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Shistosoma Mansoni-infected mice: The progression of nitric oxide and superoxide anion production in activated peritoneal macrophages.

Mayssaa M.Wahby[a*], Kamal M.Kandeel[a], Eman Rashwan,[b] Enas R. Mostafa[a]

[a] Department of Biochemistry, Faculty of Science, Alexandria University
[b] Department of Immunology, Medical Research Institute, Alexandria University

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ABSTRACT

Background: The cell mediated immunity has revealed the importance of activated macrophages as key immune effector cells. Activated macrophages have the ability to generate reactive oxygen and nitrogen species (ROS and RNS) to kill parasite. ***Objectives:*** The current study aimed to investigate the progression of nitric oxide (NO) and superoxide anion (O_2^-) production in activated macrophages, isolated from Schistosoma Mansoni-infected mice during innate immune response. ***Methods:*** Hundred male albino mice were divided into two main groups control and infected. The mice of the infected group were injected subcutaneously via the tail with one hundred cercariae /mouse. *In vivo* production of NO and the O_2^- were estimated in the isolated peritoneal macrophages with the progression of post infection time. ***Results:*** The number of the isolated peritoneal macrophages and the production of NO and O_2^- were significantly increased exponentially with time interval throughout eight weeks of infection compared to the control. The highest levels of both the peritoneal macrophages and NO as well as the lowest O_2^- value were shown at the 8th week post infection. The correlation analysis showed significant positive relationship between the number of peritoneal macrophages and NO production and non-significant positive correlation between their numbers and O_2^- production. In contrast, the NO and O_2^- production showed significant negative correlation in the activated cells. ***Conclusion:*** The activated macrophages are important immune effector cells that are capable of generating cytotoxic molecules such as NO and O_2^- during the prepatent period of Schistosoma Mansoni infection. However, the NO plays the key role in this innate immune response..

Introduction:

Nitric oxide (NO), a molecular mediator, is involved in inflammation, tissue damage, and infections [(1)]. Liberation of NO by cells of the innate immune system contributes to early control of infection [(2)]. NO is important cytotoxic mediator of activated immune effector cells [(3)]. It is formed in mammalian cells from the oxidation of L-arginine in a reaction mediated by the enzyme nitric oxide synthase [(4)]. Inducible nitric oxide synthase (iNOS) is the immune /inflammatory isoform and its high output functions as an effector component of the cell mediated immune response [(1)]. NO, secreted by activated cells, is particularly relevant to cell injury [(5)]. The phagocytic leukocytes consume oxygen and generate reactive oxygen species in a process designated respiratory burst as a response to appropriate stimuli. This dramatic increase in the consumption of oxygen and activation of membrane-associated oxidase is dependent on the reduced

Corresponding author: Mayssaa M.Wahby, Department of Biochemistry, Faculty of Science, Alexandria University.

NADPH [6]. The reactive superoxide anion (O_2^-) that is generated near the cell surface and within the phagocytic vacuole exerts antimicrobial and antitumor cell effects [7]. Reactive nitrogen species (RNS) and reactive oxygen species (ROS) act in concert to inhibit the key metabolic enzymes, cause lipid peroxidation and DNA strand breaks that result in permanent cell injury and death [8].

The antimicrobial effects of NO and O_2^- have been documented as the earliest demonstrations of macrophage cytotoxicity toward the larvae of *Schistosoma mansoni* [9]. Macrophages are morphologically and functionally heterogeneous immune cells that are regarded as a bridge between innate and adaptive immunity [10, 11]. Macrophages offer the first line of defense against invading pathogens due to their abilities of phagocytosis and mount various anti-microbial mechanisms [12]. The activated macrophages have the ability to produce reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) that have potent antimicrobial activities [13]. Accordingly, we aim to investigate the immune response against the larva of Schistosoma Mansoni. To accomplish our goal, the progression in the production of NO and O_2^- was assessed in the activated macrophages at time intervals post infection. We also analyzed the correlation between the number of peritoneal macrophages and their capability to produce NO and O_2^- ; the cytotoxic molecules toward the larvae of helminthic parasite.

Materials and Methods:

Chemicals:

Sulfanilamide (P-Amino benzene sulfonamide), N-[1-naphthyl] ethylene diamine dihydro chloride, E. coli Lipopolysaccharide (LPS, serotype 0128:B12) and Cytochrome C M.W. 11700 Dalton were obtained from Sigma Chemical Company (Sigma, St, Louis, MO). Orthophosphoric acid (H_3PO_4) and sodium chloride (NaCl, sterilized solution) were obtained from EL Nasr Pharmaceutical Chemicals Co. Sodium nitrite ($NaNO_2$) was obtained from Gestund-heitsschadlich, bensondersbein Verschlucken.

Animals and experimental design:

Hundred male albino mice weighing 20-25 g (eight weeks old) were obtained from Theodor Bilharz Research Institute, Imbaba-Cairo. All the mice were fed with standard rodent diet and water *ad libitum*. Mice were randomly divided into two main groups, twenty for control and eighty for infected group. Infected group; animals were infected subcutaneously via tail-injection with one hundred cercariae of Schistosoma mansoni per mouse by Theodor Bilharz Research Institute Laboratories. Animals of both groups were selected randomly (five controls and twenty infected mice) and were euthanized under anesthesia at the end of the 2^{nd}, 4^{th}, 6^{th} and 8^{th}week post infection. The local committee accepted the experimental design and the protocol follows the guidelines of the National Institutes of Health (NIH).

Isolation of peritoneal macrophages and cell counts:

Immediately after euthanasia, cells of peritoneal macrophages were harvested from the peritoneal cavity using 0.9 N sodium chloride then centrifuged for 10 min at 1500 rpm using a centrifuge (Model Hettich model EBA12R, Germany). Cells were washed and centrifuged for twice, the supernatant was discarded and the cells were suspended in 1ml 0.9 N sodium chloride. Cell counts were estimated using a Bürker haemocytometer. The number of viable cells were calculated and kept constant at 1×10^6 cell/ml using sodium chloride 0.9 N for dilution [14, 15].

Determination of nitric oxide production:

The isolated peritoneal macrophages from the control and infected mice after 2, 4, 6 and 8 weeks post infection were subjected to in vitro activation with LPS [16]. Diluted cell suspensions (500 µl) were incubated for 18 h with 1 µg/ml LPS at 37°C and in a humidified 5% CO_2. The nitric oxide concentrations were determined in the peritoneal macrophages after being activated by the method of Green et al. [17]. Briefly, 100 µl of the supernatants were added to 100 µl of Griess reagent in each well of 96-well culture plates and the mixture was incubated at room temperature for 15 min. The developed pink color was measured at 550 nm using spekol II spectrophotometer. The concentration of nitric oxide was calculated in µmole/10^6 cells using standard curve of sodium nitrite ($NaNO_2$).

Determination of superoxide anion production:

The superoxide anion concentration was determined in the isolated peritoneal macrophages from the control and infected mice at time intervals 2, 4, 6 and 8 weeks post infection. The O_2^- was determined by cytochrome-*c* reduction test [15]. Briefly, 500 µl of cell suspension of macrophages (1 **x** 10^6 cells/ml) were added to 100 µl of horse heart cytochrome-c (0.12 mmol/L) and the mixture was incubated at 37°C for 60 min. The reduced cytochrome-c was measured at 550 nm using spectrophotometer (spekol II), at zero time and after 60 min of incubation. The O_2^- concentration was quantified as n mole of cytochrome reduced/min using extinction coefficient 2.1×10^4 M^{-1} cm^{-1}.

Statistical Analysis:

Data were analyzed as a completely randomized design and the obtained data were subjected to one-way analysis of variance (ANOVA) **[18]** using the statistical software SPSS, version 16.0. Differences between groups were considered significant at $P < 0.05$. Data were statistically analyzed by LSD Multiple Range Test.

Results

The nitric oxide production:

The nitric oxide generated in the activated peritoneal macrophages of the control and infected mice at the 2nd, 4th, 6th and 8th week post infection were illustrated in Figure (1). In comparison to control, the NO production in the activated peritoneal macrophages of the infected groups was significantly increased ($P<0.05$) after two, six and eight weeks post infection indicating the highest value at the 8th week .While, the NO production was non-significantly ($P> 0.05$) increased at the 4th week post infection as compared to the control. The generated NO in the activated macrophages overall the eight weeks post infection was significantly increased ($P<0.05$) in the infected groups compared to the control.

The superoxide anion production:

The O_2^- production was measured in the activated peritoneal macrophages of the control and infected mice at time intervals; two, four, six and eight weeks post infection (Figure 2). The O_2^- production in the activated peritoneal macrophages of the infected mice was significantly ($P<0.05$) increased at the 6th and 8th weeks post infection in comparison to the respective control. The lowest increase was observed at the 8th week than the 6th one. Meanwhile, the O_2^- production was non-significantly ($P> 0.05$) increased at the 2nd week post infection when compared to the control value. On the other hand, the O_2^- generation in the activated peritoneal macrophages of the infected group was

non-significantly (P> 0.05) decreased at the 4th week post infection in comparison to the control. The O_2^- production in the activated peritoneal macrophages of the infected groups was significantly increased (p< 0.05) over the eight weeks post infection compared to the control.

Count of the isolated peritoneal macrophages:

The numbers of the isolated peritoneal macrophages from the control and infected mice at time intervals; two, four, six and eight weeks post infection were presented in Figure (3). The results revealed that there was a significant increase (P<0.05) in the number of the isolated peritoneal macrophages at the 6th and 8thweeks and non-significant (p> 0.05) increase at the 2ndweek post infection compared to the control. The highest value of this increase was at the 8th week. Conversely, the number of isolated peritoneal macrophage cells of the infected group was non- significantly (p> 0.05) decreased after four weeks post infection as compared to that of the control group. The number of the isolated peritoneal macrophages was significantly increased (p<0.05) in the infected groups compared to the respective control overall the eight weeks post infection.

Correlation analysis:

The Correlation analysis was performed to study the relationship between the number of the isolated peritoneal macrophages with the production of NO and O_2^- in the infected groups overall the eight weeks post infection. Also, the correlation between the production of the NO and O_2^-in the activated peritoneal macrophages of the infected groups was studied (Table 1). The analysis indicated a significant positive correlation (r=0.47, p<0.01) between the number of the isolated peritoneal macrophages and the NO generated in the activated cells over the eight weeks post infection. Additionally, the number of the isolated peritoneal macrophages showed non-significant positive correlation with the O_2^- production in the activated cells over the eight weeks post infection period. On the other hand, there was a significant negative correlation (r =- 0.34, p<0.05) between the generated NO and O_2^- in the activated peritoneal macrophages overall the period of infection (Table 1).

Discussion

Helminthe infections are a health hazards in the tropical and subtropical areas and cause economic problem due to their high morbidity rather than mortality [(19)]. It is well-known that human monocytes and macrophages contribute to innate resistance to infection with Schistosoma mansoni [(20)]. Also, previous studies showed that the production of reactive oxygen intermediates; O_2^- and reactive nitrogen intermediates; NO by macrophages is critical to host defense [(21)]. Our study focused on the progression of NO and O_2^- production in the activated macrophages that was isolated from mice peritoneal cavity during the prepatent period of Schistosoma mansoni infection. Beside, a correlation analysis was carried out between the investigated parameters to emphasize on the antiparasitic capability of the activated macrophages in innate immune response through the production of NO and O_2^- . Our results indicated a significant increase in the NO production in the activated peritoneal macrophages of the infected mice compared to the control after two, six and eight weeks post infection. Also, the NO generation was non-significantly increased at the 4th week post infection compared to the control. Moreover, NO production was significantly increased in the infected groups over the eight weeks post infection when compared to the control. The role of NO during schistosomiasis has become particularly relevant in light of recent findings [(22)]. It has been reported that the peripheral blood mononuclear cells from infected patient are capable of NO production (*in vitro*), following exposure to Schistosoma

mansoni [23]. The non-significant increase in the NO production after four weeks post infection can be attributed to the rapid conversion of Schistosomes from the aerobic stage to a fermentative-dependent form, correlated with decreased susceptibility to nitric oxide-mediated killing by activated macrophages [24]. Also, the parasite goes through a different transient period of susceptibility to macrophage killing between two and four weeks after infection in which the parasite was gained resistance to macrophage cytotoxicity [25]. It was reported that generation of ROS and RNS contribute to Schistosoma mansoni-induced liver fibrosis [26]. In addition, previous studies reported that the deposition of Schistosome eggs in the liver increased the production of NO which induced tissue damage and morbidity by its direct cytotoxic and cytostatic activity during Schistosome-infection [22]. Production of ROS and RNS by polymorphonulear phagocytes and macrophages is critical to host defense. However, regulation of the production of these highly reactive molecules was of almost importance to host survival [21]. The inflammatory cytokines trigger monocytes and endothelial cells to release the potentially larvacidal O_2^-, oxygen, nitrogen radicals and the pro-inflammatory cytokines; IL-1, IL-6, and IL-23 [27]. Our results revealed a significant increase in the O_2^- production in the activated macrophages that were isolated at the 6th and 8th weeks from the infected groups compared to the control. While, non-significant increase was observed at the 2nd week post infection compared to the control. In comparison to the control, the generated O_2^- in the activated macrophages of the infected mice was non-significantly decreased at the 4th week post infection. Overall the eight weeks post infection, the superoxide anion production was significantly increased in the activated macrophages of the infected group comparing to the control. Cell-parasite interaction results in the production of O_2^- [28]. The activated macrophages exhibited increased capacity to generate superoxide anion, compared to resident macrophages [29]. The ability of macrophages to kill parasites intracellularly is dependent upon the oxidative burst and production of oxygen radicals [30]. Moreover, our results revealed that there was significant increase in the number of peritoneal macrophage cells of the infected groups at the 6th and 8th weeks and non-significant increase at the 2nd week post infection compared to control group. On the other hand, the number of those cells was non-significantly decreased in the infected group compared to the control after four weeks post infection. In comparison to the control, the number of isolated peritoneal macrophages of the infected groups was significantly increased over the eight weeks post infection. The inflammation intensely increases influx of blood monocytes as well as the local proliferation of tissue macrophages [31]. The percentage and size of adherent macrophages is superior in peritoneal leukocyte preparations obtained from infected animals than that from normal mice [28]. During inflammation, monocytes production is increased by expansion of the promonocyte pool, a decrease in cell cycle period, and release promptly into the circulation. Tissue macrophages arise by maturation of monocytes that have emerged from the blood by proliferation of immature macrophage in the resident macrophage population [32]. In addition, the correlation analysis revealed that the number of isolated peritoneal macrophages were significantly correlated with the NO production and non-significantly correlated with the O_2^- in a positive manner over the eight weeks post infection. On the other hand, there was a significant negative correlation between NO and O_2^- production in the isolated peritoneal macrophages over the

infection period. The initial inflammatory response to the migration of parasite in the skin of sensitized animals was accompanied by a significant increase in the number of infiltrating mononuclear cells and neutrophils. Along with the massive cellular infiltration, it has been reported that there was an increased tissue expression of inducible nitric oxide synthase mRNA [(33)]. The activation of inflammatory peritoneal macrophage cells as a result of Schistosoma mansoni infection and /or lipopolysaccharide induces the arginine-dependent production of NO which functions as the effector molecule of parasite killing [(34)]. After infection, the levels of inducible nitric oxide synthase mRNA is highly elevated in animals, suggesting that NO is being produced locally [(35)]. Plating the same number of peritoneal cells would result in relative crowding of activated macrophages and less efficient O_2^- elaboration [(28)]. The production of NO is independent on the respiratory burst in phagocytic leukocytes. In macrophages, both NADPH oxidase and inducible nitric oxide synthase was induced separately and independently regulated [(36)]. The reactivity of NO is more selective and specific than that of reactive oxygen intermediates [(21)].

Conclusion:

Our study indicated that the number of isolated peritoneal macrophages as well as the NO and O_2^- production were significantly increased over the prepatant period of Schistosoma manasoni infection. This increase revealed the importance of activated peritoneal macrophages as key immune effector cells in Schistosoma mansoni infection. Also, this increase evidenced that activated macrophages are capable of generating reactive nitrogen intermediates and reactive oxygen intermediates that enhance the ability of these cells to kill the parasite. The correlation analysis confirmed that the NO is the key effector molecule of the activated macrophages in controlling the Schistosomal infection. Although there was a significant increase in the number of peritoneal macrophages, but it was not proportional with the release of O_2^-. Moreover, the production of NO was independent on the respiratory burst.

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Table 1: Correlation between nitric oxide, superoxide anion and the number of peritoneal macrophage cells

Trait	Superoxide anion	No of macrophage cells
Nitric oxide	-0.034*	0.471**
Superoxide anion	-------	0.079

* P< 0.05. ** P< 0.01.

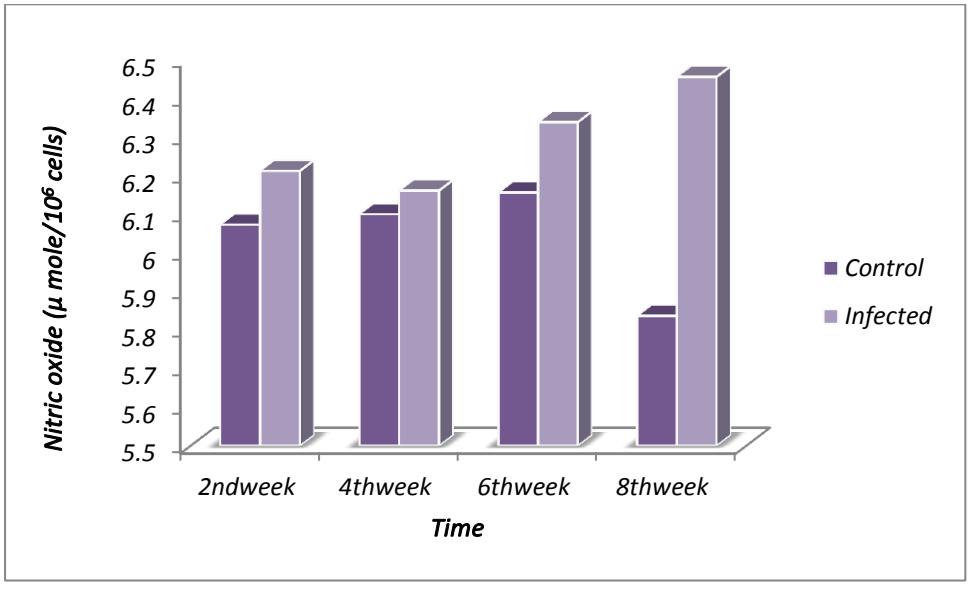

Figure (1): Production of nitric oxide by peritoneal macrophage cells under the effect of Schistosomal infection of mice.

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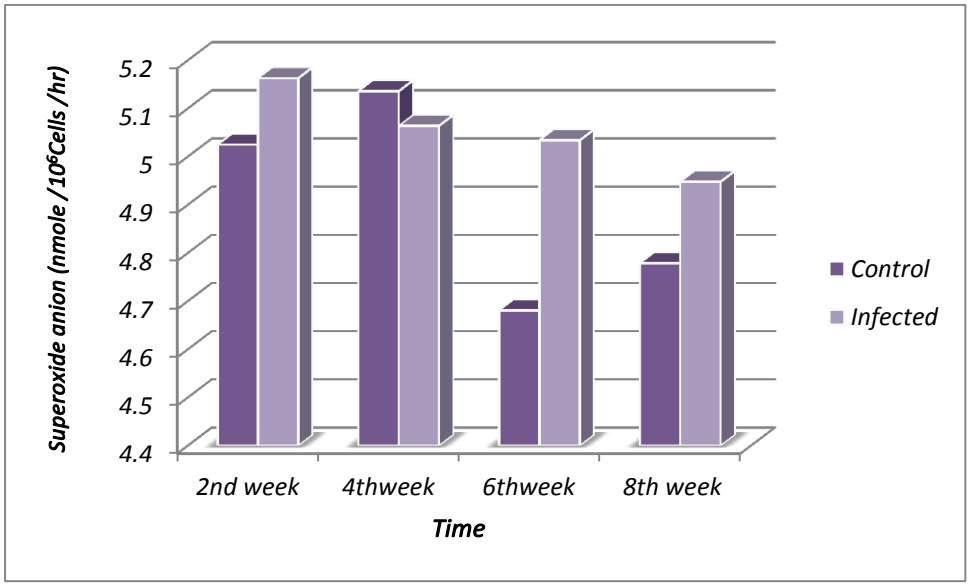

Figure (2): Production of superoxide anion by peritoneal macrophage cells under the effect of Schistosomal infection of mice.

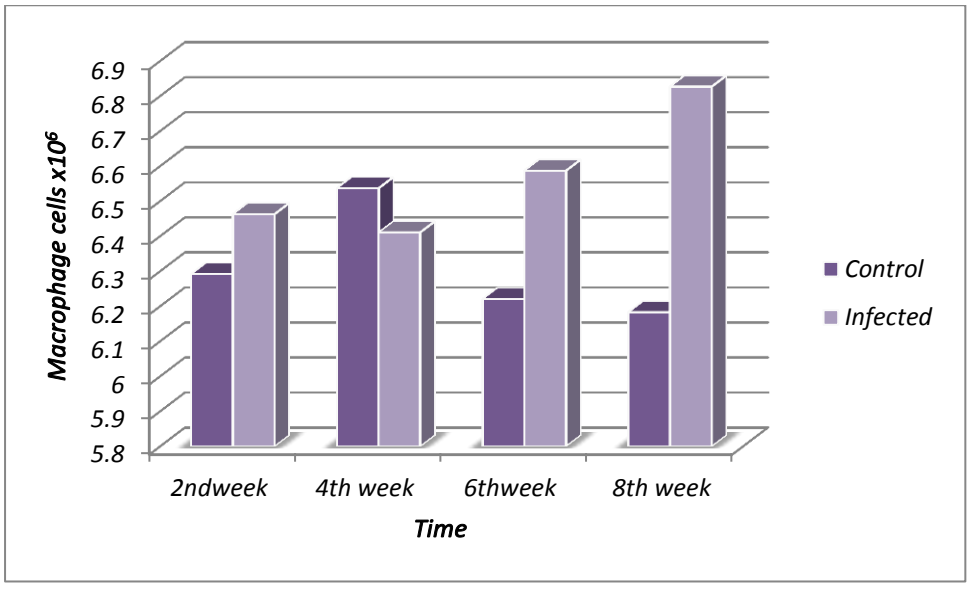

Figure (3): Number of peritoneal macrophage cells isolated from mice under the effect of Schistosomal infection