SHORT REPORT: TOTAL SERUM LEVELS OF THE NITRIC OXIDE DERIVATIVES NITRITE/NITRATE DURING MICROFILARIAL CLEARANCE IN HUMAN FILARIAL DISEASE

STEFAN WINKLER, IBRAHIM EL MENYAWI, KEN FLORIS LINNAU, AND WOLFGANG GRANINGER

Department of Infectious Diseases, Internal Medicine I, University of Vienna, Vienna, Austria

Abstract. Nitric oxide (NO) has recently been shown to be cytotoxic to both microfilariae and adult Brugia malayi in vitro and in a murine model, as well against Onchocerca lienalis microfilariae in vitro. We studied the kinetics of nitrite/nitrate, both stable end products of NO, by high-pressure liquid chromatography during microfilaricidal chemotherapy in four filariasis (three Loa loa, and one Onchocerca volvulus) patients. High serum levels of nitrite/nitrate were released during microfilarial clearance and sustained elevated levels were observed six months after chemotherapy, suggesting a role of NO in the elimination of microfilariae in human filariasis.

The microfilaricemic state of human filarial infection is associated with a parasite-specific immunologic hyporesponsiveness characterized by an expansion of T helper (Th)-2 cells and a lack of Th-1 cell responses. Microfilaricidal chemotherapy with either diethylcarbamazine (DEC) or ivermectin induces an acute inflammatory reaction accompanied by an interleukin (IL)–5 dependent increase of eosinophils, eosinophil degranulation, and elevated proinflammatory cytokine levels like IL-6, tumor necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ). Long-term effects of drug induced microfilarial clearance comprise a reversal of the parasite-specific hyporesponsiveness with restoration of Th-1 cell-mediated responses and resistance to reinfection. It is therefore assumed that host factors play an essential role in the removal of microfilariae, which is emphasized by the fact that DEC is not microfilaricidal in vitro without host factors, but induces a rapid decrease in microfilarial levels in vivo. Release of toxic granules and reactive oxygen intermediates by activated myeloid cells have all been implicated in microfilarial clearance. However, the precise immune effector mechanisms operating against the developmental stages of filarial disease remain largely unexplored.

Nitric oxide (NO) or its by-products have recently been shown to damage microfilariae of Brugia malayi and Onchocerca lienalis in vitro. In addition, inhibitory effects of NO on the development of third-stage (L3) larvae of B. malayi in a murine model and its toxicity on adult B. malayi in vitro have been demonstrated. Furthermore, the ivermectin-induced killing of microfilariae of Litomosoides carinii has been shown to be mediated by NO. To determine whether NO plays a role in the removal and the long-term suppression of microfilariae after chemotherapy in human filariasis, serum levels of its stable end products nitrite and nitrate were closely monitored during microfilarial clearance and re-examined six and 12 months after chemotherapy. Three microfilaricemic patients with Loa loa and one microfilaricemic patient with Onchocerca volvulus gave informed consent and were included in this study, as were three drug-treated amicrofilaricemic individuals from an endemic area in Gabon and three nonendemic controls from Austria (randomly assigned to either DEC or ivermectin treatment). This study was approved by the Ethics Committee of the International Foundation of the Albert Schweitzer Hospital in Gabon.

Patient 1 was a 23-year-old Austrian woman (student) who noticed a transocular migration of adult L. loa two years after a seven-week stay in Cameroon. Microfilariaemia of 2,000/ml was present and treatment with DEC was started with 0.4 mg/kg of body weight on the first day. The dosage was increased to 6 mg/kg of body weight per day until day 4 and continued for 17 days. Side effects (fever, headache, arthralgia, pruritus) due to rapid clearance of microfilariae were most pronounced on day 5 of treatment. Eosinophils were highest on day 10 (35%). Polyclonal IgE levels, measured by immunonephelometry (Behring, Vienna, Austria), were consistently lower than the detection limit. A detailed analysis of circulating cytokines and T cell phenotypes of this patient has been described previously. Patient 2 was a 27-year-old Nigerian man who complained of weakness and slight pruritus. Microfilaricemia of 600/ml was diagnosed and the patient was treated with a single dose of ivermectin (400 µg/kg body weight). Side effects consisted of elevated body temperatures (37.8°C) and headaches on days 3–5. Eosinophilia was most pronounced on day 11 (41%), and IgE levels remained consistently elevated at 800 U/ml (normal value < 200 U/ml).

Patient 3 was a 36-year-old man from Gabon presenting with intense pruritus and L. loa microfilariaemia of 6,800/ml. He was treated with ivermectin (400 µg/kg of body weight). Arthralgia and headache were reported on days 1–3 after treatment. Eosinophil counts were not available and IgE levels remained below the detection limit throughout the observation period.

Patient 4 was a 29-year-old man from Guinea-Bissau with typical skin manifestations of onchocerciasis. Ocular disease was not present but high level microfilariaemia was diagnosed by skin-snip examinations. The patient was treated with a single dose of ivermectin (150 µg/kg of body weight). Eosinophilia peaked on day 21 (35%) and IgE levels reached values up to 17,500 U/ml. No side effects due to treatment were noted. Ivermectin therapy was subsequently repeated every six months. Microfilariaemia was absent in all patients with L. loa infection 2–5 days after chemotherapy and remained so at follow-up examinations. In the onchocerciasis patient microfilariaemia was reduced to 2% of the pretreatment level at six months, and was undetectable at follow-up. Concomitant helminth infections (schistosomiasis, strongyloidesis, toxocariasis, gastrointestinal nematode disease) were ruled out as...
Figure 1. Total serum levels of the nitric oxide derivatives nitrite/nitrate during microfilarial clearance and at six and 12 months after chemotherapy for loiasis (patients 1, 2, and 3) and onchocerciasis (patient 4).

Venous blood was collected from all patients twice before treatment, then daily until day 14, on day 21, and additionally six and 12 months after chemotherapy. Follow-up of controls was terminated after three months because of sustained low nitrite/nitrate levels. After clotting, blood was immediately centrifuged and sera were stored at $-20^\circ C$ until use. Measurements for the NO derivatives nitrite/nitrate were accomplished by high-pressure liquid chromatography (HPLC) using a Shimadzu Sil-6B auto-injector-port, a Shimadzu LC-9A pump, a UV-VIS detector SPD-10 AV, and a Shimadzu LC-workstation (all instruments from Shimadzu, Tokyo, Japan). The column was an ion-exchanger based on styrol divinyl benzol and modified in our laboratory with quaternary amine. The mobile phase consisted of 10 mM methane sulfonic acid sodium salts adjusted to pH 8.5 with 0.1 M NaOH. The flow rate was 1 ml/min. The effluent was measured at a wavelength of 214 nm. Sera were deproteinized with trifluoracetic acid, vortexed for 60 sec, and centrifuged at 7,500 $\times g$ for 5 min. Five hundred microliters of supernatant were added to 100 $\mu$l of a 5% NaOH solution and 400 $\mu$l of double-distilled water. The solution was loaded onto a solid phase extraction C-18 column. The effluent was again loaded onto a solid phase ion-exchanger column and finally eluted with 200 $\mu$l of 0.5 M solution of the mobile phase. Twenty microliters were injected onto the HPLC apparatus through the auto-injector. Values are expressed as $\mu$mol/l with a detection limit of 0.1 $\mu$mol/l. Figure 1 shows the kinetics of nitrite/nitrate during microfilaricidal chemotherapy with follow-up examinations at six and 12 months after treatment. Nitrite/nitrate serum levels were always $< 2.0 \mu$mol/l in all drug-treated amicrofilaremic controls.

A similar release of high levels of the NO derivatives nitrite/nitrate was observed in the four patients, with peak levels during microfilarial clearance and sustained elevated levels six months after chemotherapy. The sharp elevation of NO immediately after treatment might indicate a cause-and-effect relationship. The maintenance of elevated NO levels occurred in the absence of other patent infectious processes in these patients. The results were not only independent of the kind of treatment used but also irrespective of the distinct tissue localization of *Onchocerca* microfilariae in the skin and *L. loa* microfilariae in the blood. These findings show stereotypic mechanisms of NO generation during drug-induced microfilarial clearance, while treatment of amicrofilaremic controls did not increase levels of NO derivatives. We cannot provide direct evidence of NO toxicity on microfilariae in our patients; nevertheless, the poor (ivermectin) or lacking (DEC) *in vitro* activities of these drugs suggest the involvement of potent host-effector-molecules in microfilarial clearance. Recently, this has been elaborated by the observation of NO as the principal mediator in ivermectin-induced killing of *L. carinii* microfilariae.

Antifilarial toxicity of NO has not only been observed against L1 but also against infective L3 larvae, as well as against adult helminths. In these experiments, cytotoxic effects were seen predominantly after prolonged exposure (e.g., 72 hr) of the parasites to IFN-γ-activated murine macrophages or NO donors, while short incubation periods resulted only in cessation of motility with its restoration after removing the helminths to cell-free culture. Cytotoxic effects on microfilariae can be expected in our patients since prolonged exposure to elevated levels of NO occurred. Potential sites of antifilarial action of NO include the mitochondria, ultrastructurally characterized by swelling and disorganization of cristae. On the other hand, it has also been reported that NO donors inhibit the *in vitro* programmed cell death of human eosinophils, and stimulate the release of TNF-α by these important anthelmintic effector cells.

The cellular sources of the NO derivatives detected in our
patients still need to be clarified, especially in the light of the apparent differences in NO generation in comparison with rodents.\textsuperscript{18} Two prerequisites, that have been associated with NO production by human monocytes, namely high levels of IFN-\(\gamma\) and up-regulation of the early activation antigen CD69 on peripheral blood mononuclear cells, have been demonstrated during clearance of microfilariaemia in loiasis.\textsuperscript{6} Yet the question of whether human monocytes/macrophages are the cellular source of the NO derivatives detected in our patients remains unanswered. Our results, however, provide evidence of a possible anti-parasitic role of NO in human filariasis.

Authors’ address: Stefan Winkler, Ibrahim El Menyawi, Ken Floris Linnau, and Wolfgang Graninger, Department of Infectious Diseases, Internal Medicine I, University of Vienna, Währingergürtel 18-20, 1090 Vienna, Austria.

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