ELSEVIER

FEMS Immunology and Medical Microbiology 25 (1999) 385–389

FEMS IMMUNOLOGY AND MEDICAL MICROBIOLOGY

Nitric oxide participates in the immune response against *Neisseria meningitidis* serogroup B

Julio Padrón *, Yanín Bebelagua, Miriam Lastre, José Lapinet, Caridad Zayas, Yanet Quintero, Miriam Diaz, Oliver Pérez

Research Div. Dept. Immunol. Finlay Inst., POB 16017, Cod 11600, Havana, Cuba

Received 1 April 1999; accepted 7 April 1999

Abstract

The present report explores the role of nitric oxide into the immune response against *Neisseria meningitidis* serogroup B. Here we show that NO mediates the αTNF increase induced by *N. meningitidis* derived lipopolysaccharides (LPS), at the same time that participates in the bactericidal activity of resting or γIFN activated macrophages and plays a role in the specific DTH and IgG response induced by a commercial anti-meningococcal vaccine. Our findings suggest a positive role for NO at the final effector mechanisms and in the early events driving the immunity against *N. meningitidis*, suggesting also an insight into its role in endotoxic shock.

Keywords: Nitric oxide; *Neisseria meningitidis*; Immune response

1. Introduction

Neisseria meningitidis (Nm) is one of the major causes of meningococcal disease in children [1]. High mortality commonly associated with meningitis is closely related with the establishment of endotoxic shock [2].

Outer membrane derived lipopolysaccharides (LPS) from Gram-negative bacteria are longly recognised as principal inducers of endotoxic shock [3]. In the same way, αTNF is widely accepted as one of the first signals driving the occurrence of death due to endotoxemia [4]. αTNF-induced severe hypotension and death was actually due to nitric oxide (NO) induction [5]. However, it should be noted as well that NO plays a crucial role in the antimicrobial response against a broad spectrum of pathogens [6]. Therefore, reasonable therapeutic strategies in this field are still requiring further understanding in the balance of all actions of NO in sepsis and endotoxic shock.

Generation of new effective vaccines, designed on the basis of the appropriate immune response according to each pathogen, are still in course. For many years vaccines came from screening procedures looking for antibodies. Today it is perfectly known that there exist two major types of immune response

* Corresponding author. Current address: Pharmacol. Dept., Lab. D21, Faculty of Medicine, UAM, c/Arzobispo Morcillo 4, Madrid 28029, Spain. Fax: +34 (91) 397 5353; E-mail: j.padron@uam.es

0928-8244/99/$20.00
PII: S0928-8244(99)00113-3

[7], and in some cases, to induce humoral response is not only ineffective, but deleterious [8].

Here the role of NO at different steps of the anti-Nm immune response was studied. First, its role was explored, not as a final effector molecule, but as an early mediator of LPS-induced αTNF induction. Next, as well its participation in the anti-Nm bactericidal activity of murine macrophages was tested. Finally, its participation in the primary events driving the establishment of anti-Nm specific humoral and cellular immune response induced by the commercial anti-meningococcal vaccine, VA-MENGOC-BC® was also studied.

2. Materials and methods

The *N. meningitidis* serogroup B (strain No. 385-83), its outer membrane derived proteoliposome, its purified LPS and the anti-meningococcal vaccine (VA-MENGOC BC®), were all obtained at the Finlay Institute, Havana, Cuba. N^G-monomethyl-L-arginine was kindly supplied by Dr. S. Moncada. All other reagents, unless specified, were purchased from Sigma.

2.1. Induction of αTNF

Induction of αTNF was studied on a described in vitro model of human whole blood culture [9]. Blood samples (5 ml) from three voluntary donors were separately diluted 1 in 5 with RPMI 1640 to be treated with Nm derived LPS (1 μg ml^{-1}) in presence (500 μM) or absence of LNMMA. αTNF induction was also studied in mice pre-treated or not with LNMMA (20 mg kg^{-1}, i.p.), and challenged with Nm derived LPS (50 μg kg^{-1}, i.v.). Cell culture supernatant and blood samples were respectively collected at different times and tested for αTNF levels by the reported L929 bioassay [10].

2.2. Induction of DTH and specific IgG levels

Induction of DTH and specific IgG levels were studied on 18–20 g and 7 weeks old Balb/c mice randomised in three groups of ten animals each. The first group was not immunised, the second was inoculated at day 0 and 35 with VA-MENGOC BC® (250 μg, i.m.), meanwhile the third group was also immunised as scheduled for group 2 but 30 min before each immunisation the mice received a single dose of LNMMA (10 mg kg^{-1}, i.p.). Finally, 56 days after the first immunisation, DTH was evaluated by specific food-pad swelling [11] 48 h after the injection of Nm derived proteoliposome (50 μg). At this point, samples of blood were obtained to evaluate the serum specific anti-proteoliposome IgG response by ELISA.

2.3. Coculture assay

Coculture assay was carried out on 96 flat well plates, using 4% (w/v) thioglycolate elicited murine peritoneal macrophages [12] plated at 2×10^5 cells/well in the presence of 2×10^3 bacteria. Two h before the addition of the suspension of bacteria, macrophages were pre-treated with LNMMA (500 μM) and/or γIFN (10 μU ml^{-1}), or left untreated as controls. Aliquots of cell culture supernatant fluid, after 24 h of coculture at 37°C and 5% CO_2, were sequentially diluted and plated (10 μl) on Franz-Agar medium in order to finally calculate the total number of colony forming units [13].

The study of the in vitro stimulatory action of the *N. meningitidis* derived proteoliposome on NO production was also carried on 4% thioglycolate elicited murine peritoneal macrophages. Cells were plated at 10^6 cells ml^{-1} and incubated, for 24 h at 37°C under 5% CO_2, with γIFN (1 μU ml^{-1}) in the presence of Nm derived proteoliposome. The Griess reaction [14] was used to quantify nitrites as an index of NO production.

The data presented represent the mean and standard errors of at least triplicate samples from three separate experiments. Student's *t*-test statistical difference was accepted for $*P<0.05$ or $**P<0.01$.

3. Results

In the first case (Fig. 1), inhibition of the NO pathway with LNMMA in the in vitro model of human whole blood culture leads to a significant diminution in the time-curve profile of Nm derived LPS-induced increase in αTNF levels. Consistently, even more drastic inhibition was observed (Fig. 2) in

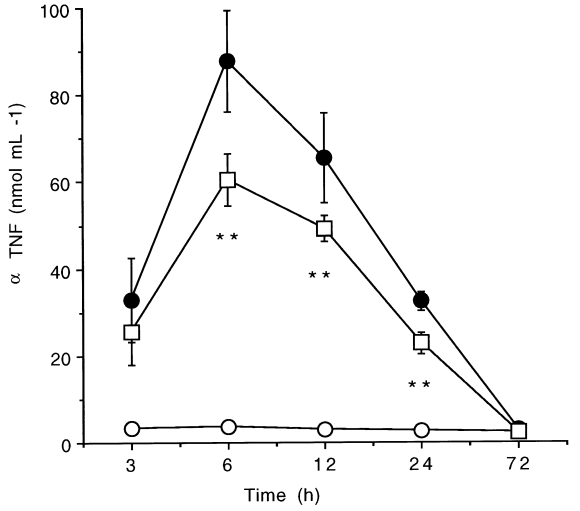

Fig. 1. Effect of LNMMA (500 μM) on LPS (1 μg ml^{-1}) induced αTNF increase in human whole blood at different times of culture (open square). For comparison, control groups of non-treated (open circles) and LPS-treated without LNMMA (closed circles) were also assayed. Shown data represent mean and standard error from three different experiments. Student's *t*-test statistical difference was accepted for $^{**}P < 0.01$.

the in vivo murine model of αTNF induction after 1 h of Nm derived LPS challenge.

Competitive inhibition of NO production with LNMMA produced a significant increase in the

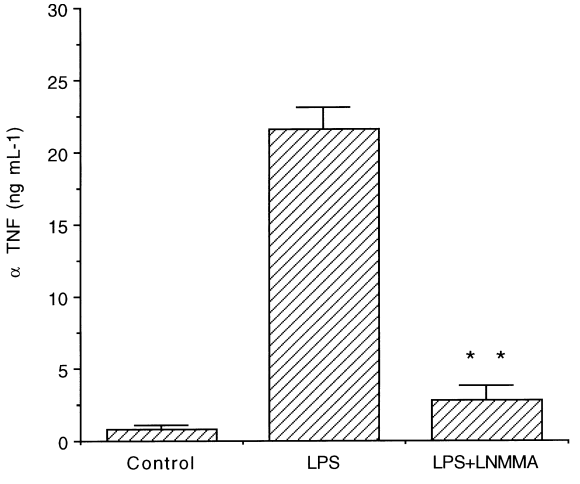

Fig. 2. Effect of LNMMA (20 mg kg^{-1}) on serum αTNF increase in mice after 1 h with challenge LPS (50 μg kg^{-1}). Shown data represent mean and standard error from 10 animals in three different experiments. Student's *t*-test statistical difference was accepted for $^{**}P < 0.01$.

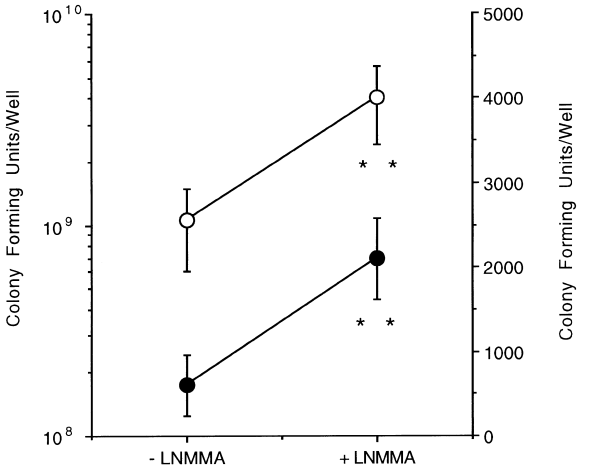

Fig. 3. Effect of LNMMA (500 μM) on the number of *N. meningitidis* colony forming units after 24 h of coculture with resting (open circles, for the left axis) or γIFN (10 μU ml^{-1}) activated murine peritoneal macrophages (closed circles, for the right axis). Shown data represent mean and standard error from three different experiments. Student's *t*-test statistical difference was accepted for $^{**}P < 0.01$.

number of colony forming units after 24 h of *N. meningitidis* coculture with either resting or γIFN activated macrophages (Fig. 3).

Additionally, murine in vivo inhibition of the NO pathway during the first hours of immunisation with an effective vaccine against *N. meningitidis* (VA-MENGOC-BC®) [13,17], leads to a significant decrease in both; the specific anti-Nm DTH response and the anti-Nm IgG level (Fig. 4).

Finally (Fig. 5), the Nm outer membrane derived proteoliposome, which constitute the essential antigenic component of VA-MENGOC-BC®, was able to potentiate the in vitro production of nitrites from murine peritoneal macrophages.

4. Discussion

As mentioned above, LPS-induced endotoxic shock has been related to a very early and transient increase in αTNF levels [4], which in turn leads to a sustained high output production of NO as a final mediator of severe hypotension and death [5]. However, the present evidence (Figs. 1 and 2), in agreement with previous reports [10,15], confirms that NO might play also a role in the signal transduction

mechanisms for αTNF induction in response to Nm derived LPS.

NO is an effector molecule from the innate immunity with a broad spectrum of action against bacteria, viruses, tumours and fungus [6]. However, all microorganisms are not equally affected [16]. In the case of *N. meningitidis* serogroup B, present results (Fig. 3) suggest that NO is indeed an important final effector mechanism from resting or γIFN activated macrophages.

Contrary to the general agreement in regard to the role of NO as a final effector mechanism of the immune system [18], much less is known about its role in the early steps driving the development of the two principal types of immune response. NO induction by different experimental adjuvants correlates with a preferential induction of the TH1 cellular immune response [19]. However, high NO production, resulted from the TH1 response, might function as a negative feedback mechanism for switching the immune system toward the TH2 antibody production [20]. In this study, inhibition of NO pathway during the early steps of anti-Nm immunisation may produce a significant decrease of both the cellular and the humoral response (Fig. 4). Taken together with previous reports, it would be possible to speculate that inhibition of NO might block the final mechanisms involved in a DTH response at the same time that suppresses the switching mechanism leading to a TH2 response.

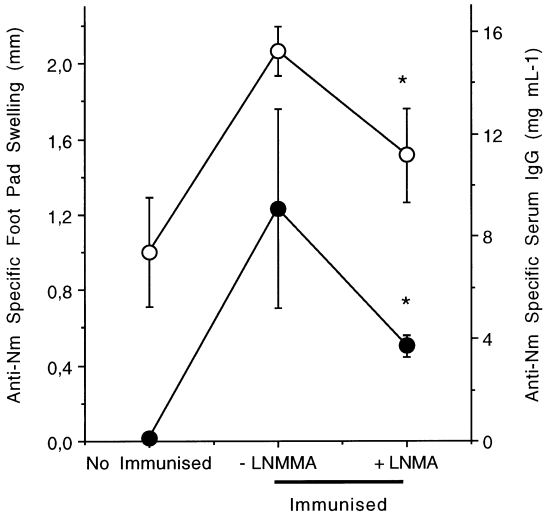

Fig. 4. Effect of LNMMA (10 mg kg^{-1}), administered 30 min before each immunisation with VA-MENGOC-BC® (250 μg) at day 0 and 35, on specific anti-*N. meningitidis* DTH (open circle, for the left axe) and IgG response (closed circles, for the right axe). Shown data represent mean and standard error from 10 animals. Student's *t*-test statistical difference was accepted for $*P < 0.05$.

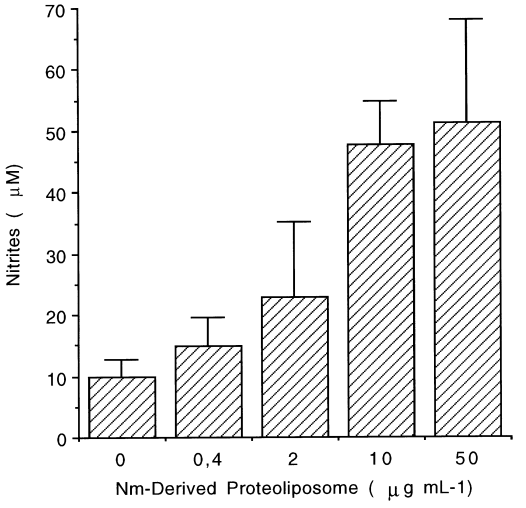

Fig. 5. Effect of *N. meningitidis* derived proteoliposome on nitrite production from murine peritoneal macrophages in presence of γIFN (1 μU ml^{-1}). Shown data represent mean and standard error from three separated experiments.

Results from Fig. 5 are not only in accordance with previous results, but also might give new approach to the mechanisms of adjuvancy of VA-MENGOC-BC®. Further studies would be required, but absence of correlation observed years ago between serum antibodies and protective efficacy [21], might be explained by more recent finding [22] which, in agreement with our present study, might suggest a concomitant and perhaps more appropriate development of a TH1 immune response.

The present report illustrates some of the complex roles of NO in the immune response against *N. meningitidis*.

In the first place it is shown that NO might play a deleterious role on the induction of αTNF due to Nm derived LPS, which in turn is known to initiate the establishment of endotoxic shock. However, at the same time, it is also shown that NO may play a beneficial role for macrophages anti-Nm bactericidal activity, so that a potential therapeutic manipulation of the NO pathway during sepsis and/or en-

dotoxic shock should keep in account those very close and controversial roles. Depending on the precise momentum, level and compartment, NO might play a protective or a pathological role. Recent reports suggest that also in humans NO may be playing a significant role in the bacterial meningococcal disease [23]. However, to understand the complexity of all actions of NO in this regard further studies would be required.

In the second place, here it is suggested that manipulation of NO pathway might be also important at the early steps of the immunisation against *N. meningitidis*. In this sense, new strategies for the screening of potential new adjuvants, and for accurate evaluation index of efficacy should be explored.

References

[1] Hoen, B. (1994) Epidemiology of primary bacterial meningitis. Rev. Prat. 44, 2148–2151.

[2] Kornelisse, R.F., Hazelzet, J.A., Hop, W.C., Spanjaard, L., Suur, M.H., Van der Voort, E. and de Groot, R. (1997) Meningococcal septic shock in children: clinical and laboratory features, outcome, and development of a prognostic score. Clin. Infect. Dis. 25, 640–646.

[3] Nishijima, H., Weil, M.H., Shubia, H. and Canilles, J. (1973) Hemodynamic and metabolic studies on shock associated with gram negative bacteremia. Medicine 52, 287–294.

[4] Cerami, A., Ikeda, Y., Le Trang, N., Hotez, P. and Beutler, B. (1985) Weight loss associated with an endotoxin induced mediator from peritoneal macrophages; the role of cachectin (tumour necrosis factor). Immunol. Lett. 11, 173–177.

[5] Kilbourn, R.G., Jubran, A. and Gross, S.S. (1990) Reversal of endotoxin-mediated shock by NG-Methyl L-Arginine, an inhibitor of NO Synthesis. Biochem. Biophys. Res. Commun. 172, 1132–1138.

[6] De-Groote, M.A. and Fang, F.C. (1995) Nitric oxide inhibitors: antimicrobial properties of NO. Clin. Infect. Dis. 2, 162–165.

[7] Lucey, D.R., Clerici, M. and Shearer, G.M. (1996) Type 1 and type 2 cytokine dysregulation in human infectious, neoplastic and inflammatory diseases. Clin. Microbiol. Rev. 9, 532–562.

[8] Nabors, G.S., Afonso, L.C., Farrell, J.P. and Scott, P. (1995) Switch from a type 1 T helper cell response and cure of established Leishmania major infection in mice is induced by combined therapy with interleukin-12 and Pentostam. Proc. Natl. Acad. Sci. USA 92, 3142–3146.

[9] Johnson, K., Aarden, L., Choi, Y., De-Groot, E. and Creasey, A. (1996) The proinflammatory cytokine response to coagulation and endotoxin in whole blood. Blood 87, 5051–5060.

[10] Rojas, A., Padron, J., Caveda, L., Palacios, M. and Moncada, S. (1993) Role of nitric oxide pathway in the protection against lethal endotoxemia afforded by low doses of lipopolysaccharides. Biochem. Biophys. Res. Commun. 191, 441–446.

[11] Singh, A.P. and Khuller, G.K. (1994) Induction of immunity against experimental tuberculosis with mycobacterial mannophospho-inositides encapsulated in liposomes containing lipid A. FEMS Immunol. Med. Microbiol. 8, 119–126.

[12] Rojas, A., Caveda, L., Romay, C., Lopez, E., Valdes, S., Padron, J., Glaria, L., Martinez, O. and Delgado, R. (1996) Effect of advanced glycosylation end products on the induction of NOS in murine macrophages. Biochem. Biophys. Res. Commun. 225, 358–362.

[13] Sifontes, S., Infante, J.F., Perez, P., Caro, E., Sierra, G. and Campa, C. (1997) The hyperferremic mouse model for the evaluation of the effectiveness of VA-MENGOC-BC against *Neisseria meningitidis* B clinical isolates. Arch. Med. Res. 28, 41–45.

[14] Padron, J., Glaria, L., Martinez, O., Torres, M., López, E., Delgado, R., Caveda, L. and Rojas, A. (1996) Nitric oxide modulates interleukin-2 induced proliferation in CTLL-2 cells. Mediat. Inflamm. 5, 324–327.

[15] Zinetti, M., Fantuzzi, G. and Delgado, R. (1995) Endogenous nitric oxide production by human monocyte cells regulates LPS-induced TNF production. Eur. Cytokine Netw. 6, 45–48.

[16] Akaike, T., Noguchi, Y., Ijiri, S., Setoguchi, K., Suga, M., Zheng, Y.M., Dietzschold, B. and Maeda, H. (1996) Pathogenesis of influenza virus-induced pneumonia: involvement of both nitric oxide and oxygen radicals. Proc. Natl. Acad. Sci. USA 93, 2448–2453.

[17] Noronha, C.P., Struchiner, C.J. and Halloran, M.E. (1995) Assessment of the direct effectiveness of BC meningococcal vaccine in Rio de Janeiro, Brazil: a case-control study. Int. J. Epidemiol. 24, 1050–1057.

[18] Nathan, C. (1997) Perspectives series: What difference does it make. J. Clin. Invest. 100, 2417–2423.

[19] Phillips, N.C. and Gagne, L. (1995) Modulation of murine macrophages NO synthesis by liposomal phospholipids: correlation with liposome immune adjuvant activity. J. Drug Target 3, 137–147.

[20] Taylor-Robinson, A.W., Liew, F.Y., Severn, A., Xu, D., McSorley, S.J., Garside, P., Padron, J. and Phillips, R.S. (1994) Regulation of the immune response by NO differentially produced by T helper type 1 and type 2 cells. Eur. J. Immunol. 24, 980–984.

[21] Milagres, L.G., Ramos, S.R. and Sacchi, C.T. (1994) Immune response of Brazilian children to a *Neisseria meningitidis* serogroup B outer membrane protein vaccine: comparison with efficacy. Infect. Immun. 62, 4419–4424.

[22] Kornelisse, R.F., Hack, C.E., Savelkoul, H.F., van der Pouw-Kraan, T.C., Hop, W.C., van Mierlo, G., Suur, M.H., Neijens, H.J. and de Groot, R. (1997) Intrathecal production of interleukin-12 and gamma interferon in patients with bacterial meningitis. Infect. Immun. 65, 877–881.

[23] Kornelisse, R.F., Hoekman, K., Visser, J.J., Hop, W.C., Huijmans, J.G., van der Straaten, P.J., van der Heijden, A.J., Sukhai, R.N., Neijens, H.J. and de Groot, R. (1996) The role of nitric oxide in bacterial meningitis in children. J. Infect. Dis. 174, 120–126.