Serum concentrations of nitrite in patients with HIV-1 infection

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Abstract

Aims—To measure circulating concentrations of nitrite in patients with HIV-1 infection.

Methods—Nitrite concentrations were measured using the Griess reaction adapted to microtitre plates in the serum of 10 asymptomatic HIV-1 positive patients, 33 patients with AIDS with cerebral disorders, 17 patients with AIDS with pulmonary involvement, and in eight patients with AIDS with other disorders. Nitrite concentrations were also measured in bronchoalveolar lavage (BAL) fluid and cerebrospinal fluid (CSF) of patients with AIDS with pulmonary involvement and cerebral disorders, respectively.

Results—Increased serum concentrations of nitrite were observed in patients with pulmonary involvement, and in particular in serum and in BAL samples of patients with interstitial pneumonia (36.2 (26.2) $\mu\text{mol/l}$ and 0.3 (0.4) $\mu\text{mol/l}$, respectively). Increased serum concentrations of nitrite were also noted in patients with retinitis caused by infection with cytomegalovirus. Serum nitrite concentrations were also raised in patients with cerebral toxoplasmosis, whereas normal serum concentrations were found in patients with HIV-1 encephalopathy and cryptococcal meningitis. Nitrite concentrations in CSF were not raised in patients with cerebral disorders.

Conclusions—These results suggest that production of nitrite in patients with AIDS with concomitant opportunistic infections may be part of the host defense against opportunistic organisms.

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Nitric oxide (NO), a short-lived radical gas, is generated via enzymatic oxidation of a guanidino nitrogen of L-arginine in a wide variety of mammalian cells and plays a role in the cardiovascular, nervous and immune systems.¹ NO is an extremely unstable molecule and is rapidly converted in vivo and in vitro to nitrite and nitrate. NO and related reactive nitrogen intermediates exert microbiostatic and microbicidal effects on a variety of pathogens, including protozoa, fungi, bacteria, and some viruses.²⁻⁵ NO is also generated by macrophages,⁶ polymorphonuclear leucocytes⁷ and lymphocytes,⁸ although in these cells an inducible enzyme is responsible for its synthesis.

Phagocytic cells produce reactive oxygen⁹ and reactive nitrogen intermediates¹⁰, two different classes of cytotoxic oxidants which contributed to intracellular killing of pathogens. Phagocytic cells, and in particular macrophages, are important for control of intracellular pathogens such as *Toxoplasma gondii*, *Listeria monocytogenes*, *Mycobacterium tuberculosis*, and protozoa, which are responsible for opportunistic infections in patients with HIV-1 infection. In 1992, Morgan *et al*¹¹ postulated that NO released from HIV-1 infected macrophages in the brain contributes to the neuropathology associated with AIDS-dementia complex, inasmuch as NO is toxic to neurons and astrocytes. More recently, Dawson *et al*¹² demonstrated that neurotoxicity of gp120 (the HIV-1 coat protein) in primary cortical neurons involves NO and superoxide anions.

This study was designed to determine circulating concentrations of NO, detected as nitrite, in the serum of patients with AIDS with cerebral disorders, with pulmonary involvement and other disorders, including retinitis caused by cytomegalovirus infection or lymphoma.

Methods

The study population comprised 68 patients infected with HIV-1 (62 men; mean (SD) age 33.1 (2.9) years). The diagnosis of HIV-1 infection was confirmed by ELISA and western blotting. Ten patients were asymptomatic (seven men; mean age 28.7 (6.9) years), 33 had cerebral disorders (31 men; mean age 35.2 (7.9) years), 17 had pulmonary involvement (all men; mean age 34.0 (6.9) years), and eight had retinitis due to cytomegalovirus infection or lymphoma (seven men; mean age 34.0 (6.9) years). Thirty one normal healthy subjects (18 men; mean age 34.5 (10.0) years) served as controls. All controls were HIV-1 seronegative by ELISA. Diagnosis of cerebral disorders was based mainly on clinical findings, whereas a diagnosis of pulmonary involvement was also supported by culture of bronchoalveolar lavage (BAL) fluid. Control samples of cerebrospinal fluid (CSF) were obtained from patients with lower back pain, who had undergone myelography. Control samples of BAL fluid were obtained from intubated patients with cerebral arteriovenous malformations or aneurysms. All control patients were not smoking at the time of study, nor had a history of atopy. Serum concentrations of nitrite were measured in patients with lymphoma before they started chemotherapy, which included the use of cyclophosphamide, vincristine and prednisone.

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In all of the other patients nitrite concentrations in serum, BAL and CSF samples were measured after diagnosis, but before treatment commenced.

Serum samples were obtained from patients infected with HIV-1 and from 21 normal subjects. BAL samples were obtained from 17 patients with AIDS and CSF samples from 33 patients with AIDS with cerebral disorders. Serum, BAL and CSF samples were frozen at -70°C pending analysis. Prior to freezing, cells were removed from BAL and CSF samples by low speed centrifugation.

Given the short half-life of NO, it cannot be measured directly. In aqueous solution, however, NO decays to yield equal amounts of nitrite and nitrate, the concentrations of which are used as indexes of NO synthesis in vivo. NO production was assayed by determining the increase in nitrite concentration using the Griess reaction adapted to microtitre plates. Briefly, 100 µl 35% sulphosalicylic acid was added to 500 µl of each serum sample to remove protein. Samples were then centrifuged at 10 000 × g for 15 minutes. Subsequently 300 µl 5% aqueous NH₄Cl buffer and 60 µl 5% NaOH were added to 200 µl of supernatant. A standard curve was prepared with known concentrations of NaNO₂ (100 µM, 75 µM, 50 µM, 25 µM, 10 µM, 5 µM, 1 µM, 0.1 µM, 0 µM, diluted in distilled water as appropriate). Samples (100 µl) were placed in flat bottomed, 96 well plates. Griess reagent (100 µl) (0.1% naphthylethylenediamine dihydrochloride, 1% sulphonylamide, 5% phosphoric acid) was added to each well. Samples were incubated for 10 minutes at room temperature in the dark, and the absorbance measured at 550 nm in an automatic microplate reader. Sensitivity of this method was 0.1 µmol.

Data are expressed as mean (SD). Statistical analysis was performed by using the non-parametric Mann-Whitney U test; p < 0.05 was considered significant.

Results

Table 1 shows circulating serum concentrations of nitrite in patients infected with HIV-1. As shown, asymptomatic seropositive patients had normal serum nitrite concentrations. By contrast, significantly raised serum concentrations of nitrite were observed in patients with pulmonary involvement, particularly in those with interstitial pneumonia (36.2 (26.2)

Table 2 Nitrite concentrations in the BAL fluid of AIDS patients with pulmonary involvement. Results are expressed as mean (SD)

Subjects	Nitrite concentrations (µmol/l)
Controls (n = 4)	0.05 (0.04)
Interstitial pneumonia (n = 9)	0.3 (0.4)*
<i>P carinii</i> pneumonia (n = 3)	0.09 (0.03)
Mycobacterial pneumonia (n = 5)	0.2 (0.2)

*p = 0.012 v control.

Table 3 Nitrite concentrations in the CSF of AIDS patients with disorders of the central nervous system. Results are expressed as mean (SD)

Subjects	Nitrite concentrations (µmol/l)
Controls (n = 10)	0.04 (0.01)
HIV-1 encephalopathy (n = 12)	0.02 (0.001)*
Cryptococcal meningitis (n = 4)	0.02 (0.003)*
Cerebral toxoplasmosis (n = 17)	0.1 (0.4)*

*p < 0.05 v control.

µmol/l; p = 0.001). It should be noted that these patients had clinical and radiological evidence of interstitial pneumonia, but blood and BAL fluid cultures were negative for the usual pathogens, including *Pneumocystis carinii*, mycobacteria, cytomegalovirus, and Gram positive and Gram negative bacteria. However, increased concentrations of serum nitrite were also observed in patients with mycobacterial pneumonia (*M tuberculosis* was isolated from the BAL fluid from one patient with AIDS and *M avium* from four others).

Of the patients with concomitant cerebral disorders, significantly raised serum concentrations of nitrite were only observed in those with cerebral toxoplasmosis (4.0 (6.2) µmol/l; p = 0.0125); patients with HIV-1 encephalopathy and cryptococcal meningitis had normal serum concentrations. Significantly increased serum concentrations of nitrite were noted in patients with retinitis due to infection with cytomegalovirus (0.7 (0.3) µmol/l; p = 0.0003). Nitrite concentrations were not raised significantly in patients with lymphoma.

Table 2 shows the concentrations of nitrite in BAL fluid samples from patients with pulmonary involvement. As can be seen, significantly increased nitrite concentrations were seen only in patients with interstitial pneumonia (p = 0.012).

Table 3 illustrates nitrite concentrations in CSF samples from patients with disorders of the central nervous system. Concentrations of nitrite in CSF were not significantly increased, even though a moderate increase in nitrite concentrations was observed in patients with cerebral toxoplasmosis (0.1 (0.4) µmol/l).

Discussion

Production of nitrite was observed in patients with AIDS with concomitant opportunistic infections, which is consistent with its putative role as a microbiostatic or microbicidal, or both, agent.

Although NO cannot be measured directly because of its very short half-life, some indication of its production can be gleaned from measuring nitrite concentrations. However,

Table 1 Serum concentrations of nitrite in patients with HIV-1 infection. Results are expressed as mean (SD)

Subjects	Nitrite concentration (µmol/l)	p value
Controls (n = 21)	0.1 (0.1)	-
Asymptomatic patients (n = 10)	0.4 (0.5)	NS
Patients with pulmonary involvement		
Interstitial pneumonia (n = 9)	36.2 (26.2)	p = 0.001
<i>P carinii</i> pneumonia (n = 3)	9.4 (2.0)	p = 0.357
Mycobacterial pneumonia (n = 5)	17.6 (16.7)	p = 0.0079
Disorders of the central nervous system		
HIV-1 encephalopathy (n = 12)	0.2 (0.3)	NS
Cryptococcal meningitis (n = 4)	0.08 (0.001)	NS
Cerebral toxoplasmosis (n = 17)	4.0 (6.2)	p = 0.0125
Other disorders		
Cytomegalovirus retinitis (n = 4)	0.7 (0.3)	p = 0.0003
Lymphoma (n = 4)	0.4 (0.3)	NS

NS = not statistically significant.

dietary intake of nitrite may obscure any differences between samples or disease groups. Knight *et al*¹³ estimated that the mean daily intake of nitrate and nitrite from food is, respectively, 95 mg and 1.4 mg. Thus, measurement of nitrite concentrations can be used as sensitive index of endogenous nitric oxide production, and is less subject to dietary influences than nitrate. Croen⁵ has shown that NO has an inhibitory effect on DNA synthesis and cell replication of herpes simplex virus type 1. The same investigator postulated that NO has a cytotoxic effect, which probably contributes to its antiviral activity. More recently, Dighiero *et al*¹⁴ demonstrated that glial cells from AIDS patients with retinitis due to cytomegalovirus infection express an inducible NO synthase. In the present study, increased production of nitrite was also noted in AIDS patients with pulmonary infections caused by *P. carinii*, *M. tuberculosis* and *M. avium*. It is likely that NO is produced in the lung during various processes, such as vascular regulation and in host defense by several cell types, including alveolar macrophages, bronchial and alveolar epithelial cells, endothelial cells, and fibroblasts.¹⁵ There is convincing evidence that alveolar macrophages of some species may synthesise NO after exposure to various cytokines and to endotoxin, and that NO is important in activating killing of various pathogens.¹⁵ Sherman *et al*¹⁶ have demonstrated that NO production by murine alveolar macrophages is induced by cytokines and *P. carinii*. There is evidence of NO formation in human peripheral blood monocytes¹⁷; NO is also generated by an inducible NO synthase in human neutrophils.¹⁸ Recombinant gp120, the HIV envelope glycoprotein, stimulates production of NO by human monocyte derived macrophages.¹⁹

In a previous study we demonstrated that nitrite can be produced in vitro by peripheral blood mononuclear cells and polymorphonuclear leucocytes isolated from patients with AIDS, especially from those with pneumonia or disseminated mycobacterial infection.²⁰ NO is a potent vasodilator and might contribute to increased plasma loss from leaky postcapillary venules in the bronchial and alveolar spaces. This may also increase migration and accumulation of inflammatory cells such as neutrophils and mononuclear cells into the interstitial and alveolar spaces. Thus, there is compelling evidence that NO may play a role in cell-cell interactions in the lung and critical defense mechanisms of the host.

Increased production of nitrite was observed only in patients with cerebral toxoplasmosis. CSF nitrite concentrations in patients with cerebral toxoplasmosis were slightly increased, but were normal in those with HIV-1 encephalopathy and cryptococcal meningitis. Dawson *et al*¹² showed that neurotoxicity of gp120 in cerebral cortical neuronal cultures is mediated by NO in vitro. However, we were not able to

detect increased production of nitrite either in the serum or in the CSF of patients with HIV-1 encephalopathy. Thus, a plethora of variables and the inherent complexity of NO biology may hinder efforts to define the effects of NO on the HIV-1 infected brain, and generally on the various organs involved in HIV-1 infection.

In conclusion, the pathophysiological significance of increased production of NO in patients with AIDS remains unclear. Apart from its antibacterial and antiviral activity, high concentrations of NO may be toxic to endothelial cells and neurons, which may play a role in the microvascular damage to various organs, including lung, liver and spleen, and in the neurological involvement and sequelae observed in AIDS patients with disorders of the central nervous system.

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