD (250:1 dilution) *(32)*. The MnSOD immunoreactive bands were detected by a chemiluminescence ECL kit (Amersham, Arlington Heights, IL).

### *Lipid Hydroperoxide Assay*

Lipid hydroperoxide concentrations were determined using the BIOXYTECH® LPO-560 assay kit purchased from OXIS (Portland, OR). This assay is based on the oxidation of ferrous ions to ferric ions by hydroperoxides under acidic conditions. The resulting ferric ions bind with an indicator dye, xyenol orange, to form a stable, colored complex whose absorbance can be measured at 560 nm. This assay was performed following the manufacturer's instructions.

### *Protein Carbonyl Assay*

Protein carbonyl concentrations were determined using a modification of the method developed by Levine et al. *(33)*. This assay is the "general assay" of oxidative protein damage and is based on the finding that ROS attack amino acid residues in proteins (particularly histidine, arginine, lysine, and proline) to produce products with carbonyl groups. These carbonyl groups were treated with 2,4-dinitrophenylhydrazine (DNP) to form hydrazone derivatives whose concentration was determined spectroscopi-

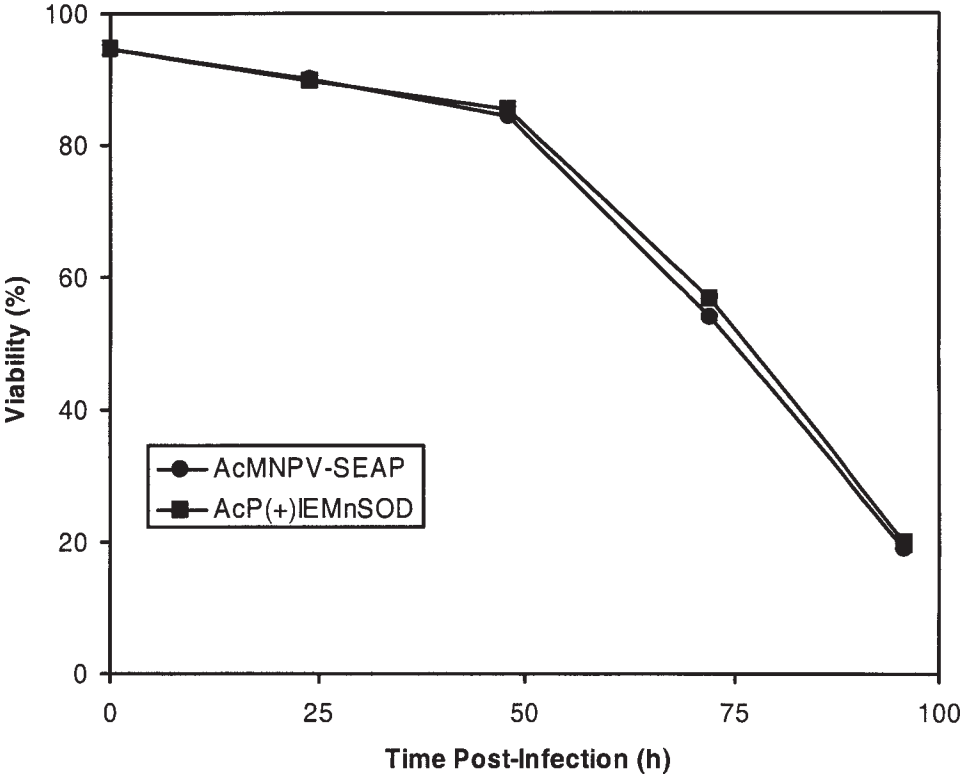


Fig. 1. Viability of Sf-9 cells infected with recombinant *A. californica* multiple nucleopolyhedroviruses expressing secreted alkaline phosphatase (AcMNPV-SEAP) or manganese superoxide dismutase (AcP(+)IEMnSOD) at a multiplicity of infection of 10 in shake-flask studies as a function of time post-infection. The error bars representing the 95% confidence levels based on four shake-flask experiments are smaller than the given symbols.

cally. Briefly, to 250-µL samples (diluted as necessary in accordance with carbonyl content) either 500 µL of DNP in 2 *M* HCl or 500 µL of 2 *M* HCl (control) was added. The samples were allowed to react at room temperature for 1.5 h with vortexing every 10–15 min. Following this reaction, 750 µL of 20% trichloroacetic acid was added, the samples were centrifuged at 16,000*g* for 3 min, and the supernatant was discarded. The resulting pellets were then washed three times with 1 mL of ethanol:ethyl acetate (1:1) to remove free reagent; the sample was allowed to stand 10 min before each centrifugation and the supernatant was discarded each time. The pellet was dissolved in 800 µL of 6 *M* guanidine solution with insolubles being removed by centrifugation. The spectrum from 355 to 395 nm was then obtained using the complementary blank. The carbonyl content was determined from the maximum absorbance in this spectral range using a molar absorption coefficient of 22,000 $M^{-1}$ $cm^{-1}$.

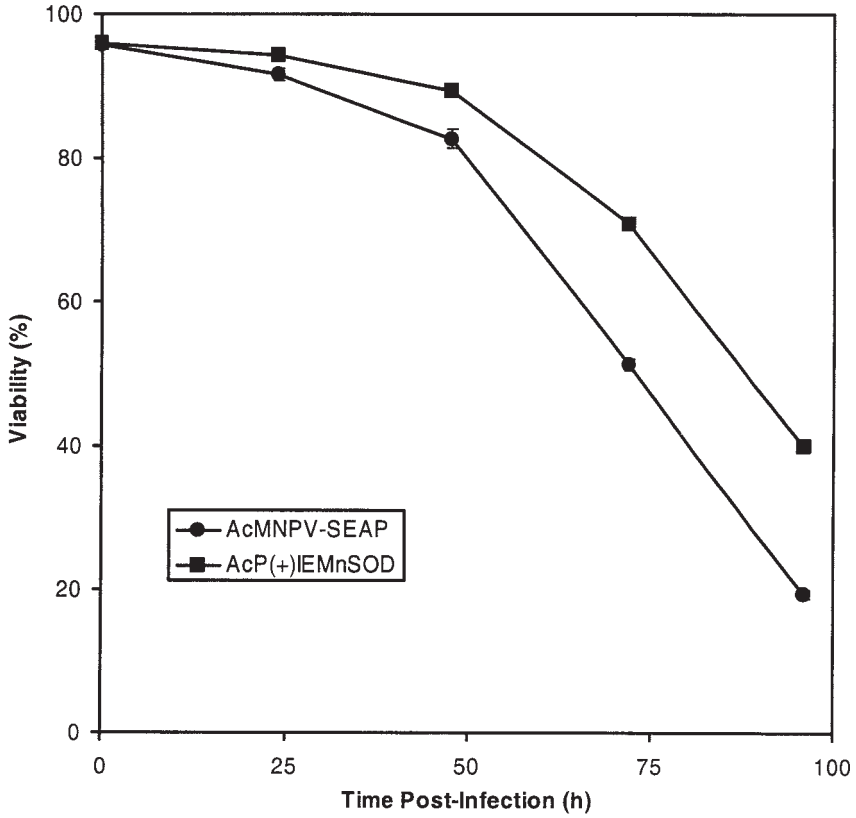


Fig. 2. Viability of Tn-5B1-4 cells infected with recombinant *A. californica* multiple nucleopolyhedroviruses expressing secreted alkaline phosphatase (AcMNPV-SEAP) or manganese superoxide dismutase (AcP(+)IEMnSOD) at a multiplicity of infection of 10 in shake-flask studies as a function of time post-infection. The error bars representing the 95% confidence levels based on four shake-flask experiments are smaller than the given symbols.

## Results and Discussion

### *Cell Death*

The viability of Sf-9 cells infected with AcP(+)IEMnSOD was not significantly improved over that of Sf-9 cells infected with AcMNPV-SEAP (Fig. 1). By contrast, the viability of Tn-5B1-4 cells infected with AcP(+)IEMnSOD was significantly improved over that of Tn-5B1-4 cells infected with AcMNPV-SEAP (Fig. 2). As discussed later, these results are consistent with the premise that the expressed MnSOD is targeted to the site of $O_2^{\bullet -}$ accumulation in Tn-5B1-4 cells, but not in Sf-9 cells. Note, however, that the decreased viability resulting from AcMNPV-SEAP infection of Sf-9 and Tn-5B1-4 cells is not likely to be a direct result of SEAP synthesis (e.g., SEAP toxicity) because wild-type AcMNPV infection of these cell lines produces similar cell viability profiles (data not shown).

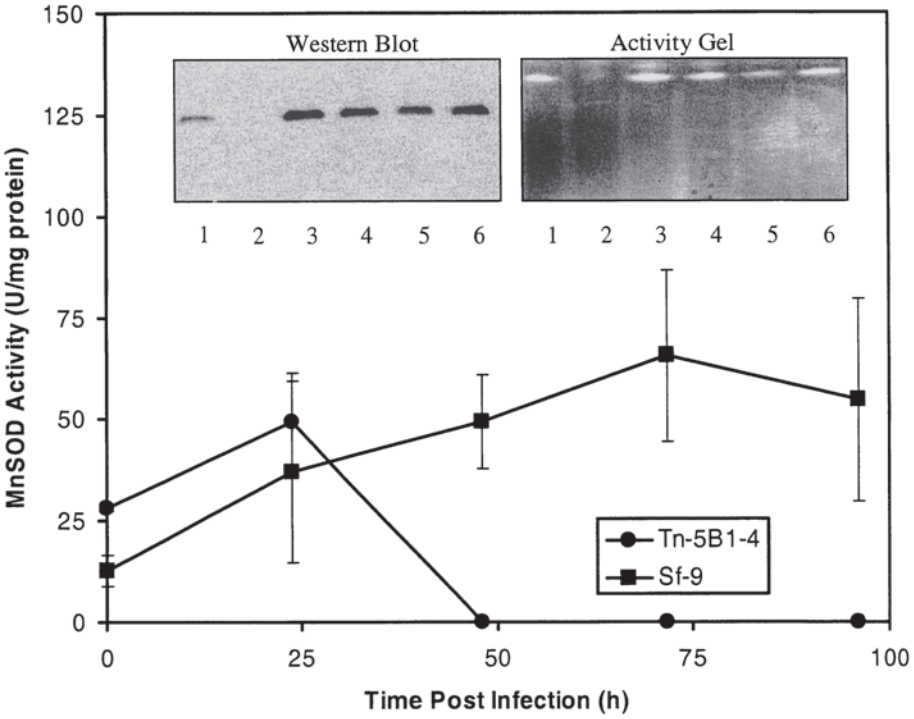


Fig. 3. MnSOD enzymatic activities of Sf-9 and Tn-5B1-4 cells infected with AcP(+)IEMnSOD at multiplicity of infection of 10 in shake-flasks as function of time post-infection (pi). The error bars represent the 95% confidence levels based on four and three shake-flask experiments for the Sf-9 and Tn-5B1-4 cells, respectively. Insets include the MnSOD Western blot (WB) and activity gel (AG) for Tn-5B1-4 cells infected with AcP(+)IEMnSOD. Column 1 in the WB and AG are the positive controls loaded with human cancer cell protein (30 and 100 µg for WB and AG, respectively). Columns 2–6 in the WB and AG were loaded with total Tn-5B1-4 cellular protein from 0, 24, 48, 72, and 96 h pi (100 and 400 µg for the WB and AG, respectively).

## *MnSOD Enzyme Activities*

The MnSOD activities of the Sf-9 and Tn-5B1-4 cell lines following infection with AcP(+)IEMnSOD as measured by the SOD enzyme assay are shown in Fig. 3. The MnSOD activity in the Sf-9 cells increased approximately fourfold from 0 to 72 h pi and then decreased slightly from 72 to 96 h pi. By contrast, the corresponding MnSOD activity in the AcP(+)IEMnSOD-infected Tn-5B1-4 cells almost doubled from 0 to 24 h pi and then dropped to zero by 48 h pi. This rapid loss of MnSOD activity in the AcP(+)IEMnSOD-infected Tn-5B1-4 cells was suspicious, and, therefore, confirmation was attempted through the use of alternative methods: the hydroethidine assay (another spectroscopic assay), a Western blot, and an activity gel. The hydroethidine assay *(34)* also indicated a rapid loss of MnSOD activity in the AcP(+)IEMnSOD-infected Tn-5B1-4 cells (data not shown). The Western blot, however, demonstrated the presence of MnSOD protein at 24, 48, 72,

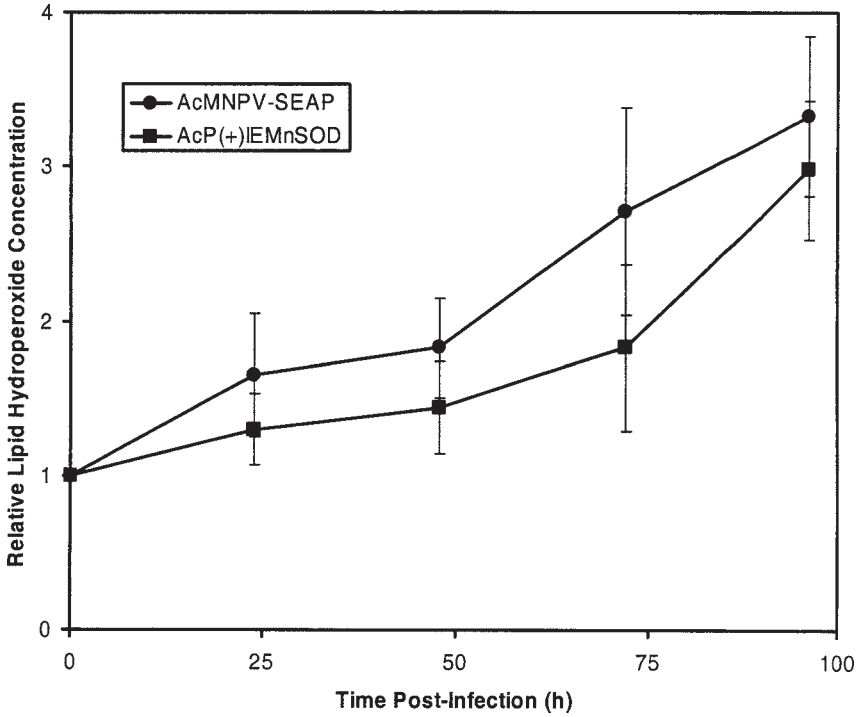


Fig. 4. Concentration of lipid hydroperoxides in Sf-9 cells infected with recombinant *A. californica* multiple nucleopolyhedroviruses expressing secreted alkaline phosphatase (AcMNPV-SEAP) or manganese superoxide dismutase (AcP(+)IEMnSOD) at a multiplicity of infection of 10 in shake-flask studies as a function of time post-infection. The error bars representing the 95% confidence levels based on four shake-flask experiments.

and 96 h pi (but not at 0 h pi). In addition, an activity gel demonstrated the presence of MnSOD activity at these same times (Fig. 3). These results are consistent with the presence of a factor in the Tn-5B1-4 cells at 48 h pi that interferes with the MnSOD spectroscopic assays and is separated from the enzyme in the activity gel, thereby resulting in the observed enzyme activity that was not present in the spectroscopic assays.

## *Lipid Hydroperoxides*

The effect of expression of MnSOD on hydroperoxide concentrations found in baculovirus-infected Sf-9 and -Tn-5B1-4 cells is shown in Figs. 4 and 5, respectively. Briefly, expression of MnSOD did not significantly influence hydroperoxide concentrations in baculovirus-infected Sf-9 cells (Fig. 4). By contrast, expression of MnSOD resulted in significantly reduced hydroperoxide concentration in baculovirus-infected Tn-5B1-4 cells, with increasing effectiveness at later times post-infection (Fig. 5). Therefore, expression of MnSOD in Tn-5B1-4 cells significantly reduces the adverse

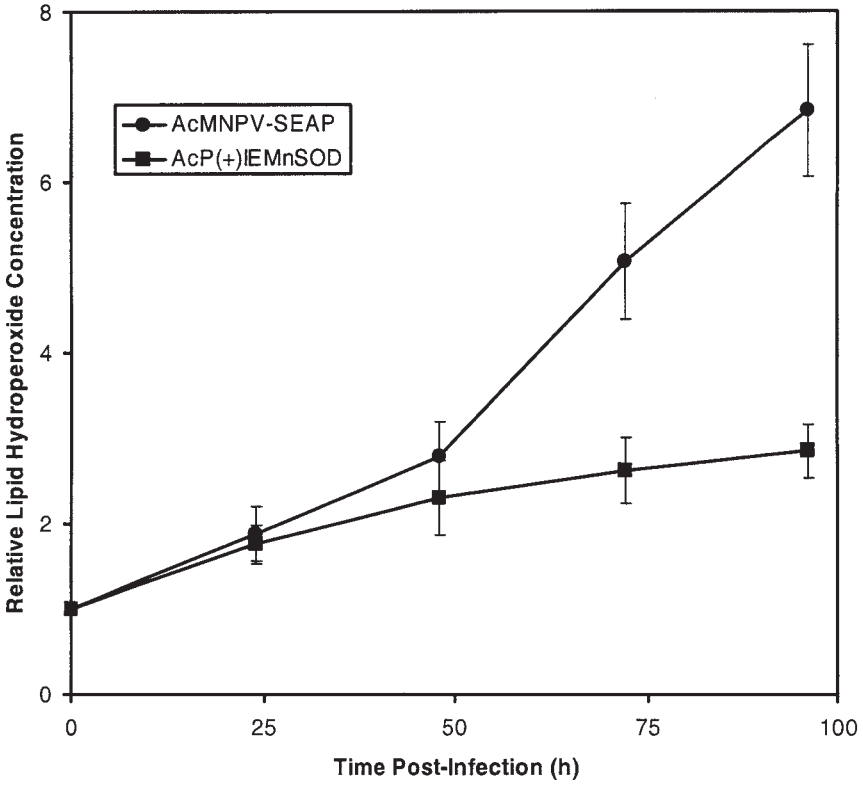


Fig. 5. Concentration of lipid hydroperoxides in Tn-5B1-4 cells infected with recombinant *A. californica* multiple nucleopolyhedroviruses expressing secreted alkaline phosphatase (AcMNPV-SEAP) or manganese superoxide dismutase (AcP(+)IEMnSOD) at a multiplicity of infection of 10 in shake-flask studies as a function of time post-infection. The error bars representing the 95% confidence levels based on four shake-flask experiments.

effects of oxidative stress (as measured by lipid oxidation) following baculovirus infection. Comparable protection from oxidative damage was not observed in Sf-9 cells.

## *Protein Carbonyls*

The effect of expression of MnSOD on protein carbonyl concentrations found in baculovirus-infected Sf-9 and Tn-5B1-4 cells is shown in Figs. 6 and 7, respectively. Consistent with the cell viability and lipid hydroperoxide results, expression of MnSOD did not significantly reduce the protein oxidation damage (as measured by protein carbonyl content) found in baculovirus-infected Sf-9 cells (Fig. 6). By contrast, expression of MnSOD in baculovirus-infected Tn-5B1-4 cells resulted in a significant reduction in protein oxidation damage, especially late in the infection cycle (>48 h pi) (Fig. 7).

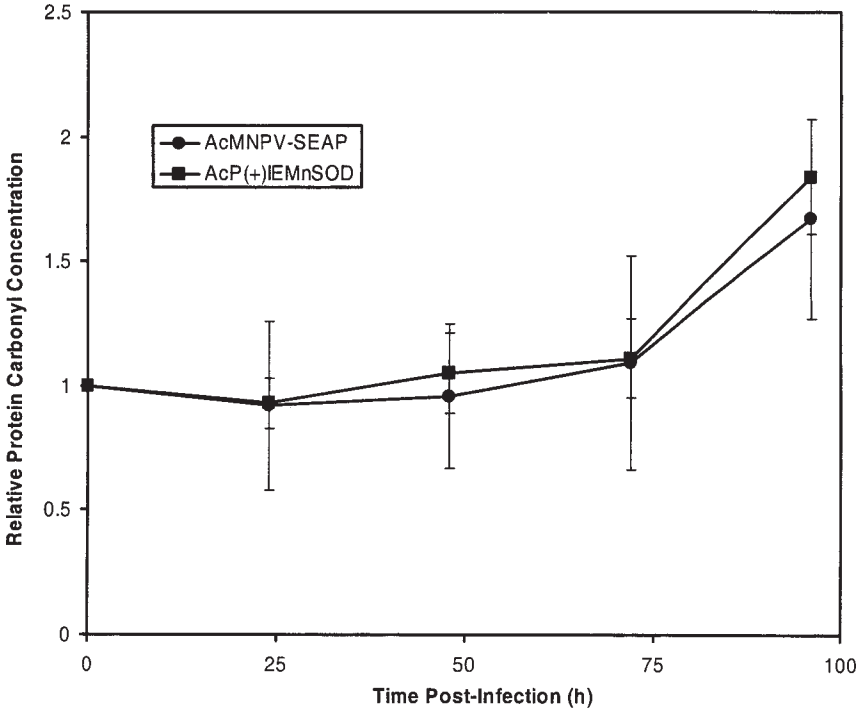


Fig. 6. Concentration of protein carbonyls in Sf-9 cells infected with recombinant *A. californica* multiple nucleopolyhedroviruses expressing secreted alkaline phosphatase (AcMNPV-SEAP) or manganese superoxide dismutase (AcP(+)IEMnSOD) at a multiplicity of infection of 10 in shake-flask studies as a function of time post-infection. The error bars representing the 95% confidence levels based on four shake-flask experiments.

## Conclusion

This study demonstrated that expression of MnSOD significantly reduces oxidative stress, as measured by reduced protein and lipid oxidation, in baculovirus-infected Tn-5B1-4 cells. By contrast, no significant reduction was observed when MnSOD was expressed in baculovirus Sf-9 cells. Since MnSOD activity increased in both cell lines, the behavior observed in Sf-9 cells may be owing to the improper targeting of the MnSOD, i.e., targeting to sites within the cell other than the sites of $O_2^{\bullet -}$ accumulation. Unfortunately, immunogold electron microscopy studies to determine MnSOD targeting were inconclusive. An alternative explanation for the results obtained in Sf-9 cells is that an imbalance existed between SOD and APOX activities comparable with SOD and GPX/CAT imbalances observed in mammalian cells *(15)*. Such an imbalance could lead to intracellular $H_2O_2$ accumulation that counteracts the positive effects of $O_2^{\bullet -}$ removal. Therefore, coexpressing MnSOD and APOX/CAT in baculovirus-infected Sf-9 cells may result in a significant reduction in oxidative damage.

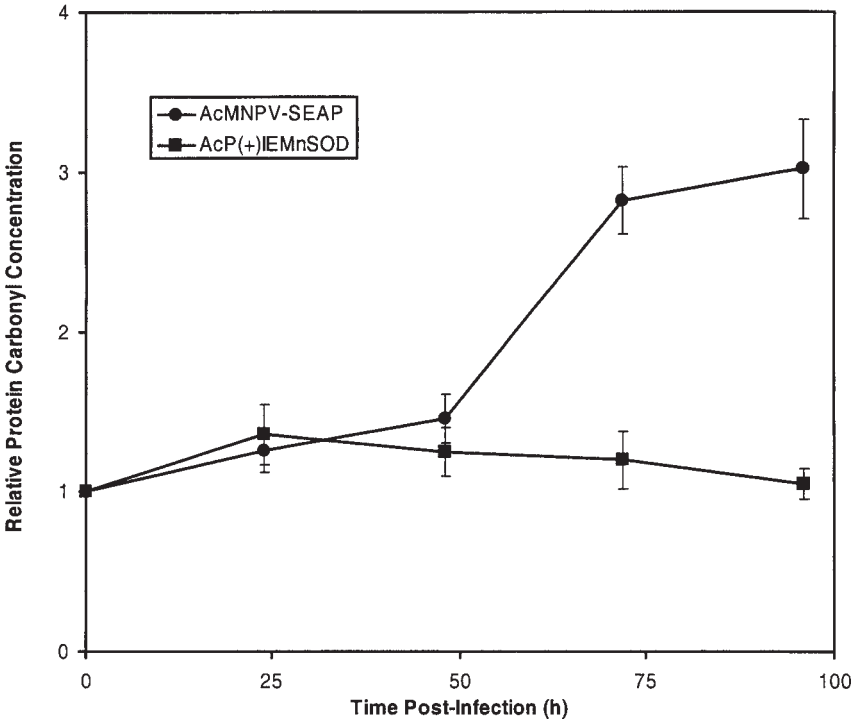


Fig. 7. Concentration of protein carbonyls in Tn-5B1-4 cells infected with recombinant *A. californica* multiple nucleopolyhedroviruses expressing secreted alkaline phosphatase (AcMNPV-SEAP) or manganese superoxide dismutase (AcP(+)IEMnSOD) at a multiplicity of infection of 10 in shake-flask studies as a function of time post-infection. The error bars representing the 95% confidence levels based on four shake-flask experiments.

Furthermore, it is possible that coexpressing MnSOD and APOX/CAT in baculovirus-infected Tn-5B1-4 cells may increase the level of protection from oxidative stress. Investigations designed to test all of these scenarios are ongoing in our laboratories.

## Acknowledgments

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