Am. J. Trop. Med. Hyg., 78(5), 2008, pp. 770–776
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Decreased Serum Paraoxonase-1 Activity during Intestinal Nematode (*Nippostrongylus brasiliensis*) Infection in Rats

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Abstract. Reduced paraoxonase-1 (PON1) activity has been observed in a number of pathological conditions; however, little is known about the effects of intestinal nematode infections, such as *Nippostrongylus brasiliensis*, on paraoxonase activity. We observed a significant reduction in serum paraoxonase and arylesterase activity after *N. brasiliensis* infection in Wistar rats from Day 6 until Day 12 post-infection (p.i.) for serum paraoxonase and from Day 3 until Day 24 p.i. for arylesterase. In addition, *N. brasiliensis* infection increased serum concentrations of pro-inflammatory cytokines (interleukin-1, interleukin-6, and tumor necrosis factor-α), with maximum concentrations observed on Day 9 p.i. These cytokines are known to inhibit the synthesis of hepatic PON1 mRNA. Thus, the observed reduction in PON1 activity during *N. brasiliensis* infection is likely associated with inflammatory reactions mounted against the parasites.

INTRODUCTION

PON1 (paraoxonase-1) [aryldialkylphosphatase (E.C.3.1.8.1)] is a 355 amino acid glycoprotein that is synthesized in the liver and secreted into the blood where it associates with high-density lipoprotein (HDL).[1–3] PON1 exhibits multiple enzyme activities, including organophosphate esterase, carboxyl esterase, lactonase, and phospholipase A2 activity.[4] It is involved in the detoxication of organophosphate insecticides, such as parathion and chlorpyrifos,[5] as well as nerve agents, such as soman, sarin, and VX.[3] PON1 may also protect against coronary artery disease by preventing oxidation of lipids within low-density lipoproteins (LDLs) during proinflammatory process.[5]

Alterations of PON1 activity, usually reductions in activity, have been observed in a number of pathological conditions affecting both human and animal species.[3] These include diabetes (types 1 and 2),[6,7] patients with chronic renal failure undergoing hemodialysis,[8] glomerulonephritis,[9] and rheumatoid arthritis.[10] Patients with chronic liver disease (chronic hepatitis and cirrhosis) show a reduction in PON1 activity, which is proportional to their degree of liver damage.[11] Administration of lipopolysaccharides (LPSs) to mice, which mimics gram-negative sepsis, has been observed to result in a 50% reduction in serum and hepatic PON1 activity, with a similar reduction in hepatic mRNA levels.[12]

On the other hand, gastrointestinal (GI) nematodes are one of the most commonly acquired infections in the world,[13] affecting up to one fourth of the world's population,[14,15] with an increased prevalence in some endemic areas in developing countries, particularly among children.[16] Furthermore, in these countries, many people work and live in close proximity to fields where organophosphorus compounds are commonly applied and stored.[17] However, previous studies have not examined the effect of nematode infections on PON1 activity.

Nippostrongylus brasiliensis is a nematode that infects mice and rats with a similar life cycle to that of the human pathogens *Ancylostoma duodenale* and *Necator americanus*.[18] *N. brasiliensis* has been extensively examined to study immune responses against helminths in rodent models.[19–21]

This study is the first to examine the effect of *N. brasiliensis* infection, as a model of nematode infection, on PON1 activity (paraoxonase and arylesterase activity), as well as the mechanism(s) by which alterations in PON1 activity occur.

MATERIALS AND METHODS

Experimental animals. Male Wistar rats (75–85 g), 4 weeks of age, were purchased from Charles River Japan (Yokohama, Japan). All animals were housed in clean cages and given a standard diet and clean water *ad libitum* in an air conditioned room (23 ± 3°C). All protocols were approved by the institutional review board for animal experiments of the University of Miyazaki.

Parasitologic techniques. The strain of *N. brasiliensis* used in this study was maintained in our laboratory by serial passage in Wistar rats using subcutaneous inoculation of 3,000–4,000 third-stage larvae (L3) prepared using the charcoal culture method.[19] The rats were infected with L3 of *N. brasiliensis* by subcutaneous inoculation into the flank region. Infection was confirmed by counting fecal egg output as eggs per day (EPD).

Serum PON1 activity. Serum PON1 activity was assayed according to the method of Beltowski and others[22] using two synthetic substrates: paraoxon (diethyl-*p*-nitrophenyl phosphate; Sigma Chemical Co., St. Louis, MO) and phenyl acetate (Nacalai Tesque, Kyoto, Japan). PON1 activity against paraoxon was determined by measuring the initial rate of substrate hydrolysis to *p*-nitrophenol by examining absorbance at 412 nm of the assay mixture (800 μL) containing 2 mM paraoxon, 2 mM $CaCl_2$, and 20 μL of plasma in 100 mM Tris-HCl buffer (pH 8.0). Enzyme activity was calculated from the E_{412} of *p*-nitrophenol (18,290/M/cm) and expressed in U/mL (where 1 U of enzyme hydrolyzes 1 nmol of paraoxon/min).

Enzyme activity toward phenyl acetate was determined by measuring the initial rate of substrate hydrolysis within the assay mixture (3 mL) containing 2 mM substrate, 2 mM $CaCl_2$, and 10 μL of plasma in 100 mM Tris-HCl (pH 8.0). Absorbance was monitored for 3 minutes at 270 nm, and

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enzyme activity was calculated from the E_{270} of phenyl acetate (1,310/M/cm) and expressed in U/mL (where 1 U of arylesterase hydrolyzes 1 μmol of phenyl acetate/min).

Measurement of serum cytokine concentrations. A multiplex bead-based immunoassay kit (Bio-Rad Laboratories, Tokyo, Japan) was used to examine serum samples for the presence of nine cytokines: interleukin-1α (IL-1α), IL-1β, IL-2, IL-4, IL-6, IL-10, tumor necrosis factor (TNF)-α, granulocyte/macrophage colony-stimulating factor (GM-CSF), and interferon (IFN)-γ. In brief, premixed standards were reconstituted in 0.5 mL of a Bio-Plex rat serum standard diluent, generating a stock concentration of 50,000 pg/mL for each cytokine. The standard stock was serially diluted in the Bio-Plex rat serum diluent to generate eight points for the standard curve. The assay was performed in a 96-well filtration plate supplied with the assay kit. Premixed beads (50 μL) coated with target capture antibodies were transferred to each well of the filtration plate and washed twice with Bio-Plex wash buffer. The samples were diluted 1:4 in the Bio-Plex serum sample diluent. Premixed standards or diluted samples (50 μL) were added to each well containing washed beads. The plate was shaken for 30 seconds at high speed (1,100 rpm) and incubated at room temperature for 30 minutes at low speed (300 rpm). After incubation and washing, premixed detection antibodies (25 μL) were added to each well. The plate was incubated for 30 minutes with a shaker at low speed (300 rpm). After incubation and washing, streptavidin-PE was added to each well. The incubation was terminated after shaking for 10 minutes at room temperature. After washing, the beads were re-suspended in 125 μL of Bio-Plex assay buffer. Beads were read, and data from the reaction were acquired and analyzed using the Bio-Plex suspension array system (Luminex 100 system) from Bio-Rad Laboratories (Tokyo, Japan), and concentrations of each cytokine were determined using Bio-Plex Manager Version 4.1 software.[23] Data are expressed in terms of picograms cytokine per milliliter serum.

Experimental design. Sixteen male Wistar rats were randomly allocated into three groups (four to six animals per group). The groups were as follows: (1) control group, (2) low-dose group infected with 400 L3 of *N. brasiliensis* (400 L3), and (3) a high-dose group infected with 4,000 L3 of *N. brasiliensis* (4,000 L3). Blood for serum samples was collected from the jugular vein on Days 3, 6, 9, 12, 18, 24, and 36 p.i.

Statistical analysis. Statistical analysis was performed with the statistical software package SPSS for Windows (version 15.0; SPSS, Chicago, IL). The significance of differences between groups was evaluated by nonparametric tests. Mann-Whitney *U* test was used for comparing between the infected groups and the control. Results are expressed as mean ± SEM. $P < 0.05$ was considered significant.

RESULTS

Monitoring of *N. brasiliensis* infection by fecal egg counts. Infection with two different quantities of *N. brasiliensis* was monitored as shown in Figure 1. After subcutaneous infection with *N. brasiliensis*, eggs became detectable in the feces of male Wistar rats from Day 5 p.i., reaching a maximum on Day 6 p.i. in both groups, and finally disappearing by Days 12 and 13 p.i. in the 400 and 4,000 L3 groups, respectively.

PON1 activity. Figure 2 shows serum PON1 activity against paraoxon (paraoxonase activity). On Day 3 p.i., non-significant changes in paraoxonase activity were observed. A 44% reduction in paraoxonase activity was observed in the 4,000 L3 infected group ($P < 0.05$) on Day 6 p.i., whereas 22% and 46% reductions were observed in the 400 ($P < 0.05$) and 4,000 L3 ($P < 0.01$) infected groups, respectively, on Day 9 p.i. A 23% reduction in paraoxonase activity was observed in the 4,000 L3 infected group ($P < 0.05$) on Day 12 p.i. compared with controls. Increases in paraoxonase activity of 29% and 35% were observed in the 4,000 L3 infected group on Days 18 ($P < 0.05$) and 24 p.i. ($P < 0.01$), respectively, compared with controls, before a return to normal levels by Day 36 p.i.

Figure 3 shows serum PON1 activity against phenyl acetate (arylesterase activity). On Days 3, 6, 9, and 12 p.i., ar-

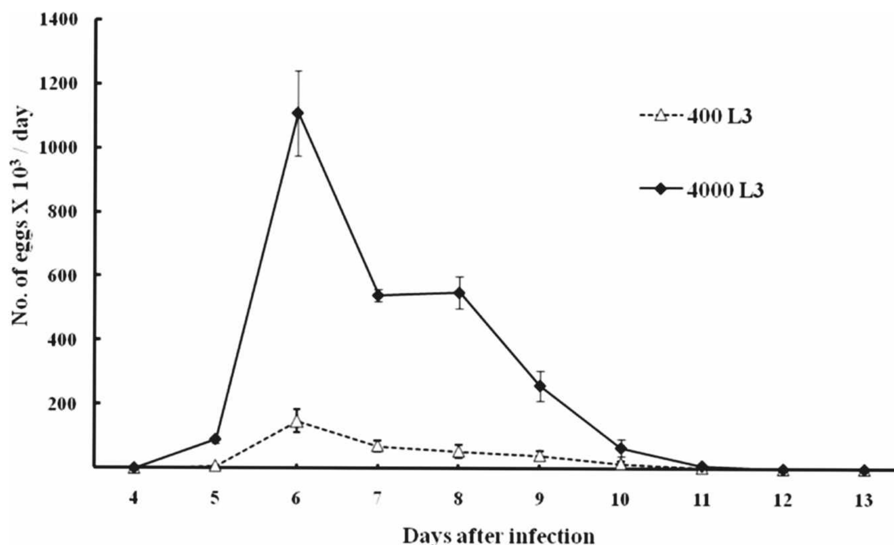

FIGURE 1. Kinetics of fecal egg output (EPD) after infection of rats with 400 and 4,000 L3 of *N. brasiliensis*. Bars represent means ± SEM (N = 4–6).

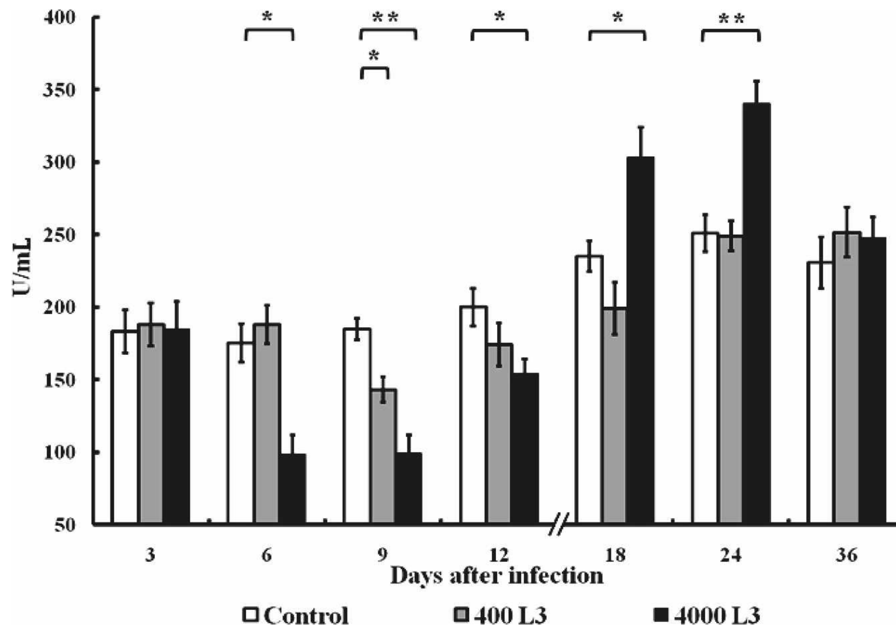

FIGURE 2. Effect of *N. brasilienis* infection on paraoxonase activity in male Wistar rats. Animals were infected with 400 and 4,000 L3 of *N. brasiliensis* and serum assays of PON1 activity were performed on Days 3, 6, 9, 12, 18, 24, and 36 p.i. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control values. Bars represent means ± SEM ($N = 4$–6).

ylesterase activity among the 4,000 L3 infected group was reduced by 15% ($P < 0.01$), 40% ($P < 0.01$), 35% ($P < 0.01$), and 20% ($P < 0.01$), respectively. On Days 3, 12, 18, and 24 p.i., arylesterase activity among the 400 L3 infected group was reduced by 6% ($P < 0.05$), 12% ($P < 0.05$), 7% ($P < 0.05$), and 11% ($P < 0.05$), respectively. Combined, these results indicate dose-dependent reductions in paraoxonase and arylesterase activity.

Changes in serum cytokine levels among rats infected with *N. brasiliensis*. IL-1α, IL-1β, IL-2, Il-4, IL-6, IL-10, GM-CSF, INF-γ, and TNF-α levels in the serum of control and *N. brasiliensis* infected rats were examined on Days 3, 9, 24, and 36 p.i. (Figs. 4–6). The IL-1α level (Figure 4A) was significantly increased on Days 3 and 9 p.i. in the 400 L3 infected group ($P < 0.05$), and a significant increase (3.3-fold) was also observed in the 4,000 L3 group ($P < 0.01$) on Day 9 p.i. The IL-1β level (Figure 4B) was significantly increased (17.8-fold) on Day 9 p.i. in the 4,000 L3 infected group ($P < 0.01$). The level of IL-2 (Figure 4C) showed non-significant changes during the different checked points, whereas IL-4 levels (Figure 4D) showed a significant increase (4.5-fold) on Day 9 p.i. in both the 400 and 4,000 L3 infected groups ($P < 0.05$).

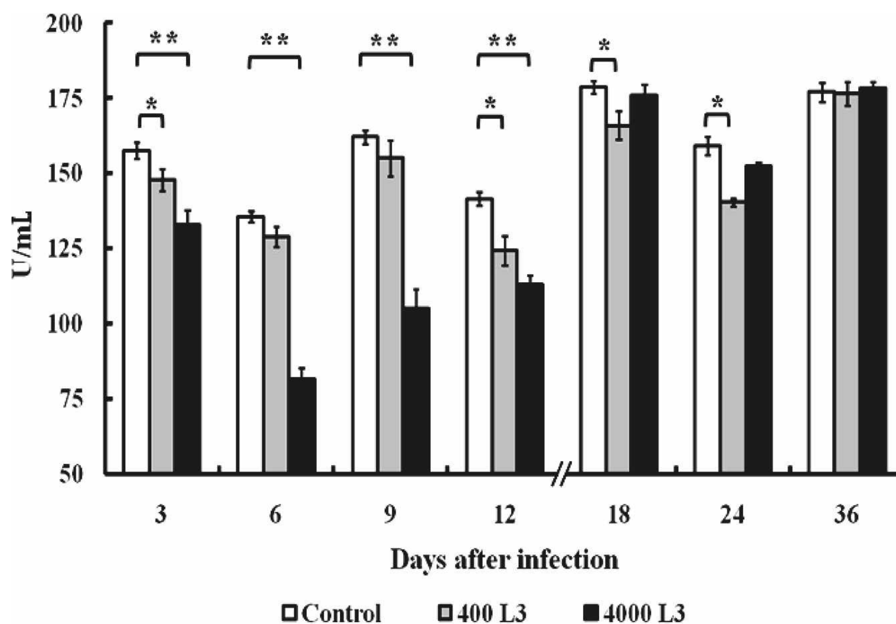

FIGURE 3. Effect of *N. brasilienis* infection on arylesterase activity in male Wistar rats. Animals were infected with 400 and 4,000 L3 of *N. brasiliensis* and serum assays of PON1 activity were performed on Days 3, 6, 9, 12, 18, 24, and 36 p.i. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control values. Bars represent means ± SEM ($N = 4$–6).

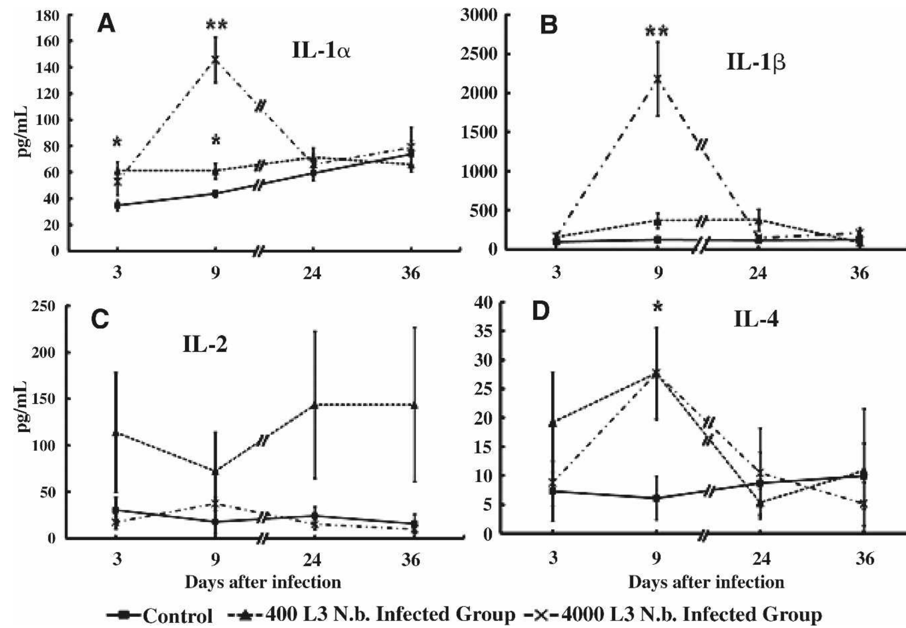

FIGURE 4. Serum concentrations of IL-1α (**A**), IL-1β (**B**), IL-2 (**C**), and IL-4 (**D**) in response to infection with 400 and 4,000 L3 *N. brasiliensis* in male Wistar rats on Days 3, 9, 24, and 36 p.i. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control values. Bars represent means ± SEM (N = 4–5).

Figure 5A shows a significant increase in the IL-6 level (3.8-fold) on Day 9 p.i. in the 4,000 L3 infected group ($P < 0.05$), whereas Figure 5B shows non-significant changes in the IL-10 level during the different checked points. GM-CSF (Figure 5C) was significantly increased on Days 3 and 9 (3-fold) p.i. in the 400 L3 infected group ($P < 0.05$), and a significant increase (4-fold) was also observed in the 4,000 L3 group ($P < 0.05$) on Day 9 p.i. INF-γ (Figure 5D) was significantly increased (5.5-fold) on Day 9 p.i. in the 400 L3 infected group ($P < 0.05$).

The TNF-α level (Figure 6) was significantly reduced in both the 400 and 4,000 L3 infected groups on Day 3 p.i. ($P < 0.01$), after which a significant increase (7-fold) was observed on Day 9 p.i. in the 4,000 L3 infected group ($P < 0.01$).

DISSCUSION

To the best of our knowledge, this study is the first to explore the effects of intestinal nematode infection with *N. brasiliensis*, a rodent model for human hook worm disease,[24] on serum PON1 activity. In this study, we showed that PON1

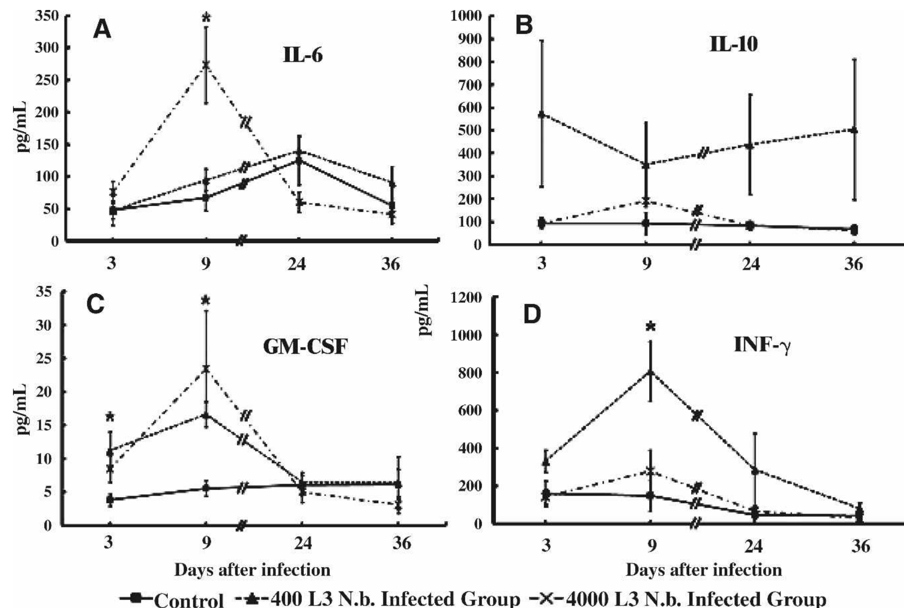

FIGURE 5. Serum concentrations of IL-6 (**A**), Il-10 (**B**), GM-CSF (**C**), and INF-γ (**D**) in response to infection with 400 and 4,000 L3 *N. brasiliensis* in male Wistar rats on Days 3, 9, 24, and 36 p.i. $^{*}P < 0.05$ compared with control values. Bars represent means ± SEM (N = 4–5).

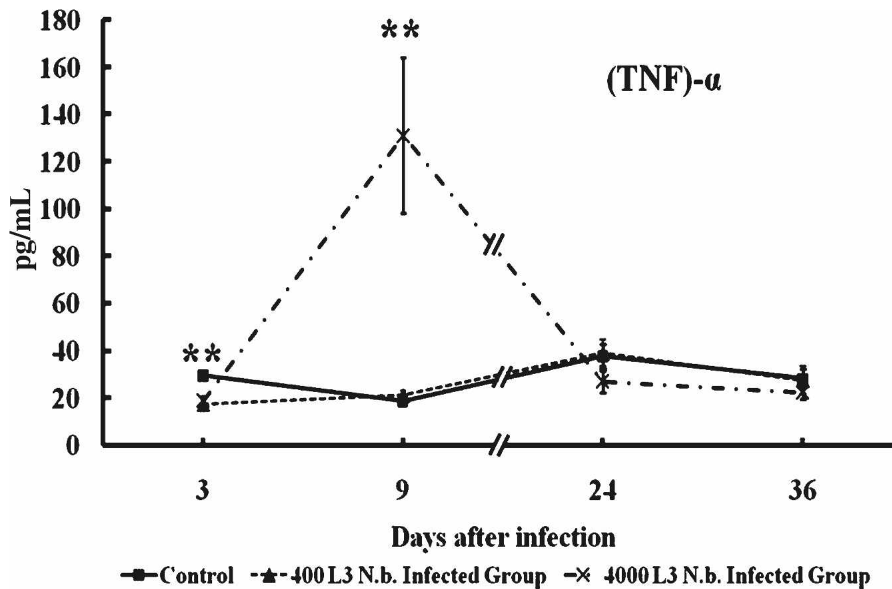

FIGURE 6. Serum concentrations of TNF-α in response to infection with 400 and 4,000 L3 *N. brasiliensis* in male Wistar rats on Days 3, 9, 24, and 36 p.i. $^{**}P < 0.01$ compared with control values. Bars represent means ± SEM (N = 4–5).

activity (paraoxonase and arylesterase activity) decreases after infection with *N. brasiliensis*. The observed reduction in paraoxonase activity started on Day 6 p.i and continued until Day 12 p.i, reaching its lowest level between Days 6 and 9 p.i. The observed reduction in arylesterase activity started on Day 3 p.i. and continued until Day 24 p.i., reaching its lowest level on Day 6 p.i. A possible mechanism of reduced serum PON1 activity is downregulation of hepatic PON1 production by pro-inflammatory cytokines.[25] We showed significant increases in various pro-inflammatory mediators, including IL-1α, IL-1β, and IL-6, on Day 9 p.i., which is supported by previous research indicating that alveolar macrophages isolated from infected rats on Day 8 p.i. secrete greater amounts of IL-1 and IL-6 than those recovered from rats on Day 2 p.i.[26] Moreover, previous research suggests that increased levels of TNF-α on Day 9 p.i. might indicate a role of TNF in host inappetance during infection with *N. brasiliensis*.[27,28] This is supported by the findings of Cohn and others,[29] which suggested that TNF-α enhances Th_2 cell transendothelial migration, thereby potentiating mucosal inflammation in the airway epithelium. This suggests that TNF-α may also be important in mucosal Th_2 responses during intestinal helminth infection. However, we observed significant reductions in TNF-α (by 41.37% and 36.03%) on Day 3 p.i. in the 400 and 4,000 L3 infected groups, respectively. This is consistent with the TNF-α levels observed in bronchoalveolar lavage fluid by McNeil and others[30] 24 hours after 2,000 L3 *N. brasiliensis* infection in Wistar rats. McNeil and others[30] have speculated that recruitment of inflammatory cells in the lung can, in some way, be delayed in response to larval invasion.

It is worth mentioning that the major source of inflammatory mediators, such as IL-1 and IL-6, may be alveolar macrophages caused by inflammatory responses in the lungs against residual worm antigens.[31] Because PON1 is predominantly, if not exclusively, produced in the liver, the observed increase in pro-inflammatory cytokines (IL-1, IL-6, and TNF-α) in this experiment likely caused the observed reduction in serum PON1 activity through suppression of hepatic production. This is supported by the findings of Feingold and others,[12] who found that both IL-1 and TNF reduce serum PON1 activity in addition to hepatic PON1 mRNA synthesis. Moreover, treatment of HepG2 cells with TNF-α and IL-1β similarly inhibits PON1 mRNA levels, suggesting that these cytokines are capable of directly suppressing hepatic PON1 mRNA production.[12,25] However, the effect of IL-6 on PON1 regulation in HepG2 cells remains controversial, because Kumon and others[25] showed upregulation of PON1 mRNA expression in HepG2 cells treated with IL-6 (1 and 10 ng/mL), which contradicts the *in vitro* results reported by Van Lenten and others,[32] who showed downregulation of PON1 mRNA expression in HepG2 cells treated with IL-6 (100 ng/mL). On the other hand, it is well known that *N. brasiliensis* powerfully induces a type 2 immune response during larval migration through the lung[33,34]; this can explain our results of increasing IL-4 on Day 9 p.i. in both *N. brasiliensis* infected groups.

It is interesting to mention that the low-dose (400 L3) infected rat group showed a significant increase in INF-γ level, whereas the high-dose group (4,000 L3) did not, and this result confirms those obtained by Uchikawa and others,[35] who found that the INF-γ production by mesenteric lymph node (MLN) cells obtained from naïve rats is significantly suppressed by addition of high dose (100 μg/mL) of *N. brasiliensis* excretory/secretory (ES) antigen to the culture medium, which led us to speculate that, in low-level infections, the ES antigen concentration in the lymphoid tissues would not attain a level as high as that which suppresses IFN-γ production, and this would lead to a rather weak type 2 response, possibly resulting in sustained infections. Because granulocyte macrophage-colony stimulating factor (GM-CSF) is a cytokine expressed by a variety of pulmonary cells, including activated T cells, macrophages, fibroblasts, and epithelial cells, and at the same time it is required for the Th_1 and Th_2 cytokines response,[36] this may explain our results of increasing its level after infection with 400 L3 on Days 3 and 9 p.i. and 4,000 L3 on Day 9 p.i. In addition, the GM-CSF is essential for airway

eosinophilia,[36] which is a prominent characteristic of the Th_2 immune response during *N. brasiliensis* infection.[37]

It is interesting that we observed significant increases in paraoxonase activity on Days 18 and 24 p.i. in 4,000 L3 infected rats, which exceeded the paraoxonase activity observed in controls. This observation is easily understood in light of a probable reduction in inflammation after complete worm expulsion with enhanced production of PON1 still occurring in response to the initial inflammation. This may explain the observed reduction in pro-inflammatory cytokines by Day 24 p.i., despite increased PON1 activity.

It is important to mention that several studies have shown a stringent association between leukocyte-generated free oxygen radicals and *N. brasiliensis* expulsion from rat's small intestine, and the onset of free radical generation corresponds with the onset of worm rejection.[38–40] Moreover, the worm rejection is inhibited with administration of antioxidant butylated hydroxyl anisole (BHA) by reducing free oxygen radicals generation by rat leukocytes.[41] Meanwhile, our results (unpublished data) indicated that administration of BHA to *N. brasiliensis*–infected rats did not ameliorate the suppressed serum PON1 activity. In addition, the maximum free radical generation in *N. brasiliensis*–infected rats occurred around Day 11 p.i.,[40] whereas our results showed a significant suppression in serum PON1 activity starting from Day 3 p.i. Combined, it is unexpected that the levels of serum PON1 activity in this study are altered because of an increased need for inactivation of oxidative stress products.

It is well known that organophosphorus pesticides, of which parathion is a typical example, are generally applied in agriculture, especially in developing countries, as the relatively non-toxic sulfur (thion) derivatives. They are activated *in vivo* by hepatic cytochrome P450-dependent microsomal monooxygenases to the highly toxic oxygen (oxon) analog by a process known as oxidative desulphuration. Knowing that, in mammals, any oxon form that escapes hepatic detoxication can be hydrolyzed in the blood by serum paraoxonase before it reaches the brain, which is the site of oragnophosphate action.[42] We can anticipate the deleterious effect of decreasing PON1 activity on organophsphate detoxication.

In conclusion, we showed that *N. brasiliensis* infection markedly decreases serum PON1 activity in Wistar rats in association with inflammation and increased levels of pro-inflammatory cytokines (IL-1, IL-6, and TNF-α). Combined with other reports examining the role of PON1 in the development of atheroscelorsis and insecticide metabolism, these results put forth the possibility that intestinal nematode infections may alter PON1 activity in humans.

Received November 2, 2007. Accepted for publication February 27, 2008.

Financial support: A.S.F. received financial support from the Egyptian government for Scholarship of PhD degree.

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