Increased Levels of Nitrite in the Sera of Children Infected with Human Immunodeficiency Virus Type 1

Donato Torre, Giulio Ferrario, Filippo Speranza, Roberto Martegani, and Claudia Zeroli

Nitric oxide (NO) is a newly discovered gas that plays an important role in cell communication and host resistance to infection. The production of NO was examined in the sera of seven children infected with human immunodeficiency virus type 1 (HIV-1) and in the sera of 14 children who became seronegative for HIV-1 during the first year of life. In addition, we determined serum levels of various cytokines, such as interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and gamma interferon (IFN-γ), as a means to monitor NO production. Production of NO, detected as circulating serum levels of nitrite, was measured with use of the Griess reagent. Serum levels of cytokines were determined by enzyme immunoassay. Increased serum levels of nitrite were observed in children with HIV-1 infection (0.4 ± 0.2 μmol/L; P = .013), and in those who became seronegative for HIV-1 during the first year of life (0.5 ± 0.3 μmol/L; P = .04). Furthermore, serum levels of IL-1β and TNF-α were significantly elevated in children with HIV-1 infection (37.5 ± 23.6 pg/mL and 91.2 ± 45.1 pg/mL, respectively). Prophylactic administration of intravenous immune globulin provoked a significant decrease of circulating levels of nitrite in children with HIV-1 infection. In conclusion, NO may play a role as a cytostatic or cytotoxic factor for invading microorganisms, and thus it is probably involved in limiting and/or reducing infection.

Patients and Methods

Study population. From January 1987 through July 1994, 21 children were born to HIV-1-infected mothers at our facility. The diagnosis of HIV-1 infection was based on the findings of ELISA and western blotting and confirmed by PCR and/or viral culture. Seven children were affected by AIDS (3 boys and 4 girls; mean age, 6.0 ± 2.8 years), and 14 children became seronegative for HIV-1 during the first year of life (7 boys and 7 girls; mean age, 3.3 ± 2.3 years). Mothers of uninfected children and of those with AIDS did not receive antiretroviral therapy during pregnancy and delivery. Children with AIDS suffered from recurrent bacterial infections (otitis, bronchitis, and pneumonia), pneumonia due to Pneumocystis carinii, and disseminated cytomegalovirus infection (Class P-2, subclasses D1 and D2). For comparison, 10 healthy children (6 boys and 4 girls; mean age, 9.7 ± 3.3 years) were evaluated. All subjects were HIV-1-seronegative by ELISA.

Medical evaluations and laboratory assessments were performed at least every 6 months during the first 2 years of life for uninfected children and every 3 months for children with AIDS. Children with more advanced disease required more frequent medical evaluations. Prophylactic treatment with intravenous immune globulin (IVIG), at the dosage of 400 mg/kg every 4 weeks, was given to all HIV-1-infected patients. Blood samples were collected from all children at their current age. Serum levels of nitrite were determined before and 1 hour after administration of IVIG.
Nitrite and cytokine assay. Levels of nitrite in the sera of our patients were determined by the Griess reaction, adapted to microwell plates. Briefly, samples were deproteinized by the addition of 100 μL of 35% sulfosalicylic acid to 500 μL of sample and then centrifuged at 10,000g for 15 minutes. Two hundred μL of supernatant, 300 μL of 5% aqueous NH₄Cl buffer, and 60 μL of 5% NaOH were combined for analysis. A standard curve was prepared with known concentrations of NaNO₂. Treated samples (100 μL) and standard curve (100 μL) were placed in a flat-bottom 96-well plate. One hundred μL of Griess reagent (0.1% naphthylethilenediaminedihydrochloride, 1% sulfanilamide, and 5% phosphoric acid) was added to each well. Samples were incubated for 10 minutes at room temperature in the dark and underwent absorbance at 550 nm in an automatic microplate reader.

Serum samples were also tested for levels of IL-1β, TNF-α, and IFN-γ by EIA. IL-1β and TNF-α levels were determined by means of Quantikine (R and D Systems, Minneapolis), whereas the IFN-γ level was determined by an ELISA based on the sandwich principle (Holland Biotechnology, Leiden, the Netherlands). Cytokine levels were determined with use of an average of duplicate values based on each standard curve. In this assay, minimum detectable levels were 12 pg/mL for IL-1β, 7.5 pg/mL for TNF-α, and 20 pg/mL for IFN-γ.

Statistical analysis. Data are expressed herein as means and SDs. Differences were evaluated for significance with a nonparametric Mann-Whitney U test and Wilcoxon’s signed rank test, when appropriate. Differences were considered significant at $P < .05$. The value of an undetectable level of each cytokine was regarded as 0 pg/mL for simplification of the analysis.

Results

Figure 1 summarizes serum levels of nitrite in the seven children with HIV-1 infection. As can be seen, increased and significant serum levels of nitrite were observed in children with AIDS (0.4 ± 0.2 μmol/L) and in children seronegative for HIV-1 during the first year of life (0.5 ± 0.3 μmol/L). Table 1 shows serum levels of IL-1β, TNF-α, and IFN-γ in six children with AIDS.

As shown, there was a significant elevation of serum levels of IL-1β and TNF-α in these patients. On the other hand, a nonsignificant increase in IFN-γ levels was observed as well. Table 2 shows serum levels of IL-1β and TNF-α in four children with AIDS who were treated prophylactically with IVIG. As shown, there was usually a significant decrease in serum levels of nitrite in these patients after prophylactic administration of IVIG.

Discussion

In this study we have shown evidence of increased circulating levels of nitrite in children with AIDS. A significant decrease in serum levels of nitrite in these patients was noted after prophylactic administration of IVIG. In addition, a concomitant increase in levels of IL-1β and TNF-α in the sera of children with AIDS was observed. Retroviruses stimulate expression of immediate-early genes and inducible NO synthase, and retrovirus-induced NO production causes damage of adjacent, uninfected cells [12, 13]. In particular, HIV-1 also stimulates NO production by human macrophages in vitro, and the level of NO is elevated in adult patients with AIDS, especially those infected with opportunistic pathogens, including T. gondii, P. carinii, M. tuberculosis, and Mycobacterium avium [15].

More recently, we have also demonstrated (in an in vitro study) that production of NO from peripheral blood mononuclear cells and polymorphonuclear leukocytes was increased in adult patients with AIDS, particularly those with opportunistic infections, including cerebral toxoplasmosis and disseminated mycobacterial infection [16]. Increased levels of nitrite were also observed in the sera of children who became seronegative for HIV-1 during the first year of life. The elevated serum levels of nitrite in these subjects may be related to the transitory presence of viral proteins of HIV-1, which may have stimulated production of NO. Several cytokines, including IFN-γ, IL-1β, and TNF-α, induce production of NO via stimulation of NO
Table 1. Serum levels of nitrite, interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and gamma interferon (IFN-γ) in children with AIDS vs. those in controls.

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Nitrite (µmol/L)</th>
<th>IL-1β (pg/mL)</th>
<th>TNF-α (pg/mL)</th>
<th>IFN-γ (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (healthy children)</td>
<td>0.2 ± 0.04</td>
<td>1.5 ± 4.1</td>
<td>2.8 ± 7.5</td>
<td>0.7 ± 1.8</td>
</tr>
<tr>
<td>(n = 7)</td>
<td></td>
<td>(n = 7)</td>
<td>(n = 7)</td>
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<tr>
<td>Children with AIDS</td>
<td>0.5 ± 0.3*</td>
<td>37.5 ± 23.6†</td>
<td>91.2 ± 45.1†</td>
<td>49.2 ± 110.5</td>
</tr>
<tr>
<td>(n = 7)</td>
<td></td>
<td>(n = 6)</td>
<td>(n = 6)</td>
<td></td>
</tr>
</tbody>
</table>

*P = .0350 (all P values are for comparisons with control values [Mann-Whitney U test]).
†P = .0029.
‡P = .0051.

The elevation of levels of IL-1β, TNF-α, and (to a lesser extent) IFN-γ in our pediatric patients with AIDS may represent strong stimulation of NO production, although HIV-1 itself induces NO production from several cells, including monocytes/macrophages [14].

Finally, we have demonstrated that administration of IVIG to our patients caused a significant reduction in serum levels of nitrite. It has been demonstrated that IVIG therapy is beneficial for HIV-1-infected children in that it reduces the number of viral and bacterial infections [17]. Although data from several clinical trials suggest its effectiveness against many infections or immune-mediated disorders [18], little is known about the mechanisms of action of IVIG [18, 19]. However, the decreased serum levels of nitrite, after administration of IVIG, may be correlated with an inhibitory effect of IVIG on production and release of NO from several types of cells, particularly monocytes/macrophages (which host HIV-1) and polymorphonuclear leukocytes. It should be noted that we have investigated a small number of patients with AIDS and that although these findings are interesting, study of a larger number of such patients is needed to confirm our data.

In conclusion, increasing evidence indicates that NO may play a part in acute and chronic inflammation and that it is therefore probably involved in limiting and/or eradicating infection. Although NO may play a role as a cytostatic or cytotoxic factor, not only for invading microorganisms but also for the cells that produce it and for neighboring cells, its potential role in immunity to HIV-1 infection warrants further investigations.

Table 2. Serum levels of nitrite in four children with AIDS, before and after administration of intravenous immune globulin (IVIG; 400 mg/kg every 4 weeks).

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Before</th>
<th>After treatment†</th>
<th>Serum level of NO₂ (µmol/L)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.50</td>
<td>0.23</td>
<td></td>
</tr>
<tr>
<td>0.26</td>
<td>0.16</td>
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<td></td>
</tr>
<tr>
<td>0.67</td>
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</tr>
<tr>
<td>0.33</td>
<td>0.33</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.15</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>0.15</td>
<td>0.13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.16</td>
<td>0.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.94</td>
<td>0.33</td>
<td></td>
</tr>
<tr>
<td>2.34</td>
<td>0.16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.33</td>
<td>0.23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.84</td>
<td>0.22</td>
<td></td>
</tr>
<tr>
<td>0.23</td>
<td>0.23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.16</td>
<td>0.23</td>
<td></td>
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</tbody>
</table>

* Determined monthly for 3 or 4 consecutive months for each patient.
† Serum levels of nitrite were measured 1 hour after treatment with IVIG; P = .0029, as determined by Wilcoxon’s signed rank test.

References
ANSWER TO PHOTO QUIZ (SEE PAGE 637)

Figure 1. Atypical dermatologic lesions in an AIDS patient with VZV infection. Cutaneous lesions evolved from small vesicular pustular papules to raised hyperkeratotic papules that were 2 cm in diameter; an ulcerated base remained after the lesions healed. Acyclovir-resistant VZV skin lesions are typically verrucous and hyperkeratotic.

Diagnosis: Disseminated acyclovir-resistant varicella-zoster virus infection in a patient with AIDS

Varicella-zoster virus (VZV) infection is a common complication of HIV infection, and disseminated VZV can occur in the late stages of AIDS. Therapy with high-dose oral acyclovir (800 mg five times a day) or iv acyclovir (10–12 mg t.i.d. for 7–14 days) can induce clearing of the lesions, but relapses occur frequently. Patients with AIDS are often given long-term maintenance therapy with oral acyclovir, but resistant virus (TK-) can emerge; in this case, treatment with foscarnet is required [1–3].

The skin lesions produced by acyclovir-resistant VZV are typically verrucous and hyperkeratotic, as is illustrated in our case (figure 1) [4]. The verrucous lesions evolved from small vesicular pustular papules to raised hyperkeratotic papules that were 2 cm in diameter. Histologic examination of specimens...
from the lesions revealed multinucleated giant cells (figure 2), and VZV was cultured from the lesions. Serum IgG and IgM antibodies to VZV were not detected in our patient.

In cases of dissemination, VZV can cause ocular infection. In our case, acute retinal necrosis occurred following retinal artery occlusion that was secondary to VZV infection (figure 3). In Spain, VZV is the fifth most common cause of infectious retinitis (after cytomegalovirus, \textit{Candida} species, \textit{Mycobacterium} species, and \textit{Toxoplasma gondii}) in patients with AIDS [5]. VZV retinitis has also been reported by several investigators in the United States [6–8].

The mild inflammatory response in the retina, aggressive systemic course, and frequent bilateralism of the ocular lesions suggest the diagnosis of retinal VZV infection; the coexistence of VZV skin lesions is also helpful in making this diagnosis [8]. VZV can cause retinitis and the acute retinal necrosis syndrome after retinal artery occlusion has occurred [9]. Retinal detachments occur frequently in AIDS patients with VZV retinitis [6, 7].

Our patient developed the acute retinal necrosis syndrome; this syndrome was associated with diffuse whitish retinal edema, macular hemorrhage, and blindness. He was treated with iv foscarnet (40 mg/kg every 8 hours) and his skin lesions partially healed. He died of bacterial pneumonia 8 weeks after admission to the hospital.

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\textbf{References}