Alterations in antioxidant defences in lung and liver of mice infected with influenza A virus

Thierry Hennet, Ernst Peterhans* and Roland Stocker†

*Present address: The Heart Research Institute, 145 Missenden Road, Camperdown, Sydney, N.S.W. 2050, Australia.

Institute of Veterinary Virology, University of Berne, Länggass-Strasse 122, 3012 Berne, Switzerland

We investigated the possible involvement of oxidative mechanisms in the pathogenesis of influenza A/PR8/34 virus infection in mice. As a biochemical marker of oxidative stress, we determined the endogenous concentrations of the antioxidants glutathione and vitamins C and E in their reduced and oxidized forms in the lungs, liver and blood plasma of control and infected animals. Following intranasal infection with 8 to 10 LD₅₀, influenza virus was detected in the lungs, but not in the plasma, liver or other organs. Infection resulted in a decrease in the total concentration of glutathione and vitamins C and E, whereas no relevant change in the ratio of oxidized to total concentration of antioxidants was observed. Changes in the concentration of hepatic antioxidants were significant in the early stages of the infection. The results suggest that hepatic alterations may be caused indirectly by mechanisms related to the host response to virus infection. The observed general decrease in the antioxidant buffering capacity may reduce the ability of tissues to protect against potential oxidative stress. Such stress can occur during bacterial superinfections, which are common in influenza, thereby rendering the host more susceptible to the pathogenic effects of such agents. In addition, reactive oxygen species produced in the lung may inactivate protease inhibitors, resulting in increased protease activity. Using an in vitro system consisting of α1-antiprotease, trypsin and HOCI as the oxidant, we have shown that the infectivity of influenza viruses can be increased up to 10000-fold by proteolytic cleavage of haemagglutinin, leading to activation of the fusogenic properties of this protein.

Introduction

Influenza A virus infection causes respiratory disease by mechanisms which are still largely obscure. The limited cytopathic effect of the virus on epithelial cells lining the respiratory tract cannot itself explain the pneumonia resulting from infection. There is increasing evidence from mouse models of influenza that suggests that tissue damage is linked to the host’s effector mechanisms, which are activated by and directed against the invading virus (Yap & Ada, 1978; Leung & Ada, 1980). Several investigators have established the importance of cytotoxic T cells in the development of pneumonia (Wyde & Cate, 1978; Suzuki et al., 1974). However, the biochemical nature of this self-inflicted damage remains to be established. Our group and others have suggested that effector mechanisms involved in the clearance of pathogens, such as the generation of reactive oxygen species (ROS) by phagocytes, could participate in the development of the disease (Peterhans et al., 1987, 1988; Oda et al., 1989). ROS could contribute to the tissue damage seen in lungs either directly by oxidizing lipids, proteins and nucleic acids (Imlay & Linn, 1988), or indirectly by activating certain proteases (Weiss, 1989).

Influenza viruses and paramyxoviruses can directly activate monocytes and polymorphonuclear leukocytes in vitro to generate ROS (Peterhans et al., 1987). To address a conceivable in vivo causal relationship between ROS production and the progression of lung tissue destruction over the course of influenza virus infection, we measured the antioxidant oxidation–reduction (ox–red) status of tissues as a marker of oxidative stress (Lunec & Blake, 1985; Stocker et al., 1986; Grootveld & Halliwell, 1987). Oxidative stress is defined as a disturbance of the prooxidant–antioxidant balance in favour of prooxidants. An array of antioxidant defence mechanisms, including enzymes like superoxide dismutase, catalase, and small molecules such as vitamins C and E, and glutathione (Halliwell & Gutteridge, 1985), protect tissues against oxidants. Linked via red–ox reactions, antioxidant defence systems are ultimately dependent on cellular energy metabolism. It follows that exposure of a tissue to oxidative stress is likely to result in an increase in the ratio of oxidized to reduced forms of important antioxidants.
We have measured the levels of vitamins C and E, and glutathione in both reduced and oxidized forms in tissues of mice infected intranasally with influenza A virus. In addition to parameters in the lung, which represents the primary target organ for virus replication, we investigated the same parameters in the liver. Although it is not a site of virus replication, this organ has been shown to be influenced to a certain extent in influenza (Ruben & Michaels, 1975; Davis et al., 1983). Our results demonstrated that over the course of infection the levels of reduced forms of pulmonary antioxidants decrease. More strikingly, we observed significant changes in the concentration of hepatic antioxidants in early stages of the infection, before the occurrence of lung consolidation. The possible relevance of these results to influenza pathogenesis is discussed.

Methods

**Virus.** A mouse-adapted strain of influenza A/PR8/34 (H1N1) was used as a concentrated lung homogenate (about 10⁶ LD₅₀/ml). Aliquots of this virus suspension were stored at −70 °C. Immediately before infection of mice, the virus suspension was thawed and diluted to the desired infectious dose in Hank’s balanced salt solution (HBSS) containing 0.5% bovine serum albumin (BSA) and 25 µg/ml gentamicin. Virus containing the inactive precursor of haemagglutinin (HA) was prepared by infection of MDBK cells at a multiplicity of approximately 10 median egg-infectious dose (EID₅₀)/cell. After adsorption at 37 °C for 1 h, the inoculum was removed and fresh medium containing 2% foetal bovine serum was added after washing the cells four times. The supernatants were harvested 36 h post-infection (p.i.). The virus was concentrated by centrifugation for 1 h at 25,000 r.p.m. in an SW28 rotor after removal of cell debris by centrifugation for 10 min at 1000 g.

**Cleavage of haemagglutinin by trypsin and oxidative inactivation of protease inhibitor.** Zl-Antiprotease was treated with HOCl as described previously (Halliwell et al., 1987). Briefly, HOCl was freshly prepared from a stock of 3% NaOCl (sextemplex; G rogg Chemie). All solutions were made in HBSS pH 7-4. The concentration of HOCl was adjusted to 100 µM using an extinction coefficient of 100 M⁻¹ cm⁻¹ at 235 nm. HBSC (200 µl; 100 µM) was added to 200 µl of zl-antiprotease (Sigma) (400 µg/ml) and the mixture was incubated for 1 h at 25 °C. Influenza virus suspensions (100 µl) were then incubated for 1 h at 37 °C with either trypsin (final concentration 10 µg/ml), trypsin and zl-antiprotease (final concentration 20 µg/ml), or trypsin and zl-antiprotease/HOCl. Cleavage of HA was monitored by assaying for haemolysis (for a review see Rott & Klenk, 1988). Typically, 250 µl of the treated virus samples was mixed with 100 µl of a 10% human erythrocyte suspension and further diluted with 1-65 ml of citrate buffer pH 5-25. After a 1 h incubation at 37 °C, the samples were centrifuged for 5 min at 10,000 g and the absorption was read using a spectrophotometer at 540 nm. Infectivity was determined by endpoint titration in embryonated eggs as described below.

**Animals.** Outbred pathogen-free female ICR-ZH mice (Institute for Animal Husbandry, University of Zürich, Switzerland) aged 4 to 6 weeks were infected intranasally with 8 to 10 LD₅₀, of influenza virus and fed ad libitum.

**Virus titration.** Mice were killed by cervical dislocation. Lungs were removed, weighed and homogenized in HBSS at 10%, (w/v), and the homogenate was further diluted serially up to 10⁻¹² with HBSS; four 11-day-old embryonated eggs were then inoculated with 100 µl of each dilution. After 3 days of incubation at 37 °C, the allantoic fluids were tested for the presence of HA. Briefly, 40 µl of PBS and 10 µl of the corresponding allantoic fluid were pipetted into the wells of a round-bottom microtitre plate (Costar). To detect HA, 50 µl of 0.7% human erythrocytes suspended in PBS containing 1% BSA was added to each well and the results were recorded after a 2 h incubation at 4 °C. The virus titre was calculated using the method of Reed & Muench (1938).

**Detection of viral antigen by immunocytochemistry.** Viral antigen was detected using the peroxidase-antiperoxidase (PAP) method of Sternberger (1979). As the primary antibody we used a rabbit antisem directed against sucrose gradient-purified influenza A virus, strain A/RI 5 (H₂N₁s subtype). The staining protocol was optimized using influenza virus-infected lung tissue.

**Tissue removal for biochemical analyses.** Mice anaesthetized with ether were exsanguinated by section of the subclavian arteries. Tissues were removed, washed in cold 10 mM-PBS, blotted dry, frozen immediately on dry ice and analysed within 60 min. For the analysis of ascorbate and dehydroascorbic acid, lungs were perfused through the right ventricle of the heart with 10 ml cold PBS. Within 15 min after the perfusion, lungs were removed, homogenized and analysed. Additional experiments showed that the trace amounts of blood left within the lungs after bleeding or perfusion were without consequence for the outcome of the analysis of pulmonary antioxidants.

**HPLC system.** The HPLC systems used for the analysis of uric acid and vitamins E and C consisted of a pump (Constametric 3000; LCD-Milton Roy) equipped with a pulse damper, a 5 µm LC-18 column (25 × 0.46 cm) with a guard-column (Supelco), a dual series glassy-carbon electrode connected to two amperometric controllers and detectors (LC-4B; BAS) and an integration system (CR-4A; Shimadzu). A second system was used to determine lipids. It consisted of a pump (Spectroflow 400; ABI Analytical), a 5 µm LC-18 column (25 × 0.46 cm) with a guard-column (Supelco), a u.v. light detector (Spectroflow 757; ABI Analytical) and an integration system (CI-10B; Milton-Roy).

**Chemicals.** HPLC solvents (Merck) were of Lichrosolv quality. The following standards were used: ascorbic acid (Goldmark from Aldrich), α-tocopherol and α-tocopherolquinone (Kodak), uric acid, reduced glutathione (GSH) and glutathione disulphide (GSSG) (all from Sigma). The lipid standards cholesteryl, cholesteryl stearate, cholesteryl oleate, cholesteryl linoleate, cholesteryl linolenate and cholesteryl arachidonate were from Sigma. Other chemicals were purchased from Merck unless specified.

**Vitamin C.** Ascorbic acid and its oxidation product dehydroascorbic acid were measured according to a combination of the methods described by Behrens & Madère (1987) and Kutnin et al. (1987). Briefly, 1 g of tissue was homogenized in 1 ml of cold 5% metaphosphoric acid. The samples were centrifuged at 10,000 g for 5 min. To 400 µl of the supernatant was then added either 115 µl of 2.58 M-K₃HPO₄ pH 9.8 (for ascorbate only) or 115 µl of 1% homocysteine (Fluka) in 2.58 M-K₃HPO₄ pH 9.8 (for total vitamin C). The samples were incubated for 30 min at 25 °C, then further diluted with the mobile phase before being subjected to HPLC. The mobile phase, containing 40 mM-sodium acetate, 0.54 mM-EDTA, 1.5 mM- ascorbic acid and 1.5 mM-dehydroascorbic acid were measured according to a combination of the methods described by Behrens & Madère (1987) and Kutnin et al. (1987). Briefly, 1 g of tissue was homogenized in 1 ml of cold 5% metaphosphoric acid. The samples were centrifuged at 10,000 g for 5 min. To 400 µl of the supernatant was then added either 115 µl of 2.58 M-K₃HPO₄ pH 9.8 (for ascorbate only) or 115 µl of 1% homocysteine (Fluka) in 2.58 M-K₃HPO₄ pH 9.8 (for total vitamin C). The samples were incubated for 30 min at 25 °C, then further diluted with the mobile phase before being subjected to HPLC. The mobile phase, containing 40 mM-sodium acetate, 0.54 mM-EDTA, 1.5 mM-dehydroascorbic acid was calculated by subtracting the value of ascorbate from that of total vitamin C.

**Vitamin E.** Blood plasma or tissues homogenized in cold 10 mM-PBS (1:10, w/v) were extracted in methanol:hexane according to
an aliquot was subjected to HPLC. HPLC conditions were as described by Grootveld & Halliwell (1987) to detect uric acid electrochemically. The analysis of vitamin E in its reduced and oxidized forms was performed simultaneously and under oxygen-free conditions, as described by Pascoe et al. (1987).

Uric acid. Blood plasma was extracted with cold 5% metaphosphoric acid (1:1, v/v) and centrifuged for 5 min at 10000 g. Samples were further acidified by adding 200 gl of 20 mM-HCl to 50 gl of extract and an aliquot was subjected to HPLC. HPLC conditions were as described by Grootveld & Halliwell (1987) to detect uric acid electrochemically.

Glutathione. The levels of GSH and GSSG in lungs and liver were analysed enzymically by the GSSG reductase assay developed by Chang et al. (1983) with the modification described in Chang et al. (1988). The changes in absorbance at 412 nm were measured with a Beckman DU 70 spectrophotometer using the kinetic program.

Thiols. Thiols were analysed according to Ellman (1959). Briefly, 100 µl of plasma was mixed with 900 µl of 200 mM-sodium phosphate buffer, 2 mM-EDTA, pH 9.0 in a cuvette and the reaction was started by the addition of 20 µl 10 mM-dithionitrobenzene dissolved in 10 mM-sodium phosphate buffer pH 7.0. The increase in absorption at 412 nm was then monitored. GSH was used as a standard for quantification.

Lipids. Blood plasma was separated by two-step centrifugation at 10000 g. Lipids were extracted as for vitamin E and analysed spectrometrically after separation by HPLC according to Yamamoto et al. (1987). We used the mobile phase methanol:butanol (50:50) isocratically at a flow rate of 1 ml/min. Lipids were detected at 210 nm and quantified by comparison of the resulting peaks to those obtained by injection of pure standards.

Liver enzymes. The concentrations of glutamate-oxalacetate transaminase (GOT) and glutamate dehydrogenase (GLDH) in blood plasma were measured by using commercially available diagnostic kits (Sigma).

Results

Mice (4 weeks old) were infected intranasally with 8 to 10 LD₅₀ of mouse-adapted influenza A/PR8/34 virus. The first symptoms of disease, such as piloerection, reduced food intake and lethargy, were apparent by 3 days p.i. By the end of day 6 all infected animals were dead (Fig. 1). The maximum virus titre in the lung was reached by day 2 p.i., i.e. before disease symptoms were observed (Fig. 1). No infectious virus was detected in liver assayed at daily intervals over the course of infection. Inspection of fixed liver sections immunostained using the PAP method (Sternberger, 1979) revealed no evidence for the presence of viral antigen (data not shown). As secondary bacterial infection often accompanies influenza, we carried out experiments to isolate bacteria from the lungs. No pathogenic bacteria or mycoplasma were observed in infected or uninfected mice. This identifies influenza A virus as the causal agent of the pathology described. Histological examination of the lungs revealed extensive infiltration of mononuclear cells. PAP immunostaining confirmed that virus replication was restricted to the epithelial cells lining the alveoli and airways (data not shown).

We measured the ratio of oxidized and reduced forms of important antioxidants as a marker of oxidative stress. Fig. 2 shows that the concentration of the reduced forms of vitamin C, glutathione and vitamin E decreased steadily by 35, 47 and 43%, respectively, during infection. However, we found no net increase in the
Table 1. *Enhancement of influenza A virus infectivity by oxidants*

<table>
<thead>
<tr>
<th></th>
<th>Virus alone</th>
<th>Virus + trypsin</th>
<th>Virus + trypsin + antiprotease</th>
<th>Virus + trypsin + antiprotease/HOCI</th>
<th>Virus + antiprotease/HOCI</th>
<th>Antiprotease/HOCI</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Infectivity</strong></td>
<td>$10^{5.1}$</td>
<td>$10^{5.4}$</td>
<td>$10^{5.4}$</td>
<td>$10^{5.1}$</td>
<td>$10^{4.1}$</td>
<td>0</td>
</tr>
<tr>
<td><strong>Haemolysis†</strong></td>
<td>0.05</td>
<td>0.25</td>
<td>0.06</td>
<td>0.33</td>
<td>0.05</td>
<td>0.03</td>
</tr>
</tbody>
</table>

* Virus titres are given in median egg infectious dose.
† Data represent extinction coefficients at 540 nm measured in the supernatant of an erythrocyte suspension mixed with a virus sample.

Table 2. *Concentrations of non-enzymic antioxidants in the blood plasma of mice infected with influenza A virus*

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
<th>Day 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic acid</td>
<td>39.7 ± 11.8</td>
<td>31.8 ± 4.6</td>
<td>35.9 ± 3.2</td>
<td>42.2 ± 3.4</td>
<td>40.6 ± 8.8</td>
</tr>
<tr>
<td>Dehydroascorbic acid</td>
<td>11.9 ± 6.4</td>
<td>5.0 ± 4.6</td>
<td>6.5 ± 5.4</td>
<td>15.8 ± 11.8</td>
<td>7.1 ± 4.4</td>
</tr>
<tr>
<td>α-Tocopherol</td>
<td>8.6 ± 1.5</td>
<td>7.2 ± 1.7</td>
<td>6.8 ± 1.6</td>
<td>4.5 ± 0.8†</td>
<td>6.4 ± 1.5†</td>
</tr>
<tr>
<td>α-Tocopherolquinone</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Uric acid</td>
<td>49.4 ± 10.2</td>
<td>45.3 ± 5.8</td>
<td>39.7 ± 14.2</td>
<td>52.5 ± 25.3</td>
<td>40.0 ± 6.0</td>
</tr>
<tr>
<td>Thiols</td>
<td>773.1 ± 127.2</td>
<td>622.1 ± 47.8</td>
<td>800.9 ± 80.8</td>
<td>810.6 ± 36.9</td>
<td>904.4 ± 219.0</td>
</tr>
</tbody>
</table>

* All values are given in μM.
† These values differed significantly from controls as determined by Student’s t-test (P < 0.01).

concentration of dehydroascorbic acid and GSSG, the oxidized forms of vitamin C and glutathione, respectively. As a result, the ratio of the oxidized to the reduced form of the antioxidants did not change during infection. The only exception was glutathione which became significantly more oxidized 6 days p.i. (data not shown). α-Tocopherolquinone, a form of oxidized vitamin E, was not detected in lung extracts from control or infected animals.

Oxidative stress could indirectly enhance the titre of virus by inactivating antiprotease which protects the alveolar lining cells against proteases. Since cleavage of HA is essential for the expression of virus infectivity, inactivation of antiproteases could result in enhancement of virus infectivity. To assess this possibility, we carried out an experiment using cell culture-grown virus known to possess the HA precursor, HA₀ (Rott & Klénk, 1988). Virus was incubated * in vitro * with either trypsin, trypsin and α1-antiprotease, or trypsin and α1-antiprotease pretreated with HOCl as oxidant. The infectivity of virus increased 10000-fold with trypsin treatment, an effect that was prevented by α1-antiprotease but not by pretreated protease inhibitor (Table 1). Trypsin treatment also enhanced the haemolytic activity of the virus measured at acidic pH, which was prevented by α1-antiprotease but not by pretreated inhibitor. Furthermore, pretreated α1-antiprotease alone was not haemolytic (Table 1).

We next determined the ox–red status of representative antioxidants found in blood plasma (Wayne et al., 1985). No decrease in water-soluble antioxidants vitamin C, uric acid and thiol groups was observed. In particular, we observed no significant change in the level of reduced and oxidized vitamin C (Table 2), which is the antioxidant most sensitive to oxidative stress in blood plasma (Frei et al., 1988). In contrast, the level of lipid-soluble vitamin E (α-tocopherol) decreased to 52% of the control value. To determine whether this change was due to an oxidative degradation or to alterations in lipid metabolism, we investigated the lipid composition of the blood plasma of mice. Vitamin E is transported in lipoproteins to its target organs and its concentration correlates with that of cholesterol in blood (Bjornson et al., 1976). We found that the concentration of circulating triglycerides in the blood of infected mice decreased steadily (data not shown). The level of free cholesterol remained unaltered, whereas those of various classes of cholesteryl esters increased over the course of infection. The changes seen in triglyceride levels might be related to the decreased food intake of infected mice, as these results also could be obtained in uninfected mice in a restricted diet model. However, by contrast to infected mice, increased cholesteryl linoleate, linolenate and arachidonate concentrations were not found in the blood of mice on a restriction diet (data not shown). It is noteworthy that α-tocopherolquinone could not be detected by the method used in either healthy or influenza virus-infected mice.

The liver is a major organ of metabolism of the antioxidants investigated in this study. In this organ, ascorbic acid and most glutathione are synthesized (Griffith & Meister, 1979; Chatterjee et al., 1975). The liver of mice sustained major changes in its antioxidant balance over the course of influenza virus infection.
These changes occurred early in infection, before the onset of disease symptoms and before infected mice ate less. The concentration of α-tocopherol and GSH decreased steadily (Fig. 3), and again we did not find any significant change in the levels of α-tocopherolquinone and GSSG (data not shown). Also, the ox-red status of vitamin E and glutathione remained unchanged. In contrast to the other antioxidants, ascorbic acid levels increased to 141% of control values during the initial stages of infection (Fig. 3). After reaching a plateau by day 3, the levels decreased to control values by the end of the infection. No significant changes were found in dehydroascorbic acid and in the ox-red status of vitamin C (data not shown).

No signs of inflammation, oedema or necrotic lesions were observed histologically in the liver during infection (data not shown). In addition, no infectious virus (Fig. 1) or viral antigen could be detected in this organ. That the integrity and function of the liver was altered during infection was indicated by the increase in plasma GOT and GLDH also. However, the amount of these enzymes increased only moderately, reaching levels approximately twofold that of the control 6 days p.i. (data not shown).

**Discussion**

We previously have proposed a role for ROS in the pathogenesis of certain viral diseases (Peterhans et al., 1987, 1988). Indeed, recent findings from our laboratory (Christen et al., 1990; G. D. Buffinton, personal communication) and others (Oda et al., 1989) have shown that increased production of ROS accompanies influenza in mice. Also, oxidative damage to macromolecules has been suggested to contribute significantly to the pathogenesis of this disease, as application of the antioxidant enzyme superoxide dismutase, conjugated to pyran polymer, protects mice infected with a lethal dose of influenza virus (Oda et al., 1989).

To assess the potential importance of oxidative stress and damage in influenza, we measured the total concentration and ox-red status of the most important tissue antioxidants ascorbic acid, glutathione and α-tocopherol. This approach has been used successfully in other disease states, such as parasitic infections (Stockter et al., 1985), rheumatoid arthritis (Lunec & Blake, 1985; Grootveld & Halliwell, 1987) and ischaemia reperfusion (Arduini et al., 1988).

Over the course of infection we noted a steady decrease in the concentration of antioxidants in total lung homogenates. In contrast, the ox-red ratios remained unchanged, except for that of glutathione 6 days p.i. As the infection progressed, more regions of the lungs became infiltrated mainly by mononuclear cells and moderate oedema was observed. Therefore, changes in the cellular composition and the increase in extracellular fluid may have contributed to these changes. In human mononuclear cells isolated from blood, the concentrations of the major antioxidants are in the mM range (Bergsten et al., 1990), i.e. similar to that observed in uninfected lung tissue (compare with Fig. 2). In plasma, the concentrations of glutathione, ascorbate and α-tocopherol are approximately 1000-, 25- and fourfold less than in tissues including lung (Fig. 2 and Table 2). This clearly argues against dilution as a cause of the observed changes in the concentrations of these antioxidants in the lungs. It is more likely that increased production of oxidants may have caused the decrease in the antioxidants. Indeed, in previous studies we have noted an increase in the activity of the superoxide-generating enzyme xanthine/xanthine oxidase (Christen et al., 1990), a decrease in superoxide dismutase and an increase in the concentration of endogenous hydrogen peroxide in the lungs of infected mice (G. D. Buffinton, personal communication). The presence of local oxidative stress in the lungs of mice suffering from influenza is also suggested by the more than 100-fold increase in the tryptophan-degrading enzyme, indoleamine-2,3-dioxygenase. This enzyme requires O₂ as a substrate and cofactor, and initiates the formation of a range of metabolites, some of which are antioxidants (Christen et al., 1990).

An unexpected finding of our studies was the
infection-induced alterations in hepatic functions, despite the apparent absence of virus (Fig. 1) and inflammation in this tissue. A functional impairment of liver was evident also from the moderate increase in plasma levels of GOT and GLDH, and decreased aminopyrine metabolism (the latter being a measure of cytochrome P450-dependent biotransformation) (M. Adé-Damilano, personal communication). The observed increase in hepatic levels of ascorbic acid (Fig. 3) could reflect an induction of vitamin C biosynthesis as an acute response of the host to the infection. Decreases in the hepatic antioxidants GSH and α-tocopherol have been reported in a mouse model of endotoxaemia, in which supplementation with antioxidants improved pathological consequences (Sugino et al., 1987, 1988). By contrast to the mouse endotoxaemia model, however, we observed a decrease in plasma triglycerides in our infection model. Disturbances in liver function occur in a number of other virus infections without evidence of virus replication in this tissue, e.g. after injection of a large dose of influenza B virus in mice (Davis et al., 1983). By contrast to that model, which is reminiscent of Reye’s syndrome, we observed no histological evidence for fatty degeneration in liver or brain oedema, nor was there a decrease in total cholesterol, as is observed in Reye’s syndrome in humans (Chaves-Carballo et al., 1979). Uninfected mice eating the same amount of food as infected ones had decreased plasma cholesterol, arguing against the possibility that the changes in lipid metabolism are simply the result of the decreased food intake observed from 3 days p.i. In the absence of detectable virus, the effects seen in the liver may be due to soluble mediator(s) released early during infection. Certain immune mediators, at high concentrations, have toxic effects on the liver. For example, interferons have been reported to have such effects (Gresser et al., 1975; Singh et al., 1982; Oberg et al., 1986), and other cytokines, such as interleukin 6 (Kishimoto, 1989) and tumour necrosis factor (Grunfeld et al., 1988), have also been recognized to act on liver metabolism. The possible roles of soluble mediators are currently being investigated in our laboratory.

Taken together, our observations argue against a direct and early contribution of oxidants to the tissue damage observed in the lungs of infected mice. However, more indirect functional consequences of oxidative stress may be of importance in the pathogenesis of influenza. Ascorbic acid and GSH serve as cofactors in enzymic reactions involved in stress responses, for example through the formation of noradrenaline (Levine, 1986) or leukotrienes (Hatzelmann & Ullrich, 1987; Peters-Golden et al., 1989). The antioxidants vitamin C and glutathione also have beneficial effects on the responsiveness of immune cells such as T lymphocytes, polymorphonuclear leukocytes and macrophages. Therefore, a decrease in the concentration of these antioxidants may result in local immunosuppression (Leibovitz & Siegel, 1981; Droge et al., 1986; Liang et al., 1989), thus facilitating the growth of opportunistic pathogens. Interestingly, a decrease in glutathione in airways has also been reported in human immunodeficiency virus-seropositive individuals and is associated with immunosuppression (Buhl et al., 1989). As an additional consequence, host-derived ROS could enhance the infectivity of the virus by inactivating α1-antiprotease, which protects the alveolar surface against the harmful effects of proteases (Weiss, 1989). In an in vitro system consisting of trypsin, α1-antiprotease and HOCl as an oxidant (also produced by neutrophils and monocytes), we were able to demonstrate that such inactivation dramatically enhances the infectivity of influenza virus possessing the inactive precursor of HA, HA0. Enhancement of the infectivity and haemolytic activity of influenza virus by trypsin-like enzymes reflects cleavage of the HA precursor protein into its active form, consisting of HA1 and HA2 (for a review see Rott & Klénk, 1988). In addition to enhancing virus infectivity, oxidative inactivation of α1-antiprotease could also contribute to proteolytic lung tissue injury, thus favouring changes in lung tissue that ultimately lead to emphysema (Clark et al., 1981). Under the conditions of oxidative stress generated by the immune response, host-derived proteases could play a detrimental role similar to that of bacterial proteases, which have been shown to exacerbate the course of influenza in mice (Tashiro et al., 1987). Protease inhibitors have been shown to protect against the lethal effects of influenza viruses in mice (Zhirkov et al., 1984; Lozitskii et al., 1987). Further experiments are needed to determine whether protection of antiproteases by antioxidants ameliorates the course of infection.

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References


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Sugino, K., Doi, K., Yamada, K. & Kawasaki, T. (1988). Changes in the levels of endogenous antioxidants in the liver of mice with...
experimental endotoxemia and the protective effects of the antioxidants. 

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