This student paper was written as an assignment in the graduate course

Free Radicals in Biology and Medicine

(77:222, Spring 2001)

offered by the

Free Radical and Radiation Biology Program
B-180 Med Labs
The University of Iowa
Iowa City, IA 52242-1181
Spring 2001 Term

Instructors:
GARRY R. BUETTNER, Ph.D.
LARRY W. OBERLEY, Ph.D.

with guest lectures from:
Drs. Freya Q . Schafer, Douglas R. Spitz, and Frederick E. Domann

The Fine Print:

Because this is a paper written by a beginning student as an assignment, there are no guarantees that everything is absolutely correct and accurate.

In view of the possibility of human error or changes in our knowledge due to continued research, neither the author nor The University of Iowa nor any other party who has been involved in the preparation or publication of this work warrants that the information contained herein is in every respect accurate or complete, and they are not responsible for any errors or omissions or for the results obtained from the use of such information. Readers are encouraged to confirm the information contained herein with other sources.

Influenza Infection

Nothing to Sneeze At

By

Patricia A. Rose

125 B Chemistry Building
Department of Chemical and Biochemical Engineering
University of Iowa
Iowa City, IA 52243

For 77:222, Spring 2001

May 3, 2001

Abbreviations:
CuZnSOD = Copper/Zinc Superoxide dismutase
NF-?B = nuclear factor ?B

Table of Contents

Abstract:

Influenza A is an enveloped RNA virus that has negatively impacted the population. When a phagocyte encounters the virus it is stimulated to release reactive oxygen and cytokines (e.g. TNF and IL-1). Virus infections are not like bacterial infections. The viral infection does not make a local foci like bacteria. ROS is very potent against bacteria but have little effect on the titer of the virus. The phagocytes are stimulated to release higher levels of ROS as the virus infection persists and this can lead to damage. The viruses can also induce lipid peroxidation, impairing many cellular processes. NO is also induced by infections and when it encounters superoxide it can form peroxynitrite, which is significantly more toxic than NO. The viral induced reactive oxygen can reduce the overall concentrations of antioxidants in the cell. Recent studies have shown that vitamin E and C can help the cell survive by inhibiting NF-?B. MnSOD is also present and it is able to dismute the superoxide to hydrogen peroxide. MnSOD has been shown to have some efficacy in treating the oxidative stress of an infection. EC-SOD has also been tested for reduction of oxidative damage. It was shown to be more effective than MnSOD when it was transfected into the mouse genome. Overall the virus is not the problem in an influenza infection, it is the reactive oxygen. Treatments in the future will no doubt include vitamin C, E and MnSOD.

Introduction

Influenza or flu viruses are from the Orthomyxoviridae family of animal RNA viruses [1]. This family contains a small number of viruses that have had a strong impact on the population. It is responsible for acute upper respiratory disease, and usually accompanied by fever and myalgia. Type A is the most common. It is responsible for the most serious flu epidemics. From 1918 to 1919 influenza A caused the death of more people than in World War I [1]. Influenza A infects humans and many species of mammals and birds. Type B only infects humans and is not extensively studied.

This paper will focus on influenza A and effects of antioxidant levels.

Physical Characteristics of Influenza A

Influenza is composed of an internal nucleocapsid and an envelope. The envelope is made of an inner matrix protein, lipid bilayer and external glycoproteins (please see figure 1) [1]. The shape of the virus particle or virion is not consistent. They range from round particles of about 100 nm in diameter, to somewhat elongated to very long (at times several thousand nanometers) filamentous particles of the same diameter [1]. There are surface glycoprotein spikes that are 10 to 12 nm long which cover the outside of the envelope. There are two different types of spikes, hemagglutinin and neuraminidase, with distinct biological activity. Hemagglutinin determines the antigen specificity [2]. Neuraminidase assists the entry of the virus into the body by breaking glucoside links in polysaccharide conjugated proteins [2]. The virus has a divided RNA genome of approximately 6×10^6 Da, which has a high content of uracil (approximately 35%) [1].

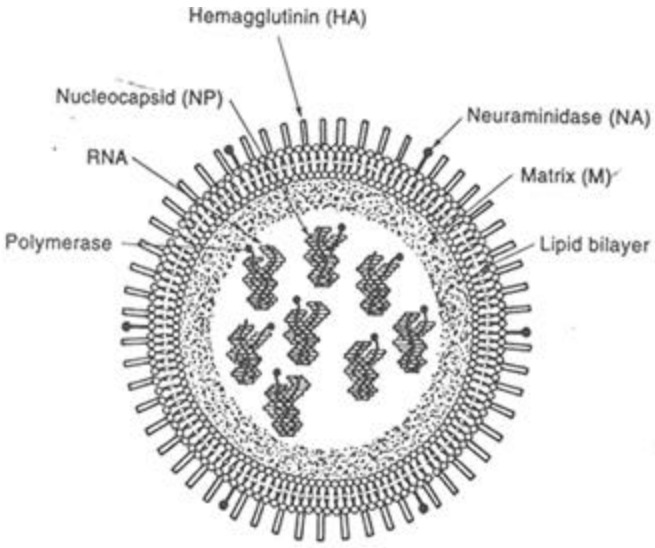


Figure 1: Schematic presentation of an Influenza Particle.

The virus RNA genome contains overlapping genes that are read during different phases of the infection to produce different proteins. The multiplicity of its genetic components allows for frequent changes in the influenza virus. When two different virus strains combine, there can be a genomic reassortment. This type of mixing can cause an antigenic shift, causing a sudden appearance of new serotypes. Change can also occur by antigenic drift that happens by spontaneous point mutations.

Virus Infection Cycle

The viral infection cycle starts with the virions adsorbing into the cell. The virion nucleic acid enters the interior of the cell with or without the other virion components. The cellular genetic replication machinery recognizes the virus genome where the virion components, including the viral genomic nucleic acid, are reproduced. The virion is then assembled and released into the milieu.

Comparison of Effect of Reactive Oxygen Species in Virus infections to other Microbial infections

Reactive oxygen species in virus infections can lead to pathogenesis but in bacterial, fungal or parasitic infection it is antimicrobial in action. The difference in result seems to be due to the difference in the mode of invasion of the various types of pathogens (please see figure 2) [3]. The most primitive host defense response is physical containment [3]. When a bacterium infects the body, it forms a foci that is distinct from normal tissues. Reactive oxygen species (ROS) can then be released in a confined area. Viruses usually attack tissue indiscriminately without forming a localized area of infection although a specific tissue tropism is well recognized for each virus [3]. Free radical effector molecules (NO and $O_2^{\bullet -}$) produced by the host will be released in a general area of infection where it can assault both normal and virus infected cells. This diffusive release of free radicals would have a counter effect on the host compared to microbial effects. The delicate balance between the host and the invader may ultimately determine consequences of free radicals. If a virus stimulates the host to produce more oxidant than the cellular buffering can accommodate, than a pathological condition may develop.

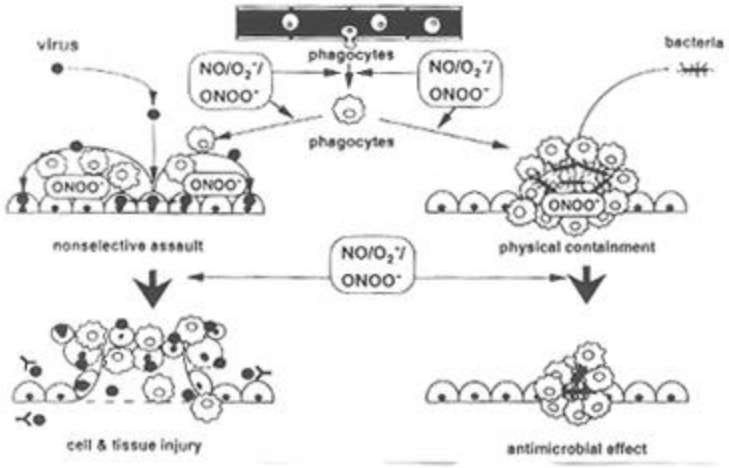


Figure 2: Schematic drawing of the different modes of the biological effects of free radicals such as $O_2^{\bullet -}$ and NO and their product peroxynitrite ($ONOO^-$) in virus and bacterial infections.

Immunological Responses to Virus Infection

Viruses are a group of organisms that obligated to enter a host to proliferate. The body can mount an immune response to this invader when an antigen-presenting cell processes the virus into pieces and displays these pieces on its surface [1]. Helper T cells recognize these antigens and help the B cells produce antibodies. Antibodies can interfere with virus-cell interactions, including adsorption, penetration, uncoating or replication. Antibodies to some components of the virus surface (hemagglutinin) neutralizes more efficiently than antibody to other components (neurminidase) [4]. Vaccination with killed whole influenza A can result in an increased ability to mount a human major histocompatibility-restricted, cytotoxic T cell response against cells infected with the same or different strains [4]. Influenza A can still be infective by avoiding the antibody response by changing the surface hemagglutinin and neuraminidase glycoprotein every few years.

Virus Activation of White Blood Cells

Viruses can activate phagocytes, leading to the release of reactive oxygen species and prooxidant cytokines, such as tumor necrosis factor (TNF) and interleukin – 1 (IL-1). These cytokines promote iron uptake by the recticuloendothelial system (RES, includes macrophages, specialized endothelial cells and reticular cells of the lymphatic tissue and bone marrow [5]). Most ROS do not diffuse more than a few fm, however the lipid peroxides resulting from the ROS induced peroxidation of membrane phospholipids, such as malondialdehyde, can transverse the circulation and cell membranes with resultant dysfunction of the vital cellular processes [6]. The TNF produced can act on host cell mitochondria, to induce a prooxidant effect by inhibiting the mitochondrial respiration at site II [6]. When respiration is disrupted superoxide can form. TNF

also acts to release NF-?B from I?B. Activated monocytes release IL-1 which stimulates neutrophils to release lysosomal proteins, including lactoferrin, which can induce hypoferremia. If the iron level exceeds the cellular binding capacity, free iron could interact with superoxide by Fenton chemistry to produce hydroxyl radicals.

A variety of viruses, including influenza A and B can produce apoptotic cytotoxicity. This induced cellular death may be a natural phenomenon evolved to contain viral infections [6].

Role of Oxygen radicals in Viral Infection

Oxygen radicals, like superoxide, are produced in a defense response. Inflammatory phagocytes, like neutrophils and macrophages, on encountering or ingesting pathogens, excrete a variety of oxidants (e.g. $O_2^{?-}$, H_2O_2, hypochlorous acid and nitrogen oxides) and are believed to be the major generators of oxidants [3]. When mice were infected with influenza A intranasally, it was shown that the level of xanthine oxidase (XO) in the brochoalveolar lavage fluid (BALF) was two to three orders of magnitude higher than the noninfected controls [3]. When allopurinol, a potent inhibitor of XO, was given to the mice prior to infection, it helped them survive a higher titer of virus [3]. This result indicates that death of the virus-infected animal is a consequence of the elevated levels of superoxide released by XO and not the virus itself.

Biological Implications of NO production in Virus Infection

Inducible nitric oxide synthase (iNOS) expression appears to be mediated through induction of proinflammatory cytokines. Induction of IFN-? and TNF-a in the lung preceded iNOS induction [3]. NO production by virus-infected macrophages can be reduced by IFN- aß. IFN-aß is a well

known antiviral effector molecule involved in the host defense mechanism [3]. Down regulation of iNOS expression can be accomplished by IL-4, IL-10 and TGF-ß. It has been suggested that NO has antiviral effects which may be because NO can block DNA synthesis by inhibiting ribonucleotide reductase [3]. This inhibition impairs the cellular energy metabolism by suppressing the mitochondrial electron transport system. Overproduction of NO together with superoxide production appears to non-selectively impair the physiological functions of the host cell regardless of the infection [3]. Inhibition of NO biosynthesis does not affect the yield of virus, indicating that the virus is not adversely affected by reactive oxygen species [3]. However NO can react with superoxide at an almost diffusion limited rate constant (6.7×10^9 M^{-1} s^{-1}) forming peroxynitrite ($ONOO^-$) [3].

Biological effect of Peroxynitrite

Peroxynitrite is much more reactive than NO. $ONOO^-$ can cause nitration of tyrosine residues that impairs intracellular signal transduction [3]. It can trigger lipid peroxidation, inactivation of aconitases and inhibition of the mitochondrial electron transport chain [3]. Peroxynitrite can induce apoptosis and has a cytotoxic effect on various cells [3]. The above list would indicate that peroxynitrite is just a destructive substance but it is also necessary in tissue remodeling. Peroxynitrite can activate neutrophil procollagenase, which is necessary in tissue degeneration under physiological and pathological conditions [3].

Role of Oxidants in Influenza Virus Induced Gene Expression

Influenza virus infection causes pathological changes predominantly in the epithelial layer of the respiratory tract. The source of ROS may be chemical or cellular. It has been shown that

xanthine oxidase is increased in influenza virus infected lungs [7]. The ROS may also be coming from activated leukocytes or the infected epithelial cells of the lung [7]. There are several defense mechanisms to control the release of ROS, including superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx) and Indoleamine 2,3-dioxygenase (IDO). Superoxide can be metabolized by the dismutation reaction ($2O_2^{?-} + 2H^+ \rightarrow O_2 + H_2O_2$) in different locations by different superoxide dismutases. CuZnSOD is a constitutively expressed cytoplasmic enzyme and MnSOD is a mitochondrial enzyme that is induced by oxidant stress [7]. The hydrogen peroxide produced in the dismutase reaction is converted to water by catalase in peroxisomes and by GPx in the cytoplasm. Indoleamine 2,3-dioxygenase (IDO) uses $O_2^{?-}$ in the oxidation of tryptophan but does not produce H_2O_2.

When cultured human airway epithelial cells were exposed to influenza virus interleukin 8 (IL-8), IDO and MnSOD mRNA expression was induced [7]. Data showed that the NF-?B was also up regulated by approximately 12 hours post-infection [7]. The IL-8 gene is influenced by the oxidant sensitive transcription factor, NF-?B [7]. IDO may be an important antioxidant but it is still under investigation and its effectiveness may be hindered by the depletion of intracellular tryptophan required as a substrate.

When the antioxidants, pyrrolidine dithiocarbamate (PDTC) and n-acetyl-L-cysteine (NAC), were added to the infected cell culture there was a reduction in oxidant production [7]. These oxidants were also shown to be able to block NF-?B activation and IL-8 gene expression in epithelial cells and this suggests an oxidant mediated intracellular signaling event after influenza virus infection [7]. PDTC and NAC are thiol compounds and may exert their antioxidant effects

through glutathione. The MnSOD gene has one NF-?B consensus sequence and multiple SP-1 and AP-2 sites [7]. Domann *et al*. have recently shown that AP-2 could specifically repress MnSOD promoter activity [8]. This result and the work by Jacoby *et al*. indicate a link between the oxidative environment and AP-2 regulation of NF-?B. AP-2 may work as a redox sensor, upregulating the NF-?B to produce MnSOD mRNA.

To investigate the levels of antioxidants *in vivo*, Jacoby et al. infected mice with influenza and followed the levels of mRNA [10]. They showed that an influenza A infection could increase the expression of genes encoding the antioxidant enzymes MnSOD, IDO, heme oxygenase I and glutathione peroxidase [9]. However, CuZnSOD and catalase mRNA were not induced [9]. The infection was also able to activate AP-1, CEBP and NF-kB, which are affected by oxidative stress [9]. When protein levels were investigated they noted that despite increased gene expression for MnSOD there was not an increase in enzyme activity [9]. This result once again reiterates the finding of Domann *et al*. in that there is another mechanism for control in MnSOD expression.

Decrease in Tissue Antioxidant levels Post Influenza A Infection

The most important tissue antioxidants are ascorbic acid, glutathione and a-tocopherol. These can repair the damage induced by ROS. Antioxidant levels were quantified in mice infected with influenza A (please see figure 3) [10]. Over the course of infection vitamin C, glutathione and vitamin E decreased steadily by 35, 47 and 43% respectively [10].

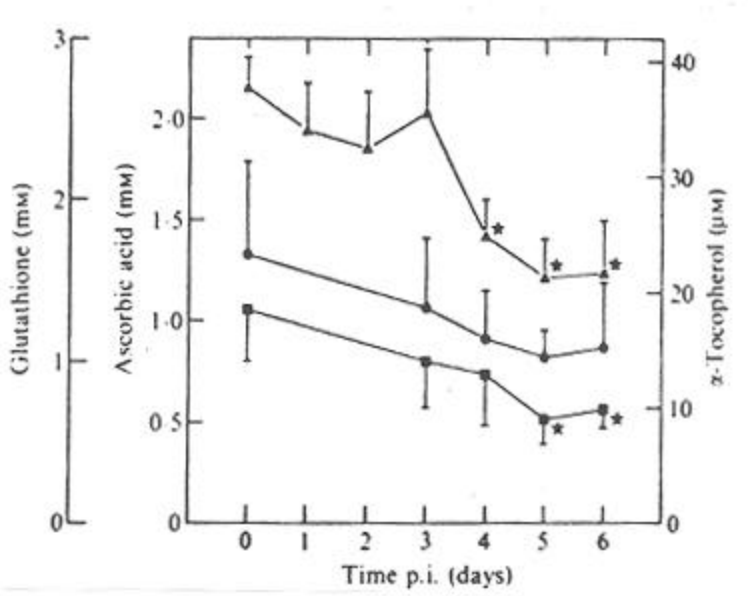


Figure 3: Levels of ascorbic acid (?), reduced glutathione (¦) and a-tocopherol (?) in homogenates of lung tissue from mice suffering from influenza. Concentrations were calculated assuming that 1 gm of wet tissue equals 1 mL. Ten control and between four and six infected mice were used at each time. Stars indicate results that are significantly different from the corresponding control values, as calculated using Student's *t*-test at P<0.01.

Even thought the overall antioxidant concentrations decrease, there wasn't a significant decrease in the ratio of oxidized to total concentration [10]. These results argue against a direct and early contribution of oxidants to the tissue damage observed in the lungs of mice [10]. Meaning that the damage observed does not occur when the antioxidants are totally exhausted but may be an indirect functional consequence of oxidative stress. However damage is a very late response to infection. There might be earlier inhibitory mechanisms that are controlled by the concentration of antioxidants and once they are lowered, cannot take place.

Vitamin E inhibits NF-?B Activation

To determine if vitamin E has an early control function Packer and Suzuki stimulated human lymphoma cells (Jurkat T cells) with TNF-a and looked for an increase in NF-?B in the nuclear extract [11]. When a-tocopherol succinate or vitamin E acetate was added to the cells there was a concentration dependent inhibition of NF-?B translocation [11]. Vitamin E might be affecting

the level of NF-?B by inhibiting the phosphorylation of I?B. When I?B becomes phosphorylated it releases NF-?B so it is free to be translocated into the nucleus and up regulate inflammatory cytokines. This inhibitory reaction of a-tocopherol succinate does not appear to be specific, however it is more potent than NAC or a-lipoic acid for the inhibition of TNF-a induction of NF-?B [11]. The inhibition may solely be due to the strong antioxidant properties of vitamin E.

Vitamin C inhibits NF-?B

To determine if Vitamin C has an early effector activity HUVECs (cells isolated from human umbilical veins) were stimulated with TNF and various proteins were qualitated. O'Neill *et al.* showed that when HUVECs were treated with Vitamin C from 0 to 20 mM one hour prior to treatment with TNF there was a dose dependent decrease in IL-8 production [12].

Activation of NF-?B by TNF was also completely abolished when the HUVECs were treated with 5 mM vitamin C for 16 hours [12]. Vitamin C could also block the upregulation of NF-?B by IL-1, PMA and H_2O_2 [12]. Vitamin C could also reduce the level of lipid peroxidation. When HUVECs were pretreated with 10 mM vitamin C there was a decrease the basal levels of TBARS to 68 +/- 4% of control values and completely blocked the TNF mediated increase (please see table I) [12].

Table I – Effect of vitamin C on basal and TNF-stimulated TBARS

Treatment	TBARS (% Control)
Vitamin C	68 ± 4 (8)[a]
TNF	123 ± 8 (6)[a]
TNF + Vitamin C	66 ± 17 (6)[a,c]

[a] Confluent monolayers of ECV304 cells were pretreated with 10 mM vitamin C for 1 h before stimulation with or without 40 ng/ml TNF for 30 min. Control cells were left untreated. TBARS levels were measured in triplicate and results were calculated as nmol malondialdehyde equivalents/mg protein. Control levels were 0.23 ± 0.02 nmol malondialdehyde equivalents/mg protein (n = 8). Results shown are mean ± SE TBARS expressed as a percentage of control. The number of experiments is indicated in parentheses.

[a] $p < 0.05$ vs control.

[c] $p < 0.05$ vs TNF.

Unlike vitamin E, the inhibition of NF-?B translocation into the nucleus is not primarily due to its antioxidant properties. When other antioxidants were tested, they were unable to mimic the general inhibitory effect of vitamin C on NF-?B [12]. Interestingly, vitamin E analogs failed to further potentate vitamin C mediated inhibition of IL-1 and TNF [12]. This result indicates that vitamin C in this instance is not acting as a protector but as an effector.

To investigate this activity of vitamin C, it was tested for activity on I?B. Vitamin C was shown to completely block the phosphorylation of I?B by Inhibitor kinase kinase (IKK) [12]. This kinase is responsible for the regulation of the general transcription factor TFIID in RNA polymerase II during NF-?B dependent gene transcription [12]. The specific target for p38 is still under investigation.

Protection by MnSOD

TNF is induced in viral infections. Several studies have shown that TNF mediated activation of either NF-?B, apoptosis or activation of various kinases requires the production of intermediates including reactive oxygen intermediates (ROI) [13]. When breast cancer cells (MCF-7) were transfected with an MnSOD construct it was first noted that these cells grew slower than the wildtype or a neomycin plamsid transfected control [13]. This growth inhibition may be due to an increased production of H_2O_2 through MnSOD. It may also be due to the cell having to divide it's cellular energy further to produce a protein at high levels that is normally significantly lowered in transformed cells.

MnSOD was shown to inhibit TNF induced NF-?B activation. When 10 pM TNF was added to the native and neomycin transformed MCF-7 cells, NF-?B was translocated into the nucleus [13]. When significantly higher levels of TNF were added to the MnSOD transfected cells (up to 1000 pM) there was a very minimal increase in band density corresponding to NF-?B [13]. TNF has also been shown to activate c-Jun kinase (JNK) [13]. JNK can activate AP-1 which in turn can upregulate IFN-ß. IFN- ß can interact with thioredoxin reductase to stimulate cell death [14]. The MnSOD transfected cells were able to keep the JNK from becoming activated [13].

The MAP kinase kinase (MEK) can also phosphorylate I?B. Phosphorylated I?B can then detach from NF-?B, allowing it to then go into the nucleus and stimulate gene transcription. MEK can become activated by TNF in a dose dependent manner [13]. The MnSOD transfected cells did not allow MEK to become activated [13].

These above effects of MnSOD on NF-?B are not specific to TNF as activation of NF-?B by okadaic acid, taxol and H_2O_2 were also blocked [13].

MnSOD as a Treatment for Influenza Infected Mice

The above studies have shown that MnSOD is able to keep NF-?B from becoming activated. These results would suggest that MnSOD might be used as a treatment to reduce the pathogenesis of influenza infections. Pathogenicity of influenza infection in mice involves, in part, an over reaction of the immune response of the host rather than a direct effect of virus multiplication. It has been shown that there is a reduction in the lethal effects by influenza infections by injection of CuZnSOD when it is conjugated to a pyran copolymer [15]. However

this data is confounded because the pyran copolymer also has antioxidative properties. When unconjugated CuZnSOD was tested it did not offer any protective effect but this result is probably due to its short half-life rather than inactivity [15]. MnSOD is a mitochondrial enzyme that can also be used extracellularly to remove superoxide and it has a plasma clearance time in mice of approximately 6 hours [15].

When MnSOD was given to influenza-infected mice by intravenous and intraperitoneal routes, it was moderately effective in inhibiting disease development [15]. There was a moderate prolongation of life (mean day of death) at the 2 highest dosages of MnSOD (100 mg/kg/day and 50 mg/kg/day, respectively [15].

Lung titers in the placebo treated controls were $10^{6.3}$ to 10^{7} per mL and those of in the MnSOD treated groups were approximately the same [15]. This result shows that the MnSOD does not have an antiviral effect but rather assists the patient in dealing with the oxidant assault generated by the virus [15].

The MnSOD treatment was able to reduce reduce lung consolidation [15]. When the lungs become consolidated there is reduction in the mean oxygen level in the blood and as the level decreases it leads ultimately to death. The attenuation of disease was slight but the disease progression in a mouse is significantly more rapid than in humans [15]. A moderate improvement might have more dramatic results in humans.

Mice Overexpressing EC-SOD

Extracellular superoxide dismutase (EC-SOD) is a dismutase that has a half-life of approximately 20 hours [16]. EC-SOD is concentrated on the cell membrane and the extracellular matrix and it is the predominant antioxidant enzyme expressed by alveolar type II pneumocytes in the lung [16]. Transgenic (TG) mice have been developed that overexpress EC-SOD in the pulmonary alveolar type II cells [16]. The TG animals show no significant alternations in other lung antioxidants such as catalase, CuZnSOD, MnSOD or glutathione peroxidase activity or the level of total, reduced or oxidized glutathione [16].

To determine if the upregulation of the dismutase would have a protective effect both wildtype and EC-SOD transgenic mice were challenged with an influenza infection. When the TG mice were compared to wildtype their total SOD activity was significantly higher in the bronchoalveolar lavage lung fluid (BALF) [16]. The increase in superoxide dismutase was mainly caused by an increase in EC-SOD [16]. The level of GSH was determined to be equivalent in both the TG and wildtype mouse before the start of the test. After the mice were infected with influenza A the TG total glutathione (GSH) levels were approximately two fold higher than in wildtype (please see figure 4) [16]. This result indicates that the oxidant levels were reduced with increased levels of EC-SOD, protecting the GSH from oxidation. When Spitz *et al*. worked with oxidative stress resistant O2R95 cells, they also saw an increase in the total glutathione (GSH) and glutathione S-transferase (GST) activity [17].

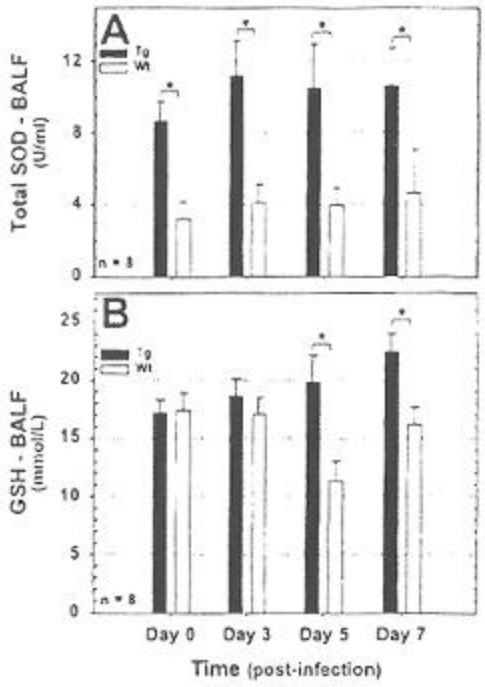


Figure 5: Levels of antioxidants in the BALF of influenza virus infected mice. A: there was a 3 fold increase in the total superoxide dismutase (SOD) activity in the BALF in TG vs. wildtype mice. B: GSH levels did not change among the TG mice throughout the experiment. Values are means of +/- S.E., n = number of mice/group. *P < 0.05.

Proinflammatory cytokines were analyzed and it was shown that IL-6, IFN-?, MIP-2a and TNF-a in wildtype mice were almost two to three times the levels seen in the transgenic infected mice [16]. This is an indication that the cells in the TG mice lungs were not as inflamed as their wildtype counterparts.

Conclusion

Influenza A is an enveloped RNA virus that is responsible for significant lost of life. When it infects a body it is able to stimulate the phagocytes to release reactive oxygen and cytokines (e.g. TNF and IL-1). Virus infections do not form a local infection like bacteria. The viral infection can attack some cells and leave their neighbors disease free. This hit and miss infection strategy confounds the immune cells into releasing large amounts of reactive oxygen that can have

pathogenic effects. The immune system can form antibodies to the influenza. These antibodies can interfere with the viral infection by disrupting virus-cell interactions of adsorption, penetration, uncoating or replication. The viruses can induce lipid peroxidation that can result in impaired cellular processes. The reactive oxygen that is generated in infections does not adversely affect the titer of the virus. NO is also induced by infections and when it encounters superoxide it can form peroxynitrite. Peroxynitrite can cause all sorts of damage in the cell but it is also a necessary component to tissue remodeling after infection. The viral induced reactive oxygen can place the cell in a stressful state (much like writing this paper), which can lower the overall concentrations of antioxidants. However recent studies have shown that vitamin E and C can help the cell (and student) survive the stress by inhibiting NF-?B. NF-?B is necessary for the production of viruses and when it is down regulated the virus is not able to properly form into a virion. MnSOD is able to dismute the superoxide to hydrogen peroxide and has shown some efficacy when used to treat the oxidative stress of an infection. EC-SOD was shown to be more effective when it was transfected into the genome of the mouse, but this is a little more aggressive than most will take to reduce the symptoms of the flu. Overall the virus is not the problem in an influenza infection, it is the reactive oxygen. Treatments in the future will no doubt include vitamin C, E and MnSOD.

Future Directions and Experiments

The different topics introduced in the paper will be briefly noted followed by ideas for further experiments.

Stocker *et al*. were able to show that the levels of antioxidants in mice were decreased after infection. The diet of these mice was not tightly controlled. The mice need to be fed a diet rich and poor in vitamin C, E and selenium. This experiment might be able show which antioxidant is a benefit to the survival of the influenza-infected mouse by specifically looking at the lung tissue.

They also noted that proteases could cleave hemagglutinin on the surface of the influenza virus, causing it to become significantly more infective. Reactive oxygen species can denature the protease inhibitors in the lung tissue. Exogenous protease inhibitors could be given directly into the lung to help lower the infectivity rate. The inhibitors could be in a nebulized form to assure that the drug would be dosed far enough into the lung to have a maximal effect, not allowing the drug to be transported into the stomach to be denatured.

Packer *et al*. were able to show that human lymphoma cells when grown with vitamin E analogs were able to inhibit NF-?B in a non-specific manner. The rate of this reaction should be investigated to determine which is the rate limiting step. Cells could be "hyper-loaded" and/or depleted of vitamin E and then to measured for the level of NF-?B activation. This maximal approach might also help to clarify how vitamin E is able to interact with the NF-?B upregulation.

O'Neill *et al*. were able to show that vitamin C inhibits NF-?B activation by TNF by the activation of p38 mitogen activated protein kinase. Vitamin C acts as a control mechanism for NF-?B, which raises the question of how NF-?B become activated in animals that can make their own vitamin C. Amphibians, reptiles and most birds can make there own vitamin C and could

be used to show how a viral infection could progress. They also showed that vitamin E did not potentate NF-?B inhibition, however only a limited range of concentrations were tried. This interaction should be further investigated with varying levels of each vitamin C and vitamin E. It was shown that the p38 mitogen activated protein kinase was key for the continued inactivation of NF-?B. This should be introduced to the cell, either in an inducible form or by fusion in a p38 filled liposome. This manipulation would allow for the ability to see if this kinase is concentration dependent.

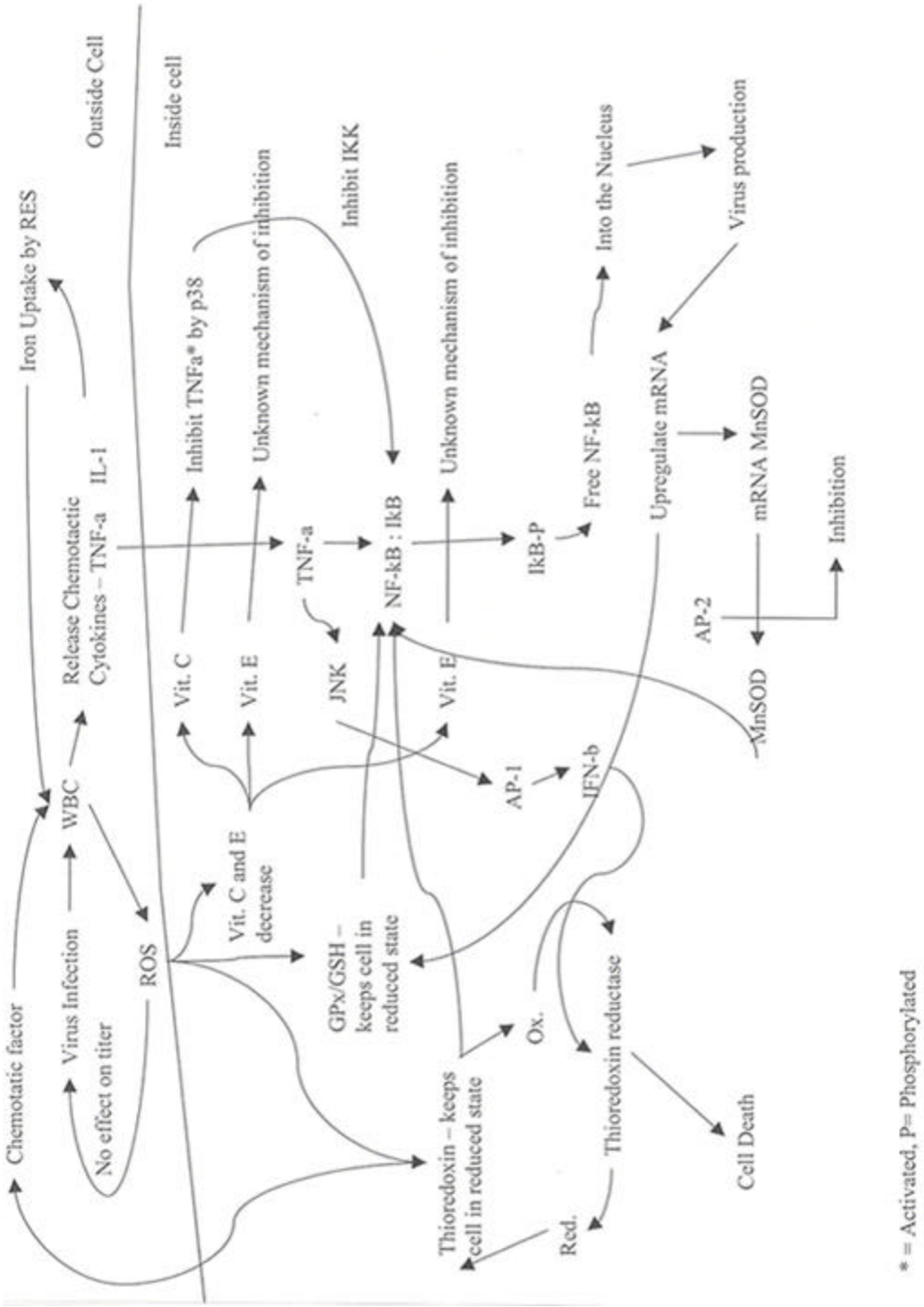
Panet *et al*. showed that when mice were dosed with MnSOD by intraperitoneal and intravenous routes there was a decrease in the influenza disease progression. The virus was dosed into the lungs. In this study the level of MnSOD was not measured in the cells of lungs or in the surfactant fluid so there is a possibility that the MnSOD was not able to get to the lungs. MnSOD needs to be dosed into the lungs in a nebulized form so that it will be able to get deep into the lungs where persistent infections can localize and cause damage.
Jacoby *et al*. have investigated levels of MnSOD. It was noted that oxidative stress increased the level of mRNA for MnSOD without an increase in activity. This result indicates that the mRNA was not available for translation. There can be several reasons for this result. The mRNA has become blocked by methylation or by binding of an inhibitor protein. Recently it was shown that MnSOD has AP-2 binding sites. AP-2 could be down regulated, possib ility by using antisense RNA and then measure levels of MnSOD protein.

Folz *et al*. developed transgenic mice that could over express the extracellular superoxide dismutase. This would not work as a therapy but it did show that the EC-SOD did not adversely

affect other antioxidants, including CuZnSOD, MnSOD and GSH. EC-SOD should be given to the wildtype mouse to determine if this enzyme could allow for the same antioxidant effects as the transgenic species. The EC-SOD mRNA could also be transfected into the cells to determine if it could offer protection to reactive oxygen species generated from influenza infections.

Aggarwal *et al*. showed that MnSOD transfected breast cancer cells could block NK-?B activation by a variety of sources. However these transformed breast cancer cells did not have the same growth pattern as the control or neomycin transformed cells. Growth inhibition or slowing is a pathway for a cell to delay replication of DNA until the conditions are favorable. One way to synchronize a culture is by serum starvation. Once all of the cells have stopped replicating, feeding the cells could restart the culture. Differences between the cultures could be minimized.

Flow Chart


Outside Cell
Inside cell
Iron Uptake by RES
Release Chemotactic Cytokines – TNF-a IL-1
Inhibit TNFa* by p38
Unknown mechanism of inhibition
Inhibit IKK
Into the Nucleus
Virus production
Chemotatic factor
Virus Infection
WBC
No effect on titer
ROS
Vit. C and E decrease
Vit. C
Vit. E
TNF-a
JNK
NF-kB : IkB
Unknown mechanism of inhibition
IkB-P
Free NF-kB
Upregulate mRNA
AP-2
mRNA MnSOD
Inhibition
MnSOD
Vit. E
AP-1
IFN-b
GPx/GSH – keeps cell in reduced state
Ox.
Thioredoxin – keeps cell in reduced state
Thioredoxin reductase
Red.
Cell Death
* = Activated, P= Phosphorylated

References:

1. Levy, J.A., Fraenkel-Conrat, H, Owens, R.A, ed. (1994) *Virology*, Englewood Cliffs, N.J.: Prentice Hall, 90-92.
2. World Wide Web.m-w.com (Dictionary).
3. Akaike, T., Suga, M., Maeda, H (1998) Free Radicals in Viral Pathogenesis: Molecular Mechanisms Involving Superoxide and NO. Proc. Soc. Exp. Biol. Med., 217, 64-73.
4. Roitt, I., Brostoff, J., Male, D. (1985) *Immunology*. Gower Medical Publishing, London, New York.16.1-16.2.
5. Worldwide web.ndif.org/terms/RES.htm.
6. Schwarz, K.B. (1996) Oxidative Stress During Viral Infection: A Review. Free Radical Biol. and Med. 21, 641-649.
7. Knobil, K., Choi, A.M.K., Weigand, G.W., Jacoby, D.B. (1998) Role of oxidants in influenza virus-induced gene expression, Am J Physiol., 274, L134-42.
8. Zhu, C.H., Huang, Y., Oberley, L.W., Domann, F.E. (2001) A Family of AP-2 Proteins Down-regulate Manganese Superoxide Dismutase Expression, J. Biol. Chem., 276, 14407-14413.
9. Choi, A.M., Knobil, K., Otterbein, S.L., Eastman, D.A., Jacoby, D.B. (1996) Oxidant stress response in influenza virus pneumonia: gene expression and transcription factor activation, Am. J. Physiol., 271, L383-391.
10. Hennet, T., Peterhans, E., Stocker, R. (1992) Alternations in antioxidant defenses in lung and liver of mice infected with influenza A virus, J. of Gen. Virology, 73, 39-46.
11. Suzuki, Y.J. and Packer, L. (1993) Inhibition of NF-?B activation by vitamin E derivatives, Biochem. Biophys. Res. Comm., 193, 277-283.
12. Bowie, A.G. and O'Neill, L.A.J. (2000) Vitamin C Inhibits NF-?B Activation by TNF Via the Activation of p38 Mitogen-Activated Protein Kinase, J. of Immunology, 7180-7188.
13. Manna, S.K., Zhang, H.J., Yan, T., Oberley, L.W., Aggarwal, B.B. (1998) Overexpression of Manganese Superoxide Dismutase Suppresses Tumor Necrosis Factor-induced Apoptosis and Activation of Nuclear Transcription Factor-?B and Activated Protein-1, J. Biol. Chem., 273, 13245-13254.
14. Hofmann, E.R., Boyanapalli, M., Lindner, D.J., Weihua, X., Hassel, B.A., Jagus, R., Gutierrez, P.L., Kalvakolanu, D.V., Hofman, E.R. (1998) Thioredoxin reductase mediates cell death effects of the combination of beta interferon and retinoic acid, Mol. Cell. Bio., 11, 6493-6504.
15. Sidwell, R.W., Huffman, J.H., Bailey, K.W., Wong, M.H., Nimrod, A., Panet, A. (1996) Inhibitory effects of recombinant manganese superoxide dismutase on influenza virus infections in mice, Antimicrob. Agents Chemother., 11, 2626-2631.
16. Suliman, H.B., Ryan, L.K., Bishop, L., Folz, R.J. (2001) Prevention of influenza-induced lung injury in mice overexpressing extracellular superoxide dismutase, Am. J. Physiol. Lung Cell Mol. Physiol., 280, L69-L-78.
17. Sullivan, S.J., Oberley, T.D., Roberts, R.J., Spitz, D.R. (1992) A stable O2-resistant cell line: role of lipid peroxidation byproducts in O2-mediated injury, Am. J. Physiol., 262, L748-L756.