MINIREVIEW

Oxygen Free Radicals as Pathogenic Molecules in Viral Diseases (43309C)

HIROSHI MAEDA[1] AND TAKAAKI AKAIKE
Department of Microbiology, Kumamoto University Medical School, Kumamoto 860, Japan

Abstract. **Oxygen free radicals such as superoxide anion (O_2^-) were generated markedly in influenza virus-infected mouse lung, and these molecular species were identified as the potent pathogenic agents. This finding has many important implications for understanding viral pathogenesis: namely, the direct viral cytotoxicity (referred cytopathic effect) is only a fraction of several types of events induced by virus infection. The toxicity and reactivity of oxygen radicals, which are presumably generated in excessive amounts by the overreaction of the host's immune response against the organs or tissues in which viruses are replicating, may explain the mechanism of tissue injuries observed not only in influenza virus infection in mice, but also in other types of viral diseases in which immunological interactions are usually involved.**

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Usually viral replication occurs in the cell, and the typical cytopathic effect caused by the virus is tissue destruction by cytolysis. In some viral diseases, more complicated interactions of virus with susceptible animal cells have been reported and the evidence suggests that immunological consequences against the replicating virus may be a prime cause of the pathogenesis of diseases (1–12). However, the details of the molecular mechanisms of these viral diseases still remain obscure.

Recently, toxicity by oxygen radicals, generated in various biological systems, has been suggested as a major cause of cancer (13), aging (14), and tissue injury (15, 16) in ischemia and inflammation. It is also generally accepted that oxygen radicals and other oxidants play an important role in defense mechanisms of hosts against various invading microbes (17). In this context, our interest here is in the pathogenic role of oxygen free radicals in the virus-host interactions in which pathological events in the host animal seem not to be a direct consequence of the presence of replicating virus. Virus diseases that generally have these pathological aspects are lymphocytic choriomeningitis (LCM) in mice (1), mouse-adapted influenza virus infection (2–4), viral hepatitis (5, 6), measles (7), dengue hemorrhagic fever/dengue shock syndrome (8), herpesvirus disease (9, 10) in humans and human acquired immune deficiency syndrome (11, 12). Among these diseases, the influenza virus infection of mice is the one in which oxygen free radicals seem to play an important role in pathogenesis (18, 19). The category of these viral infections, i.e., viral pathogenesis with oxygen radicals, will be discussed in more detail below.

Tumor viruses, which cause a very slow manifestation of cell pathology, fall in another category. However, when one considers the carcinogenic potential of oxygen radicals (13), it may be possible that oxygen radicals generated by a host reaction are involved in carcinogenic processes induced by tumor viruses. We will not discuss this issue in this article.

Deviation from Koch's Postulates

In our recent study using influenza virus A/Kumamoto/Y_5/67(H_2N_2), adapted to mice, we noticed a large gap in the time course between the peak time of

[1] To whom correspondence and requests for reprints should be addressed at Department of Microbiology, Kumamoto University Medical School, Kumamoto 860, Japan.

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virus production in the lung and the maximum death rate, which correlated with the extent of the pathological changes of the lung tissue (19). As seen in Figure 1, the virus yield in the lung reached a maximum on Day 4 after inoculation and decreased to zero (undetectable) after Days 8–10. The mortality of the infected mice was at a midpoint on Day 10 and peaked at Day 13, leaving about a 7-day gap between peak virus titer and lethality. Histological studies of the lung on Day 4, the day of maximum virus yield, revealed a weakly positive pathological change signaled by the infiltration of inflammatory cells. Pathohistological severity (by pathological score) was maximal on Day 10 and, at that time, we could no longer detect the virus by plaque-forming assay (Fig. 1). In this instance, a direct cause and effect or one to one relationship was not observed between the presence of replicating virus and pathology. We feel that this represents a clear deviation from a classical theorem of Koch's postulates.

Immunity Is Not Always Beneficial for Virus-Infected Hosts

In earlier studies on influenza virus infection in mice, it was reported that suppression of the immune system resulted in longer survival spans, although no cure was observed (2–4). The experimental mice were either athymic, subjected to x-ray radiation, or injected with antilymphocyte serum; all these treatments were intended to avoid the effect of T cells that defend against invading microorganisms and collaborate with macrophages and B cells to eliminate virus particles or virus-infected cells. Artificially immunosuppressed mice lived 30–80% longer after infection than did immunocompetent mice. This finding indicates that the host's immune system does not necessarily work to eradicate the virus, but instead may damage the host's tissues. The involvement of immunopathological mechanisms has also been demonstrated in the pathogenesis of LCM in mice (1), viral hepatitis (5, 6), measles (7), dengue hemorrhagic fever/dengue shock syndrome (8), herpesvirus disease (9, 10), and acquired immune deficiency syndrome in humans (11, 12). A recent report showed a paradoxical effect of some kind of recombinant vaccine for LCM virus infection in mice in which vaccination caused a disease dependent upon induction of virus-specific T cells (20).

The pathogenic role of various cytokines, i.e., tumor necrosis factor or γ-interferon, which can activate phagocytic cells to increase the activity of O_2^- generation (21, 22), has been shown in LCM in mice (23, 24) and in simian varicella virus in monkey (25). In addition, it has been reported that complement and virus-specific antibody enhanced production of active oxygens by granulocytes against herpes simplex virus (26). Through these findings, we envisaged the possible role of oxygen radicals in the pathogenesis of viral diseases.

On the basis of our studies of the deleterious effect of oxygen free radicals in mice infected with influenza virus (18, 19), we believe we have data that are important for understanding the missing link between viral infection and cell death as described above.

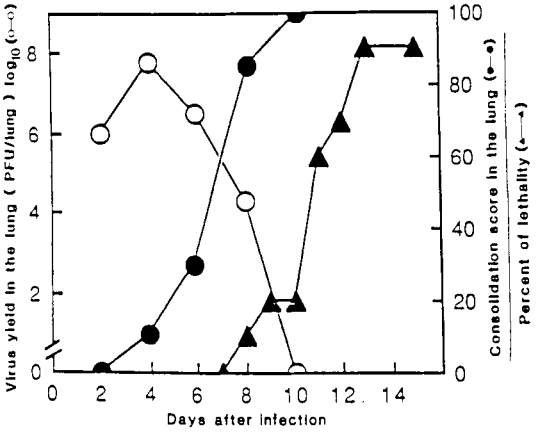

Figure 1. Time course of viral replication, score of consolidation in the lung, and survival rate of influenza virus-infected mice. Mice were inoculated by inhalation with the influenza virus A/Kumamoto/67(H_2N_2) at twice the LD_{50} dose. Virus yield was quantitated by plaque-forming assay, and the amount of consolidation was scored as described in our report (19). Reprinted from Ref. 19 with permission.

Generation of Oxygen Free Radicals in Virus Infection

For some time, it has been known that the host's defense against invading microorganisms can be effectively destroyed by the host's own macrophages and granulocytes (17, 27). These cells, upon encountering or ingesting invading bacteria or particles, excrete a number of reactive oxygen species; superoxide anion radical (O_2^-) and H_2O_2 in a "respiratroy burst" (28). The respiratory burst is a common phenomenon, and neutrophils and activated macrophages are assumed to be the major generators of oxygen radicals.

We examined neutrophils and macrophages, the most likely agents for the effects seen in the influenza

Table I. O_2^- Generation by Alveolar Phagocytic Cells Obtained from Influenza Virus-Infected Mice

Day after virus infection	O_2^- generation (nmol/30 min/1 × 10^6 cells)[a]	
	PMA(−)	PMA(+)
0	1.0 ± 0.3	3.3 ± 0.7
2	4.2 ± 1.3	18.6 ± 1.9
4	4.4 ± 1.4	12.5 ± 2.9
6	5.3 ± 1.8	21.0 ± 2.1
8	7.6 ± 1.5	21.7 ± 3.5

[a] Release of O_2^- from phagocytic cells was determined on the basis of SOD-inhibitable reduction of cytochrome *c* in the presence (+) or absence (−) of phorbol myristate acetate (PMA). Values are shown as means ± SE. The number of mice used was three for each group. Reprinted from Ref. 18 with permission.

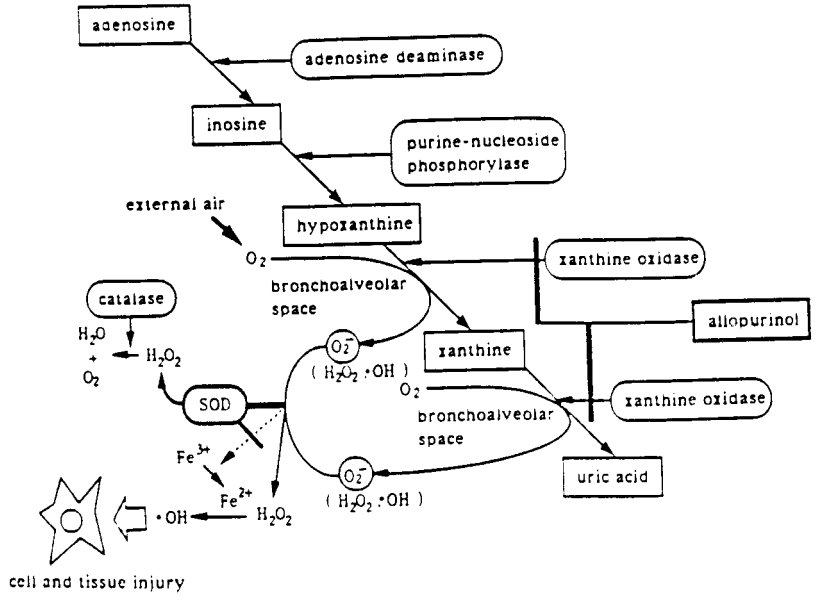

Figure 2. Adenosine catabolism and generation of oxygen free radicals in influenza virus-infected lung. Xanthine oxidase, which is the final enzyme in purine catabolism, transfers electrons to molecular oxygen to form superoxide anion (O_2^-). O_2^- can be converted into highly toxic hydroxyl radicals by the iron-catalyzed Haber-Weiss reaction. Reprinted from Ref. 19 with permission.

Table II. Xanthine Oxidase Activity in Plasma, Supernatant of Bronchoalveolar Lavage Fluid, and Lung Tissue

Day after infection	Xanthine oxidase activity[a] (nmol isoxanthopterin/min)		
	In plasma	In s-BALF	In lung tissue
0	0.229 ± 0.004	0.001 ± 0.001	0.758 ± 0.179
	(0.003 ± 0.000)	(0.001 ± 0.000)	(0.095 ± 0.019)
3	0.262 ± 0.007	0.015 ± 0.005	1.043 ± 0.091
	(0.004 ± 0.000)	(0.018 ± 0.006)	(0.123 ± 0.010)
6	0.240 ± 0.025	0.179 ± 0.029	15.7 ± 3.94
	(0.004 ± 0.001)	(0.041 ± 0.007)	(0.924 ± 0.230)
8	0.766 ± 0.090	0.419 ± 0.033	22.4 ± 1.38
	(0.009 ± 0.001)	(0.044 ± 0.003)	(0.963 ± 0.060)

[a] Xanthine oxidase activity is expressed as nmol isoxanthopterin/min/ml of plasma, supernatant of bronchoalveolar lavage fluid (s-BALF), or total lung homogenates. The corrected values expressed as per mg protein are shown in parentheses. Values are shown as means ± SE. The number of mice was three for each group. Reprinted from Ref. 19 with permission.

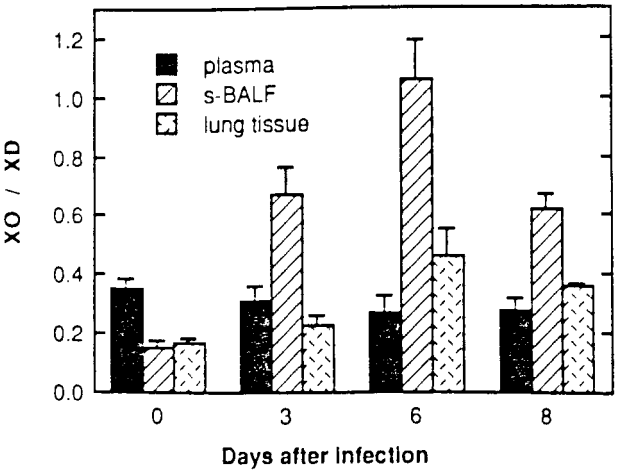

Figure 3. The xanthine oxidase (XO) to xanthine dehydrogenase (XD) ratio in plasma, supernatant of bronchoalveolar lavage fluid (s-BALF), and lung tissue of virus-infected mice. XO and XO + XD activities were determined fluorometrically, as described in our report (19). The ratio is calculated as XO activity to XD activity. Data are means ± SE. The number of mice for each group was three. Reprinted from Ref. 19 with permission.

infection of mice, by counting the number of infiltrated cells in the lung and their capacity to generate O_2^-. We found that the O_2^--generating capacity of alveolar phagocytic cells increased about 8-fold compared with noninfected control cells (Table I) (18).

The second route of oxygen radicals formation uses the enzyme xanthine oxidase (15, 19) to catalyze two similar reactions; one uses hypoxanthine and molecular oxygen (O_2) as substrates to generate xanthine and O_2^- (and H_2O_2) and the other uses xanthine and O_2 as substrates to generate uric acid and O_2^- (and H_2O_2) (Fig. 2). In order to generate active oxygen species effectively, xanthine dehydrogenase itself needs to be converted to xanthine oxidase either by limited proteolysis or oxidation of the sulfhydryl moiety (29).

When we measured the activity of xanthine oxidase in bronchoalveolar lavage fluid of infected mice, its level was higher by several orders of magnitude than that in noninfected controls (Table II). We also tested the ratio of activity of xanthine oxidase to that of xanthine dehydrogenase in virus-infected mice and we found that the conversion of xanthine dehydrogenase to xanthine oxidase was taking place in the lavage fluid

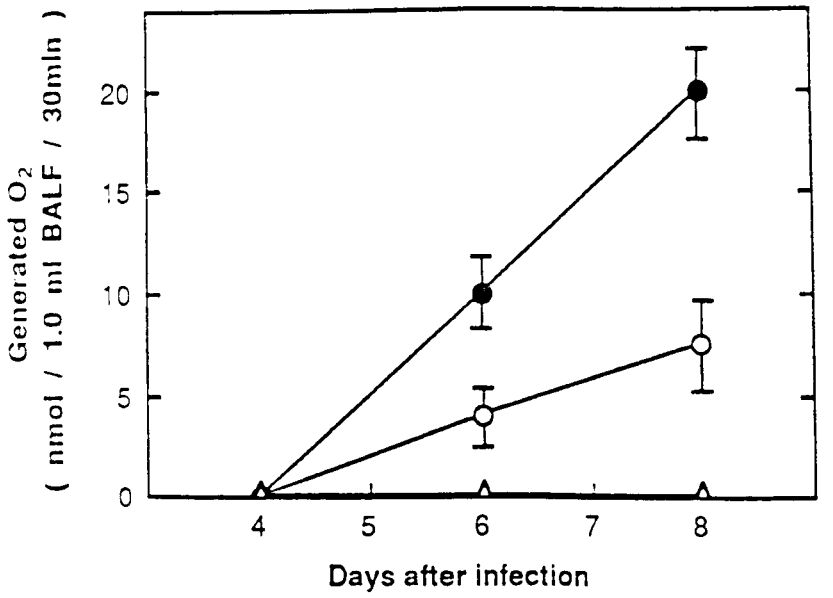

Figure 4. O_2^- generation in supernatant of bronchoalveolar lavage fluid (s-BALF). Generated O_2^- was determined spectroscopically on the basis of SOD inhibitable reduction of ferricytochrome *c*. ●, O_2^- generation with 22.5 nmol of adenosine; ○, without adenosine. O_2^- generation was inhibited completely by 45 μM of allopurinol (△). Data are means ± SE. The number of mice used for each group was three. Reprinted from Ref. 19 with permission.

Table III. Quantitation by High Performance Liquid Chromatography of Catabolic Metabolites of Adenosine in Serum and Supernatant of Bronchoalveolar Lavage Fluid

Purine metabolites	Concentration of metabolite measured[a] (mg/ml)		
	Day 0	Day 4	Day 8
Serum			
Adenosine	1.96 ± 0.34	1.76 ± 0.19	1.74 ± 0.10
Inosine	24.8 ± 0.9	42.3 ± 1.3	44.4 ± 0.5
Hypoxanthine	1.35 ± 0.58	3.66 ± 0.20	4.95 ± 0.61
Xanthine	2.31 ± 0.29	3.77 ± 0.90	7.23 ± 0.93
Uric acid	20.8 ± 3.0	50.9 ± 3.1	105.5 ± 9.8
Supernatant of bronchoalveolar lavage fluid			
Adenosine	—[b]	—	—
Inosine	0.03 ± 0.01	0.09 ± 0.00	0.82 ± 0.15
Hypoxanthine	—[b]	—	—
Xanthine	—[b]	—	—
Uric acid	0.22 ± 0.04	0.22 ± 0.05	1.06 ± 0.14

Note. Reprinted from Ref. 19 with permission.
[a] Values are shown as means ± SE of three determinations.
[b] Undetectable (below 10 ng/ml).

and the lung tissue (Fig. 3). Simultaneously, the excessive amount of O_2^- generated from this enzyme in virus-infected lungs was confirmed (Fig. 4). The very rapid purine catabolism almost depleted the substrates of xanthine oxidase (either hypoxanthine or xanthine), while levels of other metabolites of adenosine increased markedly after virus infection (Fig. 2; Table III). Adenosine added to the lavage fluid started the catabolic cascade and was converted very rapidly to uric acid in a stoichiometric manner (Fig. 5).

We also found a marked elevation of adenosine deaminase activity in the bronchoalveolar lavage fluid and, to some extent, in serum after virus infection (Table IV). We observed that a dramatic increase in O_2^- generation results from a highly elevated supply of hypopoxanthine and xanthine, which are catabolic products located downstream of the adenosine-to-inosine conversion step (Fig. 2). The rise in adenosine deaminase and xanthine oxidase activity is more closely linked to the infiltration of lung tissue by inflammatory cells and later pathological manifestations in lungs, but not to virus replication in the lungs (Fig. 1).

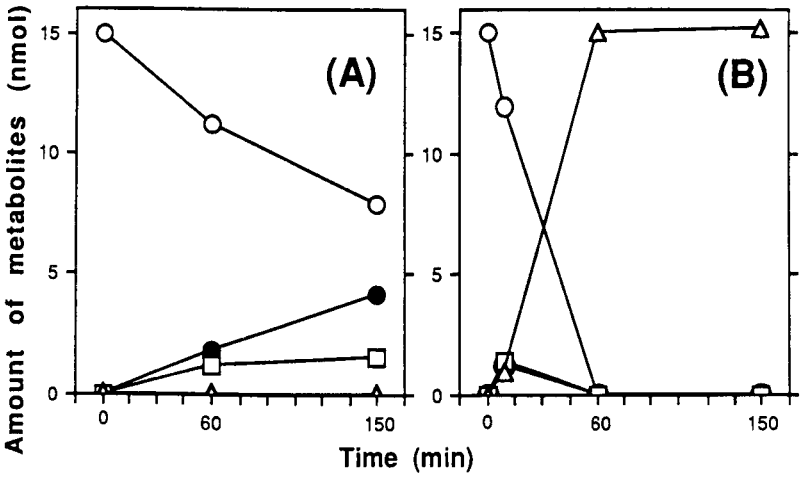

Figure 5. Catabolic profiles of adenosine in supernatant of bronchoalveolar lavage fluid (s-BALF) on (A) Day 0 and (B) Day 8 after infection. Adenosine (15.0 nmol) was added to 1 ml of s-BALF and, after various incubation periods, the level of each metabolite (○, adenosine; ●, inosine; □, hypoxanthine; △, uric acid) was quantitated by high performance liquid chromatography.

Table IV. Adenosine Deaminase Activity in Serum, Supernatant of Bronchoalveolar Lavage Fluid, and Lung Tissue

Day after infection	Adenosine deaminase activity[a]	
	In serum	In s-BALF
0	0.144 ± 0.021	0.002 ± 0.000
	(0.003 ± 0.001)	(0.012 ± 0.000)
4	0.464 ± 0.060	0.054 ± 0.001
	(0.008 ± 0.001)	(0.148 ± 0.001)
6	0.919 ± 0.192	0.149 ± 0.001
	(0.015 ± 0.003)	(0.135 ± 0.001)
8	1.81 ± 0.53	0.353 ± 0.132
	(0.024 ± 0.007)	(0.146 ± 0.054)

[a] Adenosine deaminase activity is expressed as mU/ml of serum or supernatant of bronchoalveolar lavage fluid (s-BALF). The corrected values expressed as per mg protein are shown in parentheses. Values are shown as means ± SE. The number of mice was three for each group. Reprinted from Ref. 19 with permission.

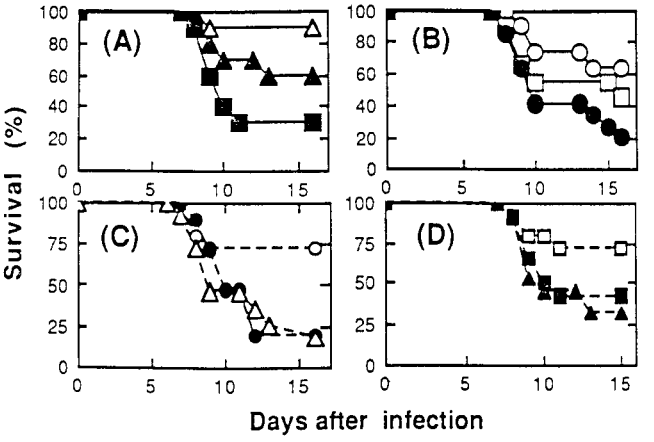

Figure 6. The effects of SOD, allopurinol, adenosine, and SBTI on influenza virus-infected mice. (A) SOD (1000 units of SOD (-▲-) or 200 units of pyran copolymer-conjugated SOD (-△-)/mouse) was intravenously administered daily from Days 5 to 8. Control, vehicle alone (-■-). (B) Allopurinol (1.0 mg/mouse (-□-) or 2.0 mg/mouse (-○-)) was orally administered daily, from Days 4 to 7. Control, vehicle alone (-●-). (C) Adenosine (0.1 mg (---●---) or 0.5 mg (---△----)/mouse) was given intraperitoneally daily from Days 4 to 7. Control (--○----). At this dose, adenosine has no toxic effect to normal mice. (D) SBTI (0.5 mg (---■---) or 1.0 mg (---□---)/mouse) was administered intraperitoneally every 12 hr from Days 2 to 5. Control (---▲----). In these experiments, each group contains 10 to 15 mice. Significant differences were observed among treated groups (2.0 mg of allopurinol, 200 units of pyran copolymer-conjugated SOD, 0.1 mg and 0.5 mg of adenosine, and 1.0 mg of SBTI) and each control ($P < 0.05$, Fisher's exact test).

An important role of adenosine deaminase in the immune response, especially T cell activity, has been described (30, 31). In addition, in mice, xanthine oxidase is known to be induced by interferon or its inducer (32). In the light of these facts, the pronounced elevation of adenosine deaminase and xanthine oxidase may reflect a hyperimmune reaction in influenza virus infection.

Are Free Radical Diseases Treatable?

We have at least verified the increased O_2^--generating potential of phagocytic cells and the excessive O_2^- generation by xanthine oxidase, coupled with an enhanced level of catabolic activity that delivers the substrate (hypoxanthine/xanthine). If O_2^- causes an animal's death it seems reasonable that an animal would survive if O_2^- was removed from its system. To test this hypothesis, we prepared a tailor-made superoxide dismutase (SOD), by which both plasma half-life and biocompatibility are improved by attaching appropriate polymers to the enzyme (33). SOD is a metaloenzyme that effectively removes O_2^- from the system [$O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$]. The abundant catalase *in vivo* activity converts hydrogen peroxide into water and the less active molecular oxygen (O_2). *In vivo*, however, intravenously injected Cu, Zn-type SOD ($M_r = 32{,}000$) will be cleared within 10 min, and, thus, very little

pharmacological effects are expected (33, 34). Since most proteins weighing less than M_r of 50,000 are effectively cleared from the kidney, we decided to increase the size of the SOD molecule. Based on our experiences with the macromolecular anticancer agent styrene-maleic acid copolymer-conjugated neocarzinostatin, in which anticancer agent neocarzinostatin was conjugated with styrene-maleic acid/anhydride copolymer (34, 35), we were able to conjugate Cu, Zn-SOD with a biocompatible divinylether maleic acid/anhydride copolymer (pyran copolymer) for the above purpose (33).

When the pyran copolymer-conjugated SOD was injected into mice intravenously once daily, beginning 5 days after the initiation of infection, when the pathological effect was apparent, the survival rate was greatly improved (Fig. 6A) and supported our hypothesis that the oxygen radical is the principal toxic agent.

We observed that inhibition of xanthine oxidase was also beneficial in suppressing the production of active oxygens (Fig. 2). The significant protective effect of allopurinol, a potent inhibitor of xanthine oxidase, was seen as dose dependent (Fig. 6B). Conversely, when we administered adenosine, the lethal effect of the infection increased significantly (Fig. 6C). We conclude that the main cause of death relates to the cytopathicity, which in turn is apparently a consequence of the elevated levels of O_2^- generated by xanthine oxidase.

Different Routes of Free Radical Generation

We have demonstrated that O_2^- produced by xanthine oxidase is a predominant effect leading to the pathological effects of this viral infection. However, our knowledge of the role of other free radical-generating systems (mitochondrial, lipid peroxidation) is limited. When cells are damaged, an influx of calcium may occur and protease (calpain) is activated. Calpain, in turn, inactivates Mn-type SOD in mitochondria, making the cells more vulnerable to the effects of free radicals (15). Recently, we found an increased amount of the thiobarbituric acid-reacting substance in the lungs of mice infected with influenza virus (unpublished observation). Although the pathological significance of the increase of thiobarbituric acid-reacting substance still remains unclear in this model, lipid peroxidation may be another source of free radicals, among which alkyl peroxyl radicals are found to have strong cytotoxic effects (36, 37).

It is known that O_2^- itself is not particularly toxic to some cells or pathogens. Its major role in tissue injuries is to reduce ferric iron to ferrous, which in turn catalyzes a Fenton reaction and results in generation of the more toxic and reactive $\cdot OH$ from H_2O_2 (38). Therefore, the conversion of O_2^- to $\cdot OH$ may be operative in the pathogenesis of influenza virus infection in mice.

Involvement of Proteases in Augmenting Pathogenesis

Recently, we pointed out that addition of *Serratia marcescens* protease (M_r = 56,000) intranasally to influenza-infected mice markedly enhanced the lethal effect of this infection and increased the virus titer about 100-fold (39). In this system, we found that a number of protease cascades (Factor XII, kallikrein, and plasmin generation) are greatly enhanced by proteases. Furthermore, the treatment of virus-infected mice with soybean trypsin inhibitor (Kunitz type) significantly increased the mouse survival rate (Fig. 6D). The increased survival rate is most likely a suppressive effect on viral activation, as Zhirnov *et al.* (40) have demonstrated previously. There are, however, several reports which show that soybean trypsin inhibitor inhibits the conversion of xanthine dehydrogenase to xanthine oxidase *in vivo* (41, 42). Therefore, it is conceivable that soybean trypsin inhibitor improved the mortality of virus-infected mice by inhibiting this conversion as well.

These findings suggest that the elevated level of protease(s) augments viral infectivity and, thus, may also be important in the formation of free radicals in lungs infected by influenza virus.

Conclusion

It has become clear that O_2^- is the prime toxic molecule generated in the viral infection. The generation of O_2^- is sustained by an enhanced supply of catabolic products of ATP and the much-elevated activity of the enzymes involved in this catabolic cascade. In fact, this suggestion was supported by the improved survival of mice when xanthine oxidase was inhibited. Likewise, Cu, Zn-SOD with longer plasma half-life and better biocompatibility, which removes toxic O_2^-, conferred a remarkably protective effect on the infected mice. A similar clinical consequence may be found in the symptoms of other viral diseases.

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