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OXIDATIVE STRESS IN LUNGS OF MICE INFECTED WITH INFLUENZA A VIRUS

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As oxidative stress has been implicated in the pathogenesis of certain viral diseases we determined antioxidant and prooxidant parameters in lungs and bronchoalveolar lavage fluid (BALF) of mice infected with a lethal dose of influenza A/PR8/34 virus. Viral infection was characterized by massive infiltration of leukocytes, mainly polymorphonuclear leukocytes, into the alveolar space. The total number of BALF cells increased up to 8-fold (day 3 post-infection) and these cells appeared activated as judged by their increased rates of superoxide anion radical ($O_2^{-\cdot}$) generation upon stimulation. Maximal rates of radical generation by BALF cells during the early stages of infection were 15- or 70-fold higher than those of cells from control animals when expressed per cell or total BALF cells, respectively. At the terminal stages of infection the total capacity of BALF cells to release $O_2^{-\cdot}$ declined to $\approx$ 35-fold the control values. Infection also resulted in increased *in vivo* formation of hydrogen peroxide (H_2O_2) within the lungs at a time that coincided with the maximal capacity of BALF cells to release $O_2^{-\cdot}$.

Whereas pulmonary activities of glutathione peroxidase and reductase remained unaltered, levels of ascorbate in the cell-free BALF decreased significantly during the early stages of the infection and then returned to normal levels and above, late in infection. The oxidation state of the dehydroascorbic acid/ascorbate couple increased concomitantly with the decrease in ascorbate concentrations early in infection and remained elevated throughout the infection. As assessed by the prevention of peroxyl radical-induced loss of phycoerythrin fluorescence, the total antioxidant capacity present in lung tissue homogenate from terminally ill animals was not diminished when compared to that prepared from lungs of control mice. We conclude that although early stages of influenza infection are associated with the presence of oxidative stress in the lung tissue and alveolar fluid lining the epithelial cells, this stress does not appear to overwhelm local antioxidant defenses. The results therefore do not support a direct causative role of oxidative tissue damage in the pathogenesis of influenza virus infection.

KEY WORDS: reactive oxygen species, antioxidants, ascorbate, redox status, inflammation, influenza

ABBREVIATIONS: BALF, bronchoalveolar lavage fluid; HBSS, Hanks balanced salt solution; H_2O_2, hydrogen peroxide; PMN, polymorphonuclear leukocytes; ROS, reactive oxygen species; $O_2^{-\cdot}$, superoxide anion.

INTRODUCTION

Both univalent and bivalent reduction of molecular oxygen takes place during normal aerobic metabolism, giving rise to superoxide anion radical ($O_2^{-\cdot}$) and hydrogen peroxide (H_2O_2). Although these oxygen reduction products, together with secondary

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more reactive oxygen species (ROS) derived from them, can exert beneficial functions such as the oxidative killing of invading microorganisms by activated immune phagocytes, they also have the potential to cause nonselective damage to host tissues. To prevent the latter, eukaryotic tissues possess an array of antioxidant defenses including enzymes, metal-binding proteins, and various small molecules.[1] However, it is becoming increasingly recognized that an imbalance in production of ROS and host antioxidant defenses in favour of the former, i.e., oxidative stress, can play a significant role in the pathogenesis of a number of diseases including atherosclerosis, chronic inflammation, post-ischemic reoxygenation injury, certain parasitic infections, and others.[1]

Acute influenza infection is characterized by oedema, haemorrhage, massive infiltration of inflammatory cells in the upper respiratory tract and the lungs, and can cause death to humans and experimental animals by mechanisms that remain largely obscure. For example, a direct cytopathic effect of the virus on epithelial cells lining the respiratory tract cannot by iteself explain pneumonia and the pathology associated with the infection.[2] Several studies have shown that certain paramyxoviruses (e.g., Sendai, parainfluenza-3 and Newcastle disease virus) and influenza viruses can directly activate appropriate immune cells (e.g., polymorphonuclear leukocytes, PMN) to produce ROS and to degranulate.[3,4] In the light of these findings and the known involvement of activated phagocytes as both initiators and potentiators of tissue damage via oxidative and proteolytic processes,[5] we have proposed previously a role for ROS in the pathogenesis of influenza infection.[2,6]

There is indirect evidence supporting the presence of local oxidative stress during the natural course of influenza infection. Hayaishi and co-workers reported that during a lethal infection of mice with influenza virus, indoleamine 2,3-dioxygenase, the tryptophan-degrading enzyme that requires $O_2^{-\cdot}$ for catalytic activity,[7] increased more than 100-fold in the trachea and lungs but not other tissues.[8] We have recently confirmed these results and also showed that some of the products derived from oxidative metabolism of tryptophan are very powerful antioxidants and present at elevated concentrations in the lungs of infected animals.[9] We proposed that these metabolites may function as additional local antioxidants during infection to counteract both the observed increase in activity of the $O_2^{-\cdot}$-producing enzyme xanthine oxidase and the decrease in the $O_2^{-\cdot}$-removing superoxide dismutase.[9] Further indirect support for a role of ROS in the pathogenesis of influenza is based on the observation that the infection-induced increase in pulmonary activity of xanthine oxidase is due mainly to the oxidative conversion of xanthine dehydrogenase to the oxidase,[9] and that administration of superoxide dismutase conjugated to a pyran polymer suppressed the lethal infection in mice.[10]

The aim of this study was to more directly investigate possible influenza-induced alterations in both production of ROS and antioxidant status of the lungs, the principle site of viral replication. We therefore assessed *in vivo* formation of H_2O_2 within the lung tissue of mice infected with a lethal dose of influenza virus, and the capacity of bronchoalveolar lavage fluid (BALF) cells to produce $O_2^{-\cdot}$. The antioxidant parameters examined over the course of infection included pulmonary activities of important antioxidant enzymes, total peroxyl radical-scavenging capacity present in the lungs, as well as the content of reduced and oxidized forms of vitamin C within BALF. The results indicate that although early stages of a lethal infection of mice with influenza A/PR8/34 virus are accompanied by local oxidative stress this stress does not appear to be directly responsible for the pathogenesis of this disease.

MATERIALS AND METHODS

1,2,4-Aminotriazole, bovine serum albumin, cytochrome c (type III), reduced and oxidized glutathione, mouse haemoglobin, superoxide dismutase, and urea were obtained from Sigma (St. Louis, MO). Ascorbic acid (Goldmark) and 2,2,2-tribromoethanol were purchased from Aldrich (Steinheim, Germany). Dodecyltriethylammonium phosphate (Q_{12}) was from Regis Chemicals (Morton Grove, IL), *B*-phycoerythrin from Calbiochem, flavine adenine dinucleotide, NADPH, and Triton X-100 (peroxide free) from Boehringer (Mannheim, Germany), and benzidene, homocysteine and H_2O_2 from Fluka (Buchs, Switzerland). All other chemicals and HPLC solvents (Lichrosolv quality) were from Merck (Darmstadt, Germany).

Animals, Virus Suspension and Infection

Outbred pathogen free female ICR-ZH mice (Institut für Tierzucht, University of Zürich, Switzerland) aged four to six weeks were used for all experiments. Mice were infected intranasally with 8-10 LD50 of a mouse-adapted strain of influenza A/PR8/34 (H1N1) virus in the form of a lung homogenate (about 10^8 LD50/ml). Aliquots of this virus suspension were stored at $-70°C$. Immediately before infection, the virus suspension was thawed and diluted to the desired infectious dose in Hanks' balanced salt solution (HBSS), containing 0.5% bovine serum albumin and 25 μg/ml gentamycin. Using this protocol, the first symptoms of disease were apparent by day three, and death of all infected animals occurred by day five to six post-infection. During the course of infection the food intake decreased significantly from day 3 onward and decreased to 87% at the terminal stage of infection. As a result the body weight of the animals decreased by up to 20% with significant changes observed at day 4 post-infection. In contrast to body weight, the weights of lungs of influenza virus-infected animals increased up to 2.5-fold over the course of infection. Due to these complications, we corrected data appropriately for potential differences in protein concentrations.

Tissue Removal, Bronchoalveolar Lavage and BALF Cells

Animals were anaesthetized by i.p. injection of 0.5 ml Avertin (150 mM NaCl, containing 2 vol.% of each 2,2,2-tribromoethanol and 2-methyl-2-butanol) and exsanguinated by section of the subclavicular artery and aorta. Lungs were perfused, removed, washed in ice-cold 100 mM potassium phosphate buffer pH 7.0, blotted dry and homogenized in the same buffer (1 : 5–1 : 10 w/v). For biochemical assays, the homogenate was centrifuged at 10000 × *g* for 15 min at 4°C. BALF cells were harvested from the alveolar cavity as described.[11] Contaminating erythrocytes were removed by hypotonic lysis, the leukocytes washed with HBSS containing 5 mM glucose and finally resuspended at 5×10^5 viable cells/ml HBSS. Cell viability was judged by trypan blue exclusion and the number of cells determined after staining with gentian violet (0.01% in 2% acetic acid). An aliquot of the cell suspension was used for a thin smear prepared with a cytospin (Shandon Southern), and the cells stained with Harleco Diff-Quik Stain Set (Merz and Dase, Düdingen, Switzerland) for differential counting (300–400 cells counted). Using this staining procedure, we could not differentiate unambiguously monocytes from macrophages and therefore listed both cell types together. For the determination of ascorbate and protein, lungs were

lavaged by three successive infusions and withdrawals of the same 1 ml of HBSS containing no glucose. Cells were removed by centrifugation at 4°C (10 min at 2000 × *g*), and the cell-free supernatant analysed immediately (for ascorbate), or stored at −20°C until assayed (for protein).

Production of ROS

Phorbol myristate acetate (1 μM)-induced generation of $O_2^{-\cdot}$ by BALF cells was determined by the superoxide dismutase (20 μg/ml)-inhibitable reduction of ferricytochrome c (50 μM), using an extinction coefficient of $\varepsilon_{550nm} = 21\,mM^{-1}\,cm^{-1}$. *In vivo* formation of H_2O_2 was assessed by the degree of inhibition of endogenous catalase mediated by 1,2,4-aminotriazole.[12] This assay is based on the *in vivo* interaction of 1,2,4-aminotriazole with compound I, the latter being formed only when catalase reacts with H_2O_2.[13] The extent of complex formation, and hence of H_2O_2 production, can be quantitated by the loss in catalase activity. The assay has been used successfully for the detection of H_2O_2 in various tissues and/or cells.[12,13] Mice were injected i.p. with 200 μl of either saline (control) or saline containing 20 mg 1,2,4-aminotriazole, 60 min prior to being anaesthetized with Avertin. Following perfusion, lungs were homogenized in cold 50 mM potassium phosphate buffer pH 7.0, containing 0.5% (v/v) Triton X-100.

Antioxidants

Ascorbate and dehydroascorbic acid in BALF were analysed by HPLC with electrochemical detection according to a combination of the methods described by Behrens and Madere[14] and Kutnik *et al.*[15] Tissue activities of glutathione peroxidase, GSSG reductase (in the presence and absence of 5 mM flavine adenine dinucleotide), and catalase were determined as described,[16–18] and the values corrected for contributions derived from contaminating red cells, estimated by measuring the concentrations of haemoglobin[19] in the lung homogenates.

Total radical scavenging activity present in lung homogenates of control and terminally ill mice was assessed by their inhibitory activity on the peroxyl radical-induced oxidation of *B*-phycoerythrin.[20] As the oxidant-induced loss of phycoerythrin fluorescence is inhibited by any efficient radical scavenger and to an extent that is directly proportional to the total antioxidant activity, this assay may be used to determine the total radical trapping capacity present in biological samples. For some experiments the crude lung homogenate was centrifuged (30 min at 4°C and 5000 × *g*) in a Centricon-10 micro-concentrator (MW ⩽ 10 000; Amicon, Danvers, MA) to remove proteinaceous material, and the ultrafiltrate used immediately for the phycoerythrin assay.

Tissue concentrations of 1,2,4-aminotriazole and protein were determined as described previously.[21,22] Data were analysed by ANOVA, *t*-test and Fisher PLSD tests and were considered significant at the 95% level of confidence (i.e., $p \leqslant 0.05$).

RESULTS

BALF Cells

Infection of mice with influenza A/PR8/34 virus resulted in a massive infiltration of

TABLE I
Differential count of inflammatory cells harvested by bronchoalveolar lavage from control and influenza A/PR8/34 virus-infected mice

Days post infection	Cells[a] ($\times 10^{-5}$)	Monocytes and Macrophages[b]	PMN[b]	Lymphocytes[b]	Other[b]
0	1.3	97.7	< 0.1	2.2	ND
1	4.9[c]	65.5[c]	32.5[c]	2.4	ND
2	5.9[c]	34.5[c]	56.7[c]	6.6[c]	2.2
3	10.5[c]	42.4[c]	36.8[c]	11.6[c]	9.2
4	6.8[c]	42.9[c]	37.4[c]	13.5[c]	6.2
5	7.1[c]	40.1[c]	41.1[c]	15.6[c]	3.3

[a]The values given represent mean values of BALF cells obtained/lavage/mouse.
[b]The values are mean values of 3 independent determinations and expressed as percentage of individual cell subpopulation to the total number of cells.
[c]Values significantly different ($p \leq 0.05$) from that of day 0.
ND; not detected.

inflammatory cells into the bronchoalveolar space (Table I). Within the first three days of infection the total number of cells present in BALF increased about 8-fold over that in control animals, followed by a decrease to ≈ 5-fold control values at days 4 and 5 post-infection. In parallel with this infection-induced increase in the total number of BALF cells, the relative proportion of PMN increased dramatically within the first 24 h post-infection and reached a maximum on day 2 (Table I). As a result, the relative contribution of monocytes/macrophages to the total BALF cells decreased correspondingly while the proportion of lymphocytes remained almost unchanged within the first days post-infection. At the terminal stages of infection the relative number of lymphocytes increased to 7-fold over control with some basophil/eosinophil-like cells also present.

Capacity of BALF Cells to Generate $O_2^{-\cdot}$

The potential of BALF cells to generate $O_2^{-\cdot}$ was determined by their ability to reduce ferricytochrome c upon stimulation with phorbol myristate acetate. BALF cells from control mice possessed a measurable though weak capacity to generate $O_2^{-\cdot}$ upon the addition of the stimulus (Figure 1). The rate of stimulus-induced $O_2^{-\cdot}$ generation increased almost 10-fold on day 1 and was maximal (≈ 15-fold) at day 2 post-infection, when expressed on a per cell basis. Considering the increase in total numbers of BALF cells (see above), the total capacity for production of $O_2^{-\cdot}$ in the bronchoalveolar fluid thus increased ≈ 70-fold over that in control animals. Inclusion of superoxide dismutase almost completely prevented reduction of cytrochrome c, and reduction of cytochrome c in the absence of a stimulus was not observed by BALF cells obtained from either control or infected mice. The increase in the capacity of BALF cells to produce $O_2^{-\cdot}$ followed closely the changes observed in the proportion of PMN in BALF. While at the late stages of infection $O_2^{-\cdot}$ generation by BALF cells was 40–50% less than on day 2 it was still substantially higher than that observed with control cells.

Endogenous H_2O_2 *Generation*

The results in Table II show that i.p. injection of 1,2,4-aminotriazole 1 h before

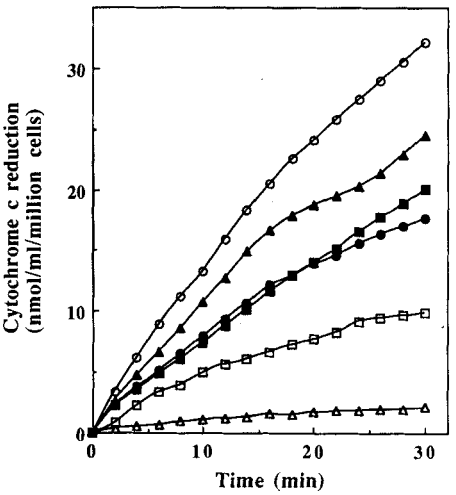

FIGURE 1 Generation of superoxide anion by BALF cells obtained from control and influenza A/PR8/34 virus-infected mice. Cells were harvested and treated as described in Materials and Methods. Data shown represent the differences between assays carried out in the presence and absence of superoxide dismutase and are mean values of 3–4 separate experiments. The standard deviations were less than 15 (days 3–5) and 35% (days 0–2) of the corresponding mean values. For each experiment cells were pooled from the lavages of 6–10 mice. Symbols: Day 0, (△); Day 1, (▲); Day 2, (○); Day 3, (●); Day 4, (□); Day 5 (■).

perfusion, removal and homogenization of the lungs, decreased pulmonary catalase activity by 65.9% in control animals. Virus infection caused a steady increase in the extent of catalase inhibition over the first three days up to 88% and subsequent slight decrease on days 4 and 5. While lung levels of aminotriazole were increased significantly from day 3 onwards, the infection-induced changes in its concentration did not follow the changes observed for catalase inhibition, indicating that the latter was

TABLE II

Catalase activity, detection of endogenous H_2O_2 and tissue levels of 1,2,4-aminotriazole (AT) in lungs from control and influenza A/PR8/34 virus-infected mice

Days post infection	Catalase[a]		Catalase inhibition[b]	AT-chromogenic material[c]	
	minus AT	plus AT		minus AT	plus AT
0	11.8 ± 2.7	4.0 ± 3.0	65.9	5.2 ± 1.5	46.4 ± 23.6
1	12.4 ± 6.7	3.4 ± 3.8	72.5	10.3 ± 10.8	35.2 ± 16.0
2	16.9 ± 14.2	3.0 ± 3.6	82.3	8.0 ± 8.8	42.9 ± 23.1
3	9.0 ± 6.3	1.1 ± 1.8	88.2	6.2 ± 3.3	89.6 ± 36.6[d]
4	6.9 ± 4.1	1.1 ± 0.9	84.5	6.9 ± 3.2	102 ± 40.2[d]
5	7.7 ± 5.4	1.7 ± 1.9	78.0	4.0 ± 1.9	91.8 ± 26.0[d]

The values given represent means ± S.D of three to six independent determinations.

[a] Catalase activity is expressed as μmol of H_2O_2 degraded/min and mg protein.

[b] Catalase inhibition is expressed as the percentage of total tissue enzyme activity inhibitable by pretreatment with 1,2,4-aminotriazole.

[c] Values are given as nmol 1,2,4-aminotriazole/mg protein.

[d] Values statistically different ($p \leq 0.05$) to that of day 0.

TABLE III
Concentrations of ascorbate (AA), dehydroascorbic acid (DHA), and protein in cell-free BALF from control and influenza A/PR8/34 virus-infected mice

Days post infection	AA + DHA (nM)	AA (nM)	DHA (%)	Protein (mg/ml BALF)
0	13.1 ± 2.3	12.7 ± 2.3	2.7 ± 1.9	0.18 ± 0.05
1	11.8 ± 1.5	11.2 ± 1.3	4.4 ± 2.3	0.11 ± 0.09
2	7.6 ± 1.5[a]	7.0 ± 1.4[a]	8.0 ± 4.5[a]	0.60 ± 0.36
3	10.6 ± 2.0	10.2 ± 2.1	3.7 ± 1.9	1.1 ± 0.6[a]
4	22.3 ± 5.6[a]	20.7 ± 5.1[a]	7.1 ± 1.8[a]	4.1 ± 1.1[a]
5	22.2 ± 4.8[a]	20.9 ± 4.7[a]	5.8 ± 3.5[a]	4.9 ± 0.9[a]

Values for ascorbate and dehydroascorbic acid (in % of total vitamin C) represent means ± S.D. of nine separate determinations.
[a]Significantly different ($p \leq 0.05$) when compared with day 0 (ANOVA or t-test).

unlikely the result of infection-induced alterations in the accessibility of 1,2,4-amino-triazole to catalase (Table II).

BALF Ascorbate

Since ascorbate appears to represent the first line on non-enzymatic antioxidant defence in plasma[23] and most likely in other extracellular fluids such as BALF,[24] its redox status may be used as a sensitive index for the presence of oxidative stress.[25] Upon reaction with oxidants, ascorbate is converted to dehydroascorbic acid, which is either hydrolyzed to diketogulouic acid or can be reduced back to ascorbate. Ascorbate and dehydroascorbic acid are biologically active and therefore collectively referred to as vitamin C. Infection of mice resulted in an initial decrease in ascorbate within the cell-free BALF (Table III), concomitant with an increase in the concentrations of its oxidation product, dehydroascorbic acid. Following this initial period of ascorbate oxidation and consumption the concentrations of both reduced and total vitamin C increased significantly to levels $\approx 70\%$ above control. In contrast, the oxidation state of vitamin C, i.e., the relative proportion of dehydroascorbic acid to total BALF vitamin C, remained elevated during late stages of infection. Protein concentrations within cell-free BALF were unaltered during the first 2 days but increased significantly at late stages of the infection.

Pulmonary Antioxidant Enzymes and Antioxidant Capacity

Activities of the pulmonary antioxidant enzymes glutathione peroxidase and reductase did not change significantly over the course of infection (Table IV), though the activities measured varied considerably. The latter was not the result of enzyme activities contributed by contaminating erthrocytes, as all values were corrected for this variable. Also, addition of exogenous flavin adenine dinucleotide did not increase the activity of the reductase, indicating that infection was not associated with changes in the flavin status with respect to this enzyme (data not shown). In contrast to glutathione peroxidase and reductase, the activity of pulmonary catalase was decreased markedly at the late stage of infection (Table II). We reported previously that the activity of superoxide dismutase was also decreased by 50% at the terminal stages of the infection.[9]

The total antioxidant capacity present in lung homogenates prepared from control

TABLE IV
Activities of glutathione peroxidase (GSH-Px) and glutathione reductase (GSSG-Red) in lung homogenates prepared from control and influenza A/PR8/34 virus-infected mice

Days post infection	GSH-Px[a]	GSSG-Red[a]
0	38.5 ± 17.8	27.0 ± 5.2
1	47.0 ± 19.2	39.8 ± 11.9
2	54.4 ± 18.6	25.7 ± 4.2
3	43.1 ± 26.4	26.2 ± 2.8
4	29.0 ± 19.1	18.1 ± 2.3
5	56.3 ± 6.6	29.3 ± 1.2

Values represent means ± S.D of three independent determinations.
[a]Enzyme activities are expressed in μmol of NADPH consumed/min and mg protein

and terminally ill mice was assessed by the ability of these homogenates to inhibit aqueous peroxyl radical-induced oxidation of the protein phycoerythrin. As shown in Figure 2A, the total peroxyl radical-trapping activity present in crude pulmonary homogenates was not decreased but rather increased at the late stages of influenza infection. To assess the contribution of small molecular weight compounds to the overall antioxidant activity, homogenates were ultrafiltered (MW < 10000 Daltons) before being tested for peroxyl radical scavenging activity. Non-proteinaceous homogenates from both control and terminally ill animals inhibited the radical-induced oxidation of phycoerythrin to the same extent (Figure 2B), indicating that influenza virus infection was not associated with significant loss in total or small molecular weight pulmonary antioxidant capacity. We do not know at present to what extent antioxidant active metabolites derived from oxidative tryptophan metabolism[9] contribute to this overall non-proteinaceous antioxidant activity.

DISCUSSION

We have proposed previously a role for ROS in the pathogenesis of certain viral diseases, including influenza,[2,6] and there is indirect evidence that indicates increased production of ROS during influenza infection.[9,10] Our results show that administration of 1,2,4-aminotriazole to mice infected with influenza A/PR8/34 virus resulted in an increased *in vivo* inhibition of pulmonary catalase activity when compared with that observed in non-infected, control animals. Since aminotriazole-mediated inhibition of catalase is observed only subsequent to interaction of the enzyme with H_2O_2,[13] and the degree of inhibition did not correlate with infection-induced increases in the pulmonary concentrations of 1,2,4-aminotriazole (Table II), our findings provide evidence for increased steady state concentrations of H_2O_2 in the lungs of influenza-infected mice. Increased formation rather than decreased removal of pulmonary H_2O_2 is likely to be responsible for this as maximal catalase inhibition was observed at a time when the activities of the H_2O_2-removing enzymes catalase and glutathione peroxidase were not decreased.

The assay used for the detection of pulmonary levels of H_2O_2 does not identify the source of its formation, and a number of possibilites may be considered. For example, we have shown previously that influenza infection causes the oxidative conversion of xanthine dehydrogenase to xanthine oxidase, and that the overall activity of the $O_2^{-\cdot}$ and H_2O_2-producing oxidase increases 3–4 fold during infection.[9] Increased intracellular generation of $O_2^{-\cdot}$ and H_2O_2 could also be caused by increased leakage of

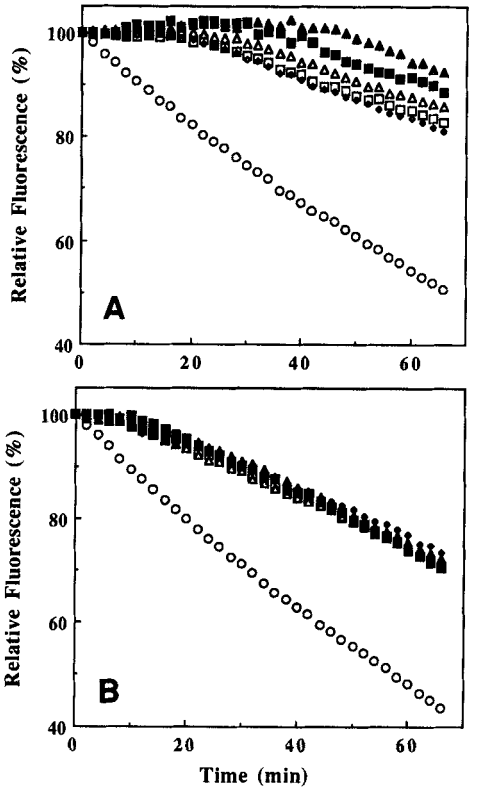

FIGURE 2 Inhibition of aqueous peroxyl radical-mediated loss of phycoerythrin fluorescence by crude homogenates (A) and ultrafiltrates (B) prepared from lungs of control and influenza A/PR8/34 virus-infected mice. Lung homogenates were prepared as described in Materials and Methods and used at 1 : 550 (v/v) (A) and 1 : 1100 (v/v) (B) final dilutions. Protein concentrations in homogenates from infected animals were unchanged when compared with controls. Phycoerythrin (17 nM) was incubated under air at 37°C in sodium phosphate buffer (75 mM) pH 7.0, containing the peroxyl radical generator 2,2′-azobis(2-amidino-propane)HCl (4 mM) in the absence (○) or presence of a homogenate prepared from control (△, ◇, and □) or infected (■ and ▲) animals.

electrons onto molecular oxygen from the electron transport chains in mitochondria, as has been shown to be the case when lungs are exposed to elevated oxygen concentrations.[26] In addition, inflammatory cells infiltrating the lungs of infected animals could contribute to the extra H_2O_2 generation. Influenza infection is known to be associated with pneumonia. The subpopulation of cells recovered from the lungs of infected mice is dependent on both virus strain and dose and the more virulent the infection the greater the numbers of infiltrating cells.[27] Furthermore, the cell types recovered in BALF from infected mice tend to correlate with those present in the lungs.[28] We also observed an influenza-induced 10-fold increase in the numbers of inflammatory cells present in BALF, particularly PMN (Table I). First signs of focal inflammation were apparent throughout the lung parenchyma at day 2 post-infection, and these sites of inflammation subsequently increased in size and spread from the peribronchial region to the adjacent lung tissue (M. Adé-Damilano, R.S. and E.P.,

unpublished work). The observation that phagocytes are present in lungs of infected animals and that these cells can produce ROS, including H_2O_2, *per se* does not allow the direct conclusion that these cells actually are responsible for the increased pulmonary H_2O_2. However, the temporal profile of 1,2,4-aminotriazole-mediated inhibition of pulmonary catalase was very similar to that of the increases in both numbers of inflammatory cells in BALF and their ability to generate $O_2^{-\cdot}$ upon stimulation (Table II, Figure 1). Thus, infiltrating phagocytes, particularly PMN, appear to be a likely major contributor to the increased levels of ROS in lungs of influenza virus-infected mice.

The increased ability of BALF cells to produce $O_2^{-\cdot}$ throughout the infection are in contrast with the deactivating effect influenza virus particles exert on cellular functions of phagocytes *in vitro*.[3,4,29] A possible explanation for this apparent discrepancy may be the presence in BALF of cytokines such as tumour necrosis factor, interleukin 1 and interferon γ[30,31] which are known to prime various types of phagocytic cells for enhanced release of ROS.[32–35]

A role for PMN as potent source of ROS during influenza infection is in analogy to such a role of these cells in other situations associated with oxidative stress to lungs, including adult respiratory distress syndrome[36] and cigarette smoking.[37] In the former condition, nanomolar concentrations of lipid hydroperoxides are present in pulmonary oedema fluid,[24] while the BALF of smokers contains increased levels of vitamin E quinone,[38] an oxidation product of the lipid-soluble antioxidant vitamin E. Similarly, our finding of increased oxidation state of the ascorbate/dehydroascorbic acid couple in the BALF of influenza virus-infected mice (Table III) indicates the presence of oxidative stress in the alveolar fluid.

While our results provide direct evidence for the presence of oxidative stress within lungs and the BALF during the early stages of influenza infection they do not support a direct causative relationship between gross oxidative tissue damage and the pathogenesis of influenza infection. This is supported by the following: at the terminal stage of the infection (i) the total antioxidant capacity of the lung towards peroxyl radicals had increased slightly; (ii) the total ultrafiltrate or low molecular weight antioxidant capacity was equal to control capacity; (iii) the oxidation state of BALF vitamin C did not increase above values obtained during the early stage of infection; and (iv) total levels of BALF ascorbic acid actually increased. The latter finding was intriguing and elevated BALF ascorbate may be derived from either infiltrating leukocytes, parenchymal cells damaged as a result of virus replication, or increased influx of pulmonary oedema fluid from blood plasma as a result of increased permeability of the alveolar capillary membranes. Regarding the latter two possibilities we noted previously that the pulmonary concentrations of ascorbate decreased steadily during influenza infection whereas plasma levels ($\approx 40\,\mu M$) did not alter significantly.[39] Independent of its origin, the presence of ascorbate in BALF is expected to prevent oxidative damage to lipids and other macromolecules in this fluid.

The presence of oxidative stress may however have indirect effects on the course of infection, in that it may influence the balance of proteases/antiproteases[5,39] or enhance the cellular release and/or interplay of cytokines such as tumour necrosis factor, interleukin 1, and interferon γ.[2] The possible roles of such mediators are currently under investigation.

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