J Microbiol Immunol Infect
2005;38:164-168

Reactive nitrogen intermediate production and tolerance variability in different mouse strains after in vivo treatment with lipopolysaccharide from *Salmonella abortus equi*

Hossein Nahrevanian[1], Jafar Gholizadeh Salmasi[2], Mahin Farahmand[1], Zohreh Aghighi[1], Mehdi Assmar[1], Mohsen Abolhassani[3]

Departments of [1]Parasitology, [2]Education and [3]Immunology, Pasteur Institute of Iran, Tehran, Iran

Received: February 1, 2005 Revised: March 20, 2005 Accepted: March 28, 2005

Lipopolysaccharide (LPS) stimulation in animal models generates a large number of immune factors including cytokines and mediators. It also acts as a potent inducer of macrophage reactive oxygen intermediates and reactive nitrogen intermediates (RNI). RNI as stable metabolites of nitric oxide (NO) are produced by cells stimulated with LPS and cytokines. In this study, LPS from *Salmonella abortus equi* was investigated as an inducer of RNI in untreated controls and test groups of white Naval Medical Research Institute (NMRI) mice. Animals were humanely killed at 30, 60, 120 and 180 min after LPS injection, and plasma RNI was measured by Griess microassay. In a further experiment, host tolerance against bacterial LPS was evaluated by sequential intravenous injection of LPS concentrations of 4, 1 and 0.5 mg/kg at 24 h intervals in NMRI and with the same schedule but via subcutaneous injection in Balb/c mice. Statistical analysis of RNI values using analysis of variance test indicated that in vivo LPS stimulation induced high levels of NO in murine hosts (p<0.001). Comparison of RNI levels at different times after administration revealed the largest amount of RNI at 180 min after inoculation. Analysis of the time course until maximum RNI induction indicated that NMRI mice had the longest delay, suggesting a difference in tolerance of NMRI and Balb/c mice to LPS stimulation dependent on LPS concentration, dose, and route of inoculation.

Key words: Inbred BALB C mice, lipopolysaccharides, nitric oxide, reactive nitrogen species

Endotoxin, with its toxic principle lipopolysaccharide (LPS), forms the major component of the outer membrane of Gram-negative bacteria with molecular masses of 2-20 kDa. LPS molecules consist of a bisphosphorylated lipid (lipid A) forming the matrix of the outermost membrane leaflet and a hydrophilic polysaccharide [1]. LPS administration is generally carried out as a standard stimulus to induce inflammation or pyrexia in experimental animals [2], which also results in the clinical features of endotoxemia, including hypotension, metabolic acidosis, hyperglycemia and hyperkalemia [3].

Previous study demonstrated that intact LPS from various bacteria was able to bind specifically to macrophages and human monocytes by recognition receptors which initiate the innate immune response [1], and to modulate enzyme activity in rabbit endothelial cells as a result of detergent action [2]. Monocytes and macrophages are highly sensitive LPS targets; Langerhans and dendritic cells also respond to LPS stimulation as antigen presenting cells, which probably represents its differential roles in the innate immunity [4]. LPS has been reported to induce production of a number of immune factors including: nitric oxide (NO), tumor necrosis factor (TNF)-α, interferon (IFN)-γ and interleukin (IL)-1 in rat hepatocytes [3,5] and mouse macrophages [6,7]; reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) in balb/c mouse macrophages and hepatocytes [8]; NO synthase (NOS) isoforms in rat aortic smooth muscle cells [9]; and inducible NOS (iNOS) and NO_2^- in rat glomerular mesangial cells [10]. However, the duration and amount of induction by LPS was varied and conflicting among different studies [9,10].

Although its optimal production requires an additional signal by TNF-α, IFN-γ, IL-1 and LPS [11-13], IFN-γ is reported to have a critical role in the NO pathway. LPS stimulates macrophages to express iNOS by induction of endogenous IFN-γ [14], resulting in the time-dependent accumulation of NO [15]. In addition, NO production is regulated in vivo by T-helper 1-associated cytokines [16]; therefore, IFN-γ seems to

Corresponding author: Dr. Hossein Nahrevanian, Department of Parasitology, Pasteur Institute of Iran, Pasteur Avenue, Tehran 13164, Iran.
E-mail: mobcghn@yahoo.co.uk

be central in the regulation of TNF-α and NO production during infection [17].

Endotoxemia is a pathophysiologic process which involves multiple mediators and cytokines [18] and leads to inflammation and injury [19]. LPS is distributed by circulation and eliminated by immune mechanisms; however, the pathology might be mediated by soluble factors [20] and cytokines [21]. Exposure to LPS alone may lead to NO tolerance, but exposure to IFN-γ plus LPS may be more effective at inducing tolerance [22]. There are some common features of the different LPS/host combinations which have been reported.

Although the pathology of the host response to LPS is heterogeneous, little is known about the basis of this variation, and therefore the type of LPS and strain of the host are important determinants of the disease profile [1]. The objective of this study was to investigate the time course of RNI production as NO response after in vivo injection of LPS from *Salmonella abortus equi* in Naval Medical Research Institute (NMRI) white mice, and also to determine whether differences in the host genetic profile are responsible for tolerance variations against bacterial LPS by comparing responses in NMRI and Balb/c mice.

Materials and Methods

Animals

Animals used in this experiment were outbred NMRI white mice and inbred Balb/c mice (4-6 weeks). All animals were supplied by the Laboratory Animal Unit of the Pasteur Institute of Iran located in Karaj. Their body weight was 20.2 ± 0.6 g, when initially measured on day zero.

Ethical declaration

Animal experiments were carried out according to the ethical standards formulated in the Declaration of Helsinki, and measures taken to protect animals from pain or discomfort are mentioned.

LPS dose and RNI assessment times

LPS 10 mg from *Salmonella abortus equi* species (Sigma Chemical Co. UK) was dissolved in sterile, pyrogen-free 0.9% normal saline. After a single intravenous dose of LPS (0.5 mg/kg), NMRI white mice were humanely killed at 30, 60, 120 or 180 min after injection. The control group received no injection and 4 sets of mice killed at different times after LPS injection were considered as the test groups (n = 5). Mice were terminally anesthetized by inhalation of diethyl ether (BDH Analar® Co., UK) and blood was collected by cardiac puncture into a 1 mL syringe containing 0.05 mL (50 IU) heparin (Monoparin, CP Pharmaceuticals Ltd., UK). After blood collection, animals were humanely sacrificed by cervical dislocation. Plasma was prepared by centrifuging blood at 1500 relative centrifugal force (RCF) [MSE; Centaur 2 Co., UK] for 10 min, collected and stored at –70°C to measure RNI by Griess microassay.

Animal tolerance to LPS

In order to evaluate the tolerance variability in the different strains of mice, descending doses of LPS including 4, 1 and 0.5 mg/kg of body weight were injected sequentially by different routes including intravenous into the tail vein of the NMRI white mice (n = 16) and subcutaneously into the abdominal area of the Balb/c mice (n = 16). Different routes of injection (intravenous, subcutaneous) were selected based on previous data showing susceptibility of mice to LPS [23]. The number of surviving mice in each group was counted during the 24 h observational period after inoculation.

Griess microassay

The Griess reaction used for the assay of nitrite was adapted from previously described methods [24]. Standard curves for both sodium nitrite and sodium nitrate (Sigma Chemical Co.) were prepared. Sixty µL samples were treated with 10 µL nitrate reductase (NAD [P]H *Aspergillus* species 5 U/mL; Sigma Chemical Co.) and 30 µL NADPH β-nicotinamide adenine dinucleotide phosphate (1.25 mg/mL, Sigma Chemical Co.). 200 µL Griess reagent [5% phosphoric acid, 1% sulfanilic acid and 0.1% N-(1-naphthyl-1)-ethylenediamine dihydrochloride, all from Sigma Chemical Co., dissolved in 100 mL d.H_2O] was then added and proteins were subsequently precipitated by 200 µL trichloroacetic acid 10% (BDH, UK). Tube contents were vortex mixed then centrifuged at 13,400 RCF (Model 1-13 Microcentrifuge, Sigma, UK). Duplicate 200 µL samples of supernatants were transferred to a 96-well flat-bottomed microplate (Costar, USA) and absorbances read at 520 nm using a microplate reader (Dynatech, MRX, USA). Values for the concentration of nitrite assayed were calculated from standard calibration plots for $NaNO_2$ (sodium nitrite) and sodium nitrate ($NaNO_3$). The reaction was based on the formation of a chromophore to form a purple red compound. Standard curves were plotted for serial dilutions of both $NaNO_2$ and $NaNO_3$ diluted in

plasma versus related absorbances at 520 nm. Values for the concentration of nitrite assayed were calculated from standard calibration plots for $NaNO_2$ ($r^2 = 0.994$) and $NaNO_3$ ($r^2 = 0.997$) following nitrate reductase action [25].

Statistical analysis

Data for RNI (NO_2^- and NO_3^-) concentrations are presented as raw values for different mice. Data are also shown as mean ± standard error of the mean for levels of NO metabolites and compared using analysis of variance test with GraphPad Prism software (Prism-Software Incorporated, San Diego, California, USA). A p value <0.001 was considered statistically significant.

Results

In this study, induction of RNI by LPS was assessed at 30, 60, 120 and 180 min after intravenous injection into white NMRI mice. GMA revealed that in vivo stimulation with LPS was able to induce the production of high levels of RNI compared to untreated controls ($p<0.001$). Comparison of RNI at different time points after a single dose (0.5 mg/kg) of LPS showed that maximum RNI induction occurred at 180 min after stimulation (813.3 ± 124.1 µM, n = 5). This time course of the RNI response to LPS is shown in Fig. 1. RNI values at 120 and 60 min after LPS injection were 209.9 ± 25 and 188.2 ± 12.5 µM, respectively. No differences of RNI induction were observed after 30 min of LPS injection (101.8 ± 12.4 µM) when compared with untreated controls (101.7 ± 12.4 µM) [Fig. 1].

The greatest RNI levels were found at 180 min after injection in NMRI mice. This response delay from LPS administration to maximum induction, was the longest in this study. As a delay of more than 180 min was observed to be fatal for animals and resulted in severe toxic symptoms, animals were humanly killed no later than 180 min after injection to prevent suffering, according to the ethical standards formulated in the Declaration of Helsinki.

The tolerance to LPS was different between NMRI and Balb/c mice. The 2 routes of injection (intravenous or subcutaneous) were selected based on previous study showing greater relative susceptibility of mice to LPS using these routes [23]. Analysis of the survival rate during the 24 h after inoculation revealed 50% mortality including 8/16, 4/8 and 2/4 Balb/c mice after each 4, 1 and 0.5 mg/kg LPS injection, respectively. Surviving mice in each group were given the next LPS injection 24 h after the previous dose. At the end of the experiments, all 16 NMRI mice had survived after the 3 sequential intravenous injections (100% survival rate). However, the 14 Balb/c mice died even after subcutaneous injection of mentioned doses and only 2 mice tolerated all injections and survived the entire experimental course (12.5% survival rate) [Fig. 2].

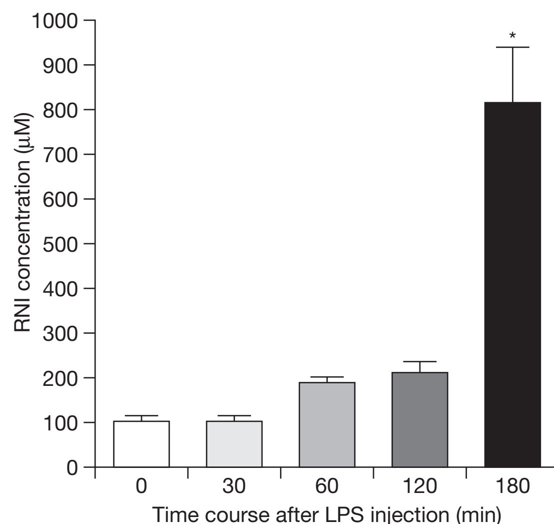

Fig. 1. Time course of nitric oxide induction after lipopolysaccharide (LPS) injection. In vivo reactive nitrogen intermediates (RNI) induction at 0, 30, 60, 120 and 180 min after intravenous injection of 0.5 mg/kg LPS in Naval Medical Research Institute mice as determined by Griess microassay. Significance of differences was determined using an analysis of variance test (*$p<0.001$, mean ± standard error of the mean, n = 5) using GraphPad Prism.

Discussion

Our results suggest the overproduction of mediators (e.g., NO) and cytokines (e.g., TNF) is toxic, but that the damage caused by this toxicity depends on the tolerance of the host. Our results also suggest that activation of mouse immune response by LPS was related to the dose and the time after administration.

In our previous study [26], the LPS dose of 0.5 mg/kg, an intravenous injection route and a time of 180 min after injection induced the greatest RNI production in MF1 mice, which supports the results of the current study. However, previous studies reported different results for several different doses, routes of injection and in different strains of mice [6,10,18,23, 27]. Our findings are in agreement with a previous study which demonstrated the level of NO induction by LPS varied depending on the drug vehicle, route of

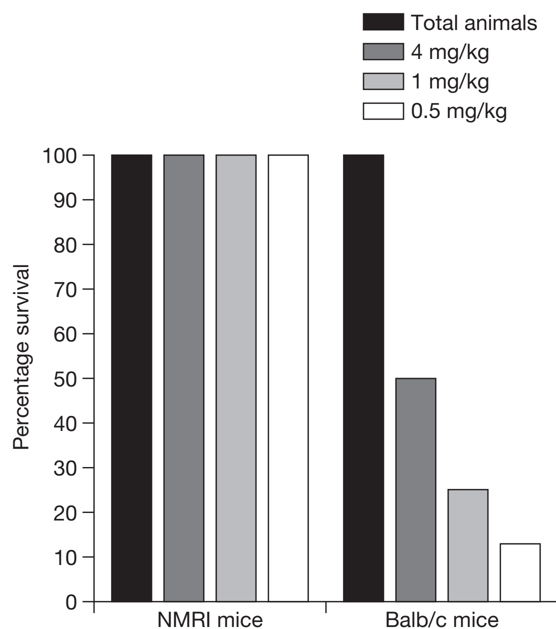

Fig. 2. Tolerance variability of Naval Medical Research Institute (NMRI) and Balb/c mice against lipopolysaccharide (LPS). Tolerance of mice to LPS after each subcutaneous LPS injection in Balb/c (n = 16) and intravenous injection in NMRI mice (n = 16) and percentage survival for all animals 24 h after each injection.

injection, type of NO inducers administered and strains of mice.

Data resulting from administration of several different types of NO donors and NO inducers showed NO donors generate NO alone, whereas NO inducers (e.g., LPS) produce other immune factors including cytokines such as TNF-α, IFN-γ and IL-1 in addition to NO [23]. Moreover, the time course and amount of NO release after treatment with NO inducers and NO donors was suggested to differ between in vivo and in vitro applications, with a shorter time course and lower amount of NO release in vivo than in vitro [28]. This might be because other host immune mechanisms may interfere and interact with the NO pathway to remove and scavenge its metabolites from the body.

In this study, the tolerance to LPS varied between NMRI and Balb/c mice using intravenous or subcutaneous routes of injection. Comparison of the survival rate at the entire experimental course revealed a remarkable tolerance variation between these 2 mice strains to LPS inoculation (12.5% in Balb/c and 100% in NMRI mice). Tolerance to LPS was induced via high-dose injection and followed by sequentially decreasing doses. Bacterial LPS acts on macrophages to release chemokines; at high levels, these chemokines may produce the syndrome of septic shock. By contrast, low quantities of released cytokines have only local effects, whereas with moderate quantities, systemic effects can be detected [29]. Our observation suggested that tolerance to high-dose LPS as a first preliminary injection was greater by healthy normal mice.

In conclusion, this study found clear differences in the tolerance of NMRI and Balb/c mice to LPS. The high death rate after each subcutaneous injection in Balb/c mice and the survival of all NMRI mice after intravenous injection suggested variability in the tolerance of bacterial LPS. Balb/c mice showed higher susceptibility than NMRI mice to LPS injection, despite subcutaneous administration, which releases LPS more slowly into the circulation. This could be associated with the genetic profiles of host strains leading to different responses against bacterial LPS. The differences in tolerance depended on the dose, route of injection and strain of mice. However, the immune response to specific antigens is complex and dependent on various factors, and needs to be clarified by complementary studies.

Acknowledgment

We thank Dr N Namvar Asl from Karaj Laboratory Animal Unit, Pasteur Institute of Iran for kind provision of animals.

References

1. Caroff M, Karibian D, Cavaillon J-M, Haeffner-Cavaillon N. Structural and functional analyses of bacterial lipopolysaccharides. Microbes Infect 2002;4:915-26.
2. Conforto A, Dudek R, Hoffmann MR, Bing RJ. The production of nitric oxide in endothelial cells by amphiphiles. Life Sci 1994;54:1143-53.
3. Asari Y, Majima M, Sugimoto K, Katori M, Ohwada T. Release site of TNF alpha after intravenous and intraperitoneal injection of LPS from *Escherichia coli* in rats. Shock 1996;5: 208-12.
4. Tada Y, Asahina A, Fujita H, Mitsui H, Torii H, Watanabe T, et al. Differential effects of LPS and TGF-β on the production of IL-6 and IL-12 by Langerhans cells, splenic dendritic cells and macrophages. Cytokine 2004;25:155-61.
5. Geller DA, Nussler AK, Di Silvio M, Lowenstein CJ, Shapiro RA, Wang SC, et al. Cytokines, endotoxin and glucocorticoids regulate the expression of inducible nitric oxide synthase in hepatocytes. Proc Natl Acad Sci USA 1993;90:522-6.
6. Jacobs P, Radzioch D, Stevenson MM. Nitric oxide expression in the spleen, but not in the liver, correlates with resistance to

blood-stage malaria in mice. J Immunol 1995;155:5306-13.
7. Mizutani A, Maki H, Torii Y, Hitomi K, Tsukagoshi N. Ascorbate-dependent enhancement of nitric oxide formation in activated macrophages. Nitric Oxide 1998;2:235-41.
8. Mellouk S, Green SJ, Nacy CA, Hoffman SL. IFN-gamma inhibits development of *Plasmodium berghei* exoerythrocytic stages in hepatocytes by an L-arginine-dependent effector mechanism. J Immunol 1991;146:3971-6.
9. Paul A, Bryant C, Lawson MF, Chilvers ER, Plevin R. Dissociation of lipopolysaccharide-mediated induction of nitric oxide synthase and inhibition of DNA synthesis in RAW 264.7 macrophages and rat aortic smooth muscle cells. Br J Pharmacol 1997;120:1439-44.
10. Shultz PJ, Archer SL, Rosenberg ME. Inducible nitric oxide synthase mRNA and activity in glomerular mesangial cells. Kidney Int 1994;46:683-9.
11. Vyas P, Attur M, Ou GM, Haines KA, Abramson SB, Amin AR. Thin layer chromatography: an effective method to monitor citrulline synthesis by NOS activity. In: Moncada S, Stamler J, Gross S, Higgs EA, eds. The biology of nitric oxide. London: Portland Press Ltd; 1996:44.
12. Xie QW, Whisnant R, Nathan C. Promoter of the mouse gene encoding calcium-independent nitric oxide synthase confers inducibility by interferon gamma and bacterial lipopolysaccharide. J Exp Med 1993;177:1779-84.
13. Nussler AK, Di Silvio M, Billiar TR, Hoffman RA, Geller DA, Selby R, et al. Stimulation of the nitric oxide synthase pathway in human hepatocytes by cytokines and endotoxin. J Exp Med 1992;176:261-4.
14. Bogdan C, Rollinghoff M, Diefenbach A. Reactive oxygen and reactive nitrogen intermediates in innate and specific immunity. Curr Opin Immunol 2000;12:64-76.
15. Archer SL, Freude KA, Shultz PJ. Effect of graded hypoxia on the induction and function of inducible nitric oxide synthase in rat mesangial cells. Circ Res 1995;77:21-8.
16. Jacobs P, Radzioch D, Stevenson MM. In vivo regulation of nitric oxide production by tumor necrosis factor alpha and gamma interferon, but not by interleukin-4, during blood stage malaria in mice. Infect Immun 1996;64:44-9.
17. Yoshimoto T, Takahama Y, Wang CR, Yoneto T, Waki S, Nariuchi H. A pathogenic role of IL-12 in blood-stage murine malaria lethal strain *Plasmodium berghei* NK65 infection. J Immunol 1998;160:5500-5.
18. Liu S, Adcock IM, Old RW, Barnes PJ, Evans TW. Lipopolysaccharide treatment in vivo induces widespread tissue expression of inducible nitric oxide synthase mRNA. Biochem Biophys Res Commun 1993;96:1208-13.
19. Diehl AM. Cytokine regulation of liver injury and repair. Immunol Rev 2000;174:160-71.
20. Clark IA. Does endotoxin cause both the disease and parasite death in acute malaria and babesiosis? Lancet 1978;2:75-7.
21. Jakobsen PH, Bate CA, Taverne J, Playfair JH. Malaria: toxins, cytokines and disease. Parasite Immunol 1995;17: 223-31.
22. Liew FY. Regulation of nitric oxide synthase in macrophages. In: Moncada S, Stamler J, Gross S, Higgs EA, eds. The biology of nitric oxide; 2 Enzymology, biochemistry and immunology. London: Portland Press; 1992:223-9.
23. Dascombe MJ, Nahrevanian H. Pharmacological assessment of the role of nitric oxide in mice infected with lethal and nonlethal species of malaria. Parasite Immunol 2003;25: 149-59.
24. Rockett KA, Awburn MM, Rockett EJ, Cowden WB, Clark IA. Possible role of nitric oxide in malarial immunosuppression. Parasite Immunol 1994;16:243-9.
25. Nahrevanian H, Dascombe MJ. Nitric oxide and reactive nitrogen intermediates during lethal and nonlethal strains of murine malaria. Parasite Immunol 2001;23:491-501.
26. Nahrevanian H, Dascombe MJ. Expression of inducible nitric oxide synthase (iNOS) mRNA in target organs of lethal and non-lethal strains of murine malaria. Parasite Immunol 2002; 24:471-8.
27. Hom GJ, Grant SK, Wolfe G, Bach TJ, Maclntyre DE, Hutchinson NJ. Role of inducible nitric oxide synthase in septic shock-induced hypotension and vascular hyporeactivity in rat: tissue analysis of nitric oxide synthase mRNA and protein expression in the presence & absence of dexamethasone, N^G-MMLA or indomethacin. In: Moncada S, Stamler J, Gross S, Higgs EA, eds. Biology of nitric oxide. London: Portland Press; 1996:268-72.
28. Balmer P, Phillips HM, Maestre AE, McMonagle FA, Phillips RS. The effect of nitric oxide on the growth of *Plasmodium falciparum*, *P. chabaudi* and *P. berghei* in vitro. Parasite Immunol 2000;22:97-106.
29. Abbas AK, Lichtman AH, Pober JS. Effector mechanisms of immune responses. In: Abbas AK, Lichtman AH, Pober JS, eds. Cellular and molecular immunology. 3rd ed. USA: W.B. Saunders Company; 1997:254-391.