N-ACETYLCYSTEINE SYNERGIZES WITH OSELTAMIVIR IN PROTECTING MICE FROM LETHAL INFLUENZA INFECTION

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Many studies have shown that oxidative stress is important in the pathogenesis of pulmonary damage during influenza virus infections. Antioxidant molecules are therefore potentially useful against viral infection. Our previous studies show that N-acetylcysteine (NAC) has a protective effect in a model of lethal influenza infection in mice. NAC administration significantly decreased the mortality in infected mice. Further studies have demonstrated that NAC enhanced survival in combination with the antiviral agent ribavirin. In the present study, we report the effect of combined treatment with NAC and Oseltamivir, clinically used in the treatment and prevention of influenza virus infection, in a murine model of lethal influenza infection. NAC was given as a single daily dose of 1000 mg/Kg starting from 4 h before infection and until day 4 after infection; Oseltamivir was given twice daily at dose of 1 mg/Kg/die for 5 days, starting from 4 h before infection. End-point evaluation was 21-days' survival. NAC alone was slightly effective (20%), since a suboptimal treatment was used. Survival increased to 60% with Oseltamivir and to 100% with Oseltamivir and NAC used in combination. Since NAC alone does not show any antiviral action, the present findings suggest that antioxidant therapy increase survival by an improvement in host defense mechanisms, and/or by a direct antioxidant effect against oxidative stress associated with viral infection. Our studies demonstrate the effectiveness of combining agents acting through different mechanisms, such as antiviral drugs oseltamivir and the antioxidant NAC, indicating a possible advantage of combining the two treatments.

Various mediators contribute to the pathogenesis of pulmonary inflammation induced by infectious agents. In particular, cytokines, chemokines and reactive oxygen species (ROS) have been implicated. Cytokines and chemokines, which are both produced as part of the host immune response to bacteria (1-2), contribute to the pathogenesis of tissue damage (3-4). ROS contributes to the antibacterial response, even if overproduction can result in oxidative stress that amplifies the inflammatory response.

Many studies have shown that oxidative stress is important in the pathogenesis of pulmonary damage during infections, in organ failure and in acute respiratory distress syndrome (ARDS) associated with bacterial sepsis. Antioxidant molecules are therefore potentially useful against both viral infection and infection-associated symptoms.

Oxidative stress and its role in the pathogenesis of various infections has been documented in experimental models both *in vitro* and *in vivo* using a variety of pathogens: e.g. *Chlamydia pneumoniae* (5), *Helicobacter pylori* (6), different Gram-

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Table I. *Effects of Oseltamivir and NAC on 21-days' survival.*

Treatment[a]	Dosage (mg/Kg/die)	N° of survivors/total[b]	Survival rate (%)
Oseltamivir	1.0	6/10	60
Vehicle (control)	0	0/10	0
NAC	1000	2/10	20
Vehicle (control)	0	1/10	10
Oseltamivir + NAC	1.0 plus 1000	10/10	100
Vehicle (control)	0	0/10	0

[a] *Treatment, per os, started 4 h before infection and for the subsequent 4 days*
[b] *Mice were observed daily for 21 days for survival*

Table II. *Effects of Oseltamivir and NAC on the lethality of influenza infection in mice.*

Treatment[a]	MST (N° of days)[b]
Oseltamivir (n=10)	21.7
NAC (n=10)	8.8
Oseltamivir + NAC (n=10)	> 21 *
Vehicle (n=30)	8.7

[a] *Treatment, per os, started 4 h before infection and for the subsequent 4 days*
[b] *Results are reported as mean survival time (MST) in days, estimated by the Kaplan-Meier method*
** $p<0.05$ compared to Oseltamivir alone by Wilcoxon test*

negative bacteria (7), *Streptococcus pneumoniae* in an animal model of pneumococcal meningitis (8-9), HIV (10-12) and Hepatitis C virus (HCV) (13-16). ROS, through the induction of several inflammatory cytokines, have also been implicated in the pathogenesis of SARS (17).

The possible involvement of oxidative mechanisms in the pathogenesis of influenza virus infection has also been investigated. Intranasal instillation of influenza virus H1N1 in mice resulted in a significant decrease in the pulmonary concentrations of catalase, reduced glutathione and superoxide dismutase. Furthermore, the H5N1 virus (A/Hong Kong/483/97), when compared with human influenza virus subtype H1N1, is a more potent inducer of pro-inflammatory cytokines (e.g. tumor necrosis factor-a) and chemokines (e.g. IP-10) from primary human macrophages *in vitro*. This characteristic may contribute to the unusual severity of human H5N1 disease (18-19).

Previous studies have shown that N-acetylcysteine (NAC), a thiol antioxidant and precursor of GSH

synthesis, has a protective effect in a model of lethal influenza infection in mice. NAC administration significantly decreaseds the mortality in infected mice, presumably by limiting tissue damage (20). Further studies, using the same *in vivo* model, have demonstrated that NAC, at doses that do not improve survival of mice when given alone, enhance survival in combination with the antiviral agent ribavirin (21).

In the present study we report the effect of combined treatment with NAC and Oseltamivir, a neuroaminidase inhibitor clinically used in the treatment and prevention of influenza virus infection, in a murine model of lethal influenza infection.

MATERIALS AND METHODS

BALB/c mice (18-20 g), 10 per treatment group, were anaesthetized with ether/chloroform and infected intranasally with 2-3 × LD_{50} of an influenza virus strain adapted in mice (A/PR8/H1N1). Animals were treated as follows:

1) Oseltamivir alone, 1 mg/Kg/die, per os, twice daily for 5 days, starting from 4 h before infection and for the subsequent 4 days;
2) NAC alone, 1000 mg/Kg/die, per os, starting from 4 h before infection and for the subsequent 4 days;
3) Combined treatment with Oseltamivir and NAC as described above.

Each experimental group was associated to a respective control group, receiving only the vehicle. Mice were observed daily for 21 days for survival. Mean survival time (MST) was calculated as previously described (22).

RESULTS

The effect of treatment of influenza-infected mice with Oseltamivir and NAC used alone and in combination at 21-days' survival is shown in Table I. A control group, receiving only the vehicle, was used for each treatment.

Treatment with Oseltamivir increased the survival of infected mice from 0 to 60%. NAC alone was slightly effective (20%), since a suboptimal treatment was used, but it significantly increased survival in combination with Oseltamivir (average, 100% vs. vehicle-treated control mice).

Table II shows the estimated mean duration of survival. All control animals died 7 to 11 days after infection with influenza virus. Although NAC used alone did not increase survival, when used in combination with Oseltamivir it improved antiviral efficacy and enhanced survival time.

The difference between treatment with Oseltamivir used alone and in combination with NAC is statistically significant.

DISCUSSION

The present study confirms our previous report of a positive effect of NAC administration in combination with an antiviral drug in a murine model of influenza infection. In this report we used a neuraminidase inhibitor, Oseltamivir, clinically used in the treatment and prevention of influenza virus infection.

Since our previous paper reported that NAC alone does not show any antiviral action (20), the present finding could be explained by an improvement in host defense mechanisms, and/or by a direct antioxidant effect against oxidative stress associated with viral infection.

Since NAC is a precursor of GSH synthesis, the data indicate that GSH could play a key role in the host response to infections. Many authors have demonstrated that GSH prevents pulmonary damage induced by sepsis or bacterial endotoxins, suggesting a protective role on the pathogenesis of pulmonary damage (23-27). This is also in agreement with previous reports indicating that influenza infection decreased pulmonary antioxidants, including catalase, glutathione and superoxide dismutase (28), while increasing the activity of the superoxide-generating enzyme xanthine oxidase (XO) (29). Therefore, various antioxidant substances might prevent lung injury in a mouse model of influenza infection (20, 30).

Glutathione and NAC, might also increase the host defense against influenza infection. Our studies demonstrate no direct antiviral effect of NAC on influenza infection *in vitro*, while other authors (31) report that GSH has a dose-dependent anti-influenza effect in cultured cells. Protection was also observed with an *in vivo* mouse model. Moreover, GSH prevented apoptosis of infected cells via inhibition of viral induced caspase activation.

On the contrary, oxidative stress, induced by exposure to diesel exhaust (DE), enhanced the

susceptibility to influenza virus infection in all cell models and the addition of the antioxidant GSH reversed the effects of DE on influenza infections (32).

Studies on virus (HIV)-infected patients have shown that GSH is important for T cell-mediated immunity; in fact, the treatment with anti-oxidants caused a significant increase in all immunological functions, including an almost complete restoration of natural killer cell activity (33). In a model of peritoneal sepsis in mice, GSH depletion caused increased infection and ARDS, while NAC augmented antibacterial functions in the peritoneum, decreased infection and improved survival (34). Overproduction of ROS can be toxic to cells of the immune system and suppress the immune response. For instance, phagocyte-generated ROS suppress natural killer (NK) T cells (35), can impair phagocytic functions and bactericidal activity of macrophages through *de novo* synthesis of actin or actin oxidation in patients with inflammatory lung diseases (36).

A clinical study shows that NAC administration to influenza-infected patients reduced both local and systemic symptoms and increased cell-mediated immunity (37).

In conclusion, we are constantly exposed to ROS generated from endogenous and some exogenous sources (e.g. viral infection). These ROS react with biological molecules causing structural and functional damage.

Antioxidants limit oxidative damage to biological molecules by various mechanisms and contribute to antioxidant defense systems in humans, and may help to protect against degenerative diseases. Our studies demonstrate the effectiveness of combining agents acting through different mechanisms, such as antiviral drugs including ribavirin and oseltamivir and the antioxidant NAC, indicating a possible advantage of combining the two treatments. Further experiments and clinical studies are necessary to confirm this hypothesis.

REFERENCES

1. **Bals R. and P.S. Hiemstra.** 2004. Innate immunity in the lung: how epithelial cells fight against respiratory pathogens. *Eur. Respir. J. 23:327.*
2. **Strieter R.M., J.A. Belperio and M.P. Keane.** 2003. Host innate defenses in the lung: the role of cytokines. *Curr. Opin. Infect. Dis. 16:193.*
3. **Abraham E.** 2003. Neutrophils and acute lung injury. *Crit. Care Med. 31:195.*
4. **Demling R.H.** 1995. The modern version of adult respiratory distress syndrome. *Annu. Rev. Med. 46: 193.*
5. **Nazzal D., A.V. Cantero, N. Therville, B. Segui, A. Negre-Salvayre, M. Thomsen and H. Benoist.** 2006. *Chlamydia pneumoniae* alters mildly oxidized low-density lipoprotein-induced cell death in human endothelial cells, leading to necrosis rather than apoptosis. *J. Infect. Dis. 193:136.*
6. **Verhulst M.L., A.H.A.M. Van Oijen, H.M.J. Roelofs, W.H.M. Peters and J.B.M.J. Jansen.** 2000. Antral glutathione concentration and glutathione S-transferase activity in patients with and without *Helicobacter pylori*. *Digest. Dis. Sci. 45:629.*
7. **Saluk-Juszczak J., B. Wachowicz, E. Bald and R. Gowacki.** 2005. Effects of lipopolysaccharides from gram-negative bacteria on the level of thiols in blood platelets. *Curr. Microbiol. 51:153.*
8. **Leib S.L., Y.S. Kim, L.L. Chow, R.A. Sheldon and M.G. Tauber.** 1996. Reactive oxygen intermediates contribute to necrotic and apoptotic neuronal injury in an infant rat model of bacterial meningitis due to group B streptococci. *J. Clin. Invest. 98:2632.*
9. **Meli D.N., S. Christen and S.L. Leib.** 2003. Matrix metalloproteinase-9 in pneumococcal meningitis: activation via an oxidative pathway. *J. Infect. Dis. 187:1411.*
10. **Droge W., H.P. Eck, H. Naher, U. Pekar and V. Daniel.** 1988. Abnormal amino-acid concentrations in the blood of patients with acquired immunodeficiency syndrome (AIDS) may contribute to the immunological deficit. *Biol. Chem. Hoppe-Seyler 369:143.*
11. **Buhl R., H.A. Jaffe, K.J. Holroyd, F.B. Wells, A. Mastrangeli, C. Saltini, A.M. Cantin and R.G. Crystal.** 1989. Systemic glutathione deficiency in symptom-free HIV-seropositive individuals. *Lancet 2:1294.*
12. **Staal F.J., M. Roederer, D.M. Israelski, J. Bubp, L.A. Mole, D. McShane, S.C. Deresinski, W. Ross, H. Sussman and P.A. Rajul.** 1992. Intracellular glutathione

levels in T cell subsets decrease in HIV-infected individuals. *AIDS Res. Hum. Retroviruses 8:305.*

13. **Suarez M., O. Beloqui, J.V. Ferrer, B. Gil, C. Qian, N. Garcia, P. Civeira and J. Prieto.** 1993. Glutathione depletion in chronic hepatitis C. *Internat. Hepatol. Commun. 1:215.*
14. **Grattagliano I., G. Vendemiale, C. Sabba, P. Buonamico and E. Altomare.** 1996. Oxidation of circulating proteins in alcoholics: role of acetaldehyde and xanthine oxidase. *J. Hepatol. 25:28.*
15. **Beloqui O., J. Prieto, M. Suarez, B. Gil, C. Qian, N. Garcia, P. Civeira.** 1993. N-acetyl cysteine enhances the response to interferon-alpha in chronic hepatitis C: a pilot study. *J. Interferon Res. 13:279.*
16. **Barbaro G., G. Di Lorenzo, M. Soldini, S. Parrotto, G. Bellomo, G. Belloni, B. Grisorio and G. Barbarini.** 1996. Hepatic glutathione deficiency in chronic hepatitis C: quantitative evaluation in patients who are HIV positive and HIV negative and correlations with plasmatic and lymphocytic concentrations and with the activity of the liver disease. *Am. J. Gastroenterol. 91:2569.*
17. **Perlman S. and A.A. Dandekar.** 2005. Immunopathogenesis of coronavirus infections: implications for SARS. *Nat. Rev. Immunol. 5:917.*
18. **Chan M.C., C.Y. Cheung, W.H. Chui, S.W. Tsao, J.M. Nicholls, Y.O. Chan, R.W. Chan, H.T. Long, L.L. Poon, Y. Guan and J.S. Peiris.** 2005. Proinflammatory cytokine responses induced by influenza A (H5N1) viruses in primary human alveolar and bronchial epithelial cells. *Respir. Res. 6: 135.*
19. **Cheung C.Y., L.L. Poon, A.S. Lau, W. Luk, Y.L. Lau, K.F. Shortridge, S. Gordon, Y. Guan and J.S. Peiris.** 2002. Induction of proinflammatory cytokines in human macrophages by influenza A (H5N1) viruses: a mechanism for the unusual severity of human disease? *Lancet 360:1801.*
20. **Ungheri D., C. Pisani, G. Sanson, A. Bertani, G. Schioppacassi, R. Delgado, M. Sironi and P. Ghezzi.** 2000. Protective effect of N-acetylcysteine in a model of influenza infection in mice. *Int. J. Immunopathol. Pharmacol. 13:123.*
21. **Ghezzi P. and D. Ungheri.** 2004. Synergistic combination of N-acetylcysteine and ribavirin to protect from lethal influenza viral infection in a mouse model. *Int. J. Immunopathol. Pharmacol. 17:99.*
22. **Kaplan E.L. and P. Meier.** 1958. Nonparametric estimation from incomplete observations. *J. Am. Stat. Assoc. 53:437.*
23. **Rahman I. and I. Kilty.** 2006. Antioxidant therapeutic targets in COPD. *Curr. Drug Targets 7: 707.*
24. **Rahman I., S.K. Biswas and A. Kode.** 2006. Oxidant and antioxidant balance in the airways and airway diseases. *Eur. J. Pharmacol. 533:222.*
25. **Rahman I. and I.M. Adcock.** 2006. Oxidative stress and redox regulation of lung inflammation in COPD. *Eur. Respir. J. 28:219.*
26. **Rahman Q., P. Abidi, F. Afaq, D. Schiffmann, B.T. Mossman, D.W. Kamp and M. Athar.** 1999. Glutathione redox system in oxidative lung injury. *Crit. Rev. Toxicol. 29:543.*
27. **Bernard G.R.** 1991. N-acetylcysteine in experimental and clinical acute lung injury. *Am. J. Med. 91:54.*
28. **Kumar P., M. Khanna, V. Srivastava, Y.K. Tyagi, H.G. Raj and K. Ravi.** 2005. Effect of quercetin supplementation on lung antioxidants after experimental influenza virus infection. *Exp. Lung Res. 31:449.*
29. **Akaike T., M. Ando, T. Oda, T. Doi, S. Ijiri, S. Araki and H. Maeda.** 1990. Dependence on O_2-generation by xanthine oxidase of pathogenesis of influenza virus infection in mice. *J. Clin. Invest. 85: 739.*
30. **Suliman H.B., L.K. Ryan, L. Bishop and R.J. Folz.** 2001. Prevention of influenza-induced lung injury in mice overexpressing extracellular superoxide dismutase. *Am. J. Physiol. Lung Cell Mol. Physiol. 280:69.*
31. **Cai J., Y. Chen, S. Seth, S. Furukawa, R.W. Compans and D.P. Jones.** 2003. Inhibition of influenza infection by glutathione. *Free Radic. Biol. Med. 34:928.*
32. **Jaspers I., J.M. Ciencewicki, W. Zhang, L.E. Brighton, J.L. Carson, M.A. Beck and M.C. Madden.** 2005. Diesel exhaust enhances influenza virus infections in respiratory epithelial cells. *Toxicol. Sci. 85:990.*
33. **Droge W. and R. Breitkreutz.** 2000. Glutathione and immune function. *Proc. Nutr. Soc. 59:595.*

34. **Villa P., A. Saccani, A. Sica and P. Ghezzi.** 2002. Glutathione protects mice from lethal sepsis by limiting inflammation and potentiating host defense. *J. Infect. Dis. 185:1115.*

35. **Betten A., C. Dahlgren, U.H. Mellqvist, S. Hermodsson and K. Hellstrand.** 2004. Oxygen radical-induced natural killer cell dysfunction: role of myeloperoxidase and regulation by serotonin. *J. Leukoc. Biol. 75:1111.*

36. **O'Reilly P.J., J.M. Hickman-Davis, I.C. Davis and S. Matalon.** 2003. Hyperoxia impairs antibacterial function of macrophages through effects on actin. *Am. J. Respir. Cell Mol. Biol. 28:443.*

37. **De Flora S., C. Grassi and L. Carati.** 1997. Attenuation of influenza-like symptomatology and improvement of cell-mediated immunity with long-term N-acetylcysteine treatment. *Eur. Respir. J. 10: 1535.*