American Journal of Immunology 1 (4): 148-153, 2005
ISSN 1553-619X

In vitro Activation of Human and Rat Lymphocyte and Macrophage Functions by Norepinephrine

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Abstract: Norepinephrine (NE) has been associated not only with increasing blood pressure, atherosclerosis, heart disease, and other life threatening conditions, but also with altering immune responses by influencing leukocyte functions. In the present study, we evaluated the *in vitro* effects of NE on rat thymic lymphocyte and human peripheral blood mononuclear cells (HPBMC) functions. We observed that NE marginally, but significantly ($P < 0.01$) enhanced (1.3-fold increase) proliferation of rat thymic lymphocytes at 10^{-5} M, without altering HPBMC proliferation, as compared with untreated control. In addition, NE (10^{-5} M) significantly ($P < 0.01$) enhanced nitric oxide production (2.9 ± 0.095 nmol/well) (which reduced 20% cell viability), and stimulated ($P < 0.01$) TNF-α production (4 ± 0.16 pg/ml) by rat macrophages. NE (10^{-5} M) was also observed to induce 2-fold increase in mRNA signal of TNF-α, and stimulated that of IL-1 and IL-6 by HPBMC, as compared with untreated control. Taken together, these results indicated that NE was capable to activate *in vitro* rat and human lymphocyte and macrophage pro-inflammatory response.

Key words: Norepinephrine, immunomodulation, proliferation, nitric oxide, TNF-α, inflammatory cytokines, lymphocytes, macrophages, rat, human.

INTRODUCTION

It is well recognized that during an immune response, homeostasis is maintained by the interaction between the brain and the immune system[1], and the consequent sharing of cytokines, peptide hormones, neurotransmitters, and receptors for these molecules[2]. It is then important to understand the bi-directional communication and regulation of the immune and nervous systems. Norepinephrine (NE) is the principal neurotransmitter released from sympathetic nervous system[3], associated with regulation of autonomic activity within the body by increasing metabolism, increasing heart rate and respiration, dilating the pupils, increasing perspiration, and improving the flow of oxygen to the major muscle groups[4]. However, chronic NE release commonly relates to life threatening conditions such as high blood pressure, atherosclerosis, heart disease, and other[5]. Release of NE from the sympathetic nerve terminals within lymphoid organs, and the presence of adrenoreceptors on lymphocytes and macrophages, suggest an additional pathway of immunoregulation by the central nervous system[6,7]. Adrenergic agonists have been shown to regulate function of immune cells, leading to alteration of cytokine production, lymphocyte proliferation, lymphocyte traffic, and antibody secretion[8-10].

The present study was designed to evaluate the effects of NE on rat and human lymphocyte proliferation, and macrophage inflammatory activities following *in vitro* stimulation. We found that NE stimulated pro-inflammatory parameters of leukocyte functions.

MATERIALS AND METHODS

Reagents, culture media, and cell lines: Penicillin-streptomycin solution, L-glutamine, ficoll-hypaque solution, trypsin-EDTA solution, and RPMI 1640 and AIM-V media were obtained from Life Technologies (Grand Island, NY). Norepinephrine, fetal bovine serum (FBS), concanavalin A (Con A), phytohemaglutinin (PHA), lipopolysaccharide (LPS) from *Escherichia coli* serotype 026:B6, sodium dodecyl sulfate (SDS), N,N-dimethylformamide (DMF), dimethyl sulfoxide (DMSO), PBS, and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO). The murine fibrosarcoma L929 (clone CCL 1) was purchased from the American Type

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Culture Collection (Rockville, MD) and was maintained in RPMI 1640 medium supplemented with 10% FBS, 1% L-glutamine, and 0.5% penicillin-streptomycin solution (referred as complete RPMI 1640 medium). Extraction buffer was prepared by dissolving 20% (wt/vol) SDS at 37oC in a solution of 50% each DMF and demineralized water, and the pH was adjusted to 4.7.

Animals: Sprague-Dawley male rats (200-220g) were purchased from Harlan Sprague-Dawley Inc. (Indianapolis, IN). They were kept in a pathogen- and stress-free environment at 240C, under a light-dark cycle (light phase, 06:00-18:00 h), and given water and food *ad libitum*. Animals were euthanized by asphyxiation in 100% CO2 chamber.

Cell preparation and culture: Thymus was immediately removed after rat death. Single-cell suspensions were prepared by disrupting the thymus in RPMI 1640 medium. Cell suspensions were then washed three times in this medium, and suspended and adjusted to 1 x 10^7 cells/ml with AIM-V medium. The culture medium was changed at this step to the serum-free medium AIM-V which has been observed to support cell culture[11]. Peritoneal macrophages were prepared by lavaging the peritoneal cavity with cold RPMI 1640 medium, and washing the resulting cell suspension twice in this medium. One hundred-microliter cell suspensions at 1.7 x 10^6 cells/ml in AIM-V medium were then plated in flat-bottomed 96-well plates (Becton Dickinson, Lincoln Park, NJ) for 2 h at 37°C. Non-adherent cells were removed, and adherent cells (about 70% of the input cells or about 1 x 10^6 cells/ml) were then utilized for determining nitric oxide and tumor necrosis factor-alpha (TNF-α) production. The final adherent cell monolayer consisted of 95-99% macrophages as judged by Giemsa's stain procedures.

T cell proliferation assay: T cell proliferation was determined by a colorimetric technique using MTT[12]. Thymic cell suspensions (100 μl) were added to flat-bottomed 96-well plates (Becton Dickinson) containing triplicate cultures (100 μl) of AIM-V medium (unstimulated control) or NE at various concentrations. After incubation for 44 h at 37°C with 5% CO_2, MTT (0.5 mg/ml, final concentration) was added, and cultures were additionally incubated for 4 h. Cell cultures were then incubated for 16 h with extraction buffer (100 μl) and optical densities, resulting from dissolved formazan crystals, were then read in a microplate reader (Bio-Tek Instruments, Inc., Winooski, VT) at 540 nm.

Nitrite determination: Accumulation of nitrite in the supernatants of macrophage cultures was used as an indicator of nitric oxide production by resident or activated cells. Peritoneal macrophages were incubated for 72 h in 200 μl AIM-V medium, in the presence or absence of various concentrations of NE or LPS (20 ng/ml) in triplicates, in a total volume of 200 μl AIM-V medium. After incubation, supernatants were obtained and nitrite levels were determined with the Griess reagent[13], using $NaNO_2$ as standard. Optical densities at 540 nm were then determined in a microplate reader (Bio-Tek Instruments, Inc.). Macrophage viability was determined by the MTT reduction assay as previously described[12].

The percentage of viability was calculated as follows:

$$\% \text{ viability} = \frac{A_{540} \text{ in NE-treated cells}}{A_{540} \text{ in untreated cells}} \times 100$$

TNF-α assay: TNF-α levels in rat macrophage culture supernatants were determined by the L929 bioassay. In brief, peritoneal macrophage monolayers were incubated in the presence or absence of NE (10^{-5} M) or 20 ng/ml LPS, in a total volume of 200 μl of AIM-V medium for 4 h, after which supernatants were collected and kept at -80°C until use. TNF-α levels in the supernatants were then quantified by the L929 bioassay as described elsewhere[13]. This bioassay was performed in complete RPMI 1640 medium using 1/3 serial dilutions of the supernatants. Recombinant murine TNF-α (a gift from NCI Biological Resources Branch, Rockville, MD, lot 88/532) was used as standard. After 24 h of incubation, cell viability of L929 cells was determined by a colorimetric technique using MTT to a final concentration of 0.5 mg/ml, and incubating the cells for 1.5 h at 37°C[14]. After the incubation period, supernatants were discarded, and formazan crystals were dissolved in DMSO. Optical densities were then determined in a microplate reader (Molecular Devices Corporation) at 540 nm. TNF-α levels (pg/ml) were determined using a recombinant murine TNF-α standard curve.

Effect of NE on human leukocytes: Mononuclear leukocytes were obtained from peripheral blood (HPBMC) of a healthy 18-year old female volunteer, and centrifuged in a 1.077 g/dl ficoll-hypaque solution for 30 min at 10°C and 1600 rpm. The recovered mononuclear cells were washed twice in RPMI 1640 medium, and adjusted to 5 X 10^6 cells/ml in AIM-V medium. One hundred microliters of the leukocyte suspension was then incubated in the presence or absence of various concentrations of NE. Cell cultures were then incubated for 44 h at 37°C in 5% CO_2. After incubation, MTT (0.5 mg/ml, final concentration) was

added, and cultures were additionally incubated for 4 h. Cell cultures were then incubated for 16 h with extraction buffer (100 µl) and optical densities, resulting from dissolved formazan crystals, were then read in a microplate reader (Bio-Tek Instruments, Inc.) at 540 nm.

Inflammatory cytokine gene expression in HPBMC: Whole HPBMC were incubated for 16h in the presence or absence of PHA (20 µg/ml). PHA-treated samples were then incubated for 5 additional hours at 37°C, 95% CO2-5% air, in the presence or absence of NE (10^{-5} M). After this, mononuclear cells were obtained by centrifugation on a ficoll-hypaque gradient. The recovered mononuclear cells were washed twice in RPMI 1640 medium, and adjusted to 5 X 10^6 cells/ml in AIM-V medium. Next, trizol was added to the cells to extract total RNA. Gene expression for inflammatory cytokines was then detected by RT-PCR as reported elsewhere[15].

Statistical analysis: The results were expressed as mean ± SEM of the response of 3 separate rat thymuses or HPBMC samples to each treatment (3 replicate determinations per treatment) from three independent experiments. Level of significance was assessed by Student's *t* test and analysis of variance (ANOVA).

RESULTS

Effect of NE on lymphoproliferation: As observed in Figure 1, NE marginally (1.3-fold increase), but significantly (**P** < 0.01) enhanced proliferation of rat thymic lymphocytes at the highest concentration tested (10^{-5} M). There was no effect of NE on HPBMC. For comparison, rat thymic lymphocyte proliferation indexes to concanavalin A at concentrations of 0.6, 1.2, and 2.4 µg/ml were 2.6, 3.4 and 1.05 respectively (data not shown).

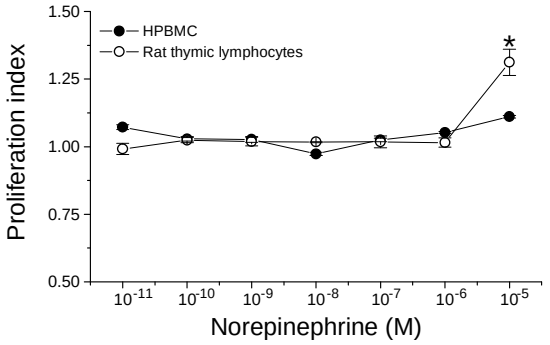

Fig. 1: Rat thymic leukocyte and HPBMC proliferation induced by NE. Thymic cells and HPBMC were cultured in the presence or absence of AIM-V medium (unstimulated control) or NE at various concentrations for 44 h at 37°C in 5% CO_2, after which leukocyte proliferation was determined by a colorimetric technique using MTT, as detailed in the text. Data represent mean ± SEM of triplicates from a representative experiment. ***P** < 0.01 as compared with untreated control. Proliferation index indicates the cell response of NE-treated cells divided by that of untreated cells. Optical densities at 540 nm for untreated rat thymic lymphocytes and HPBMC were 0.484 ± 0.02 and 0.366 ± 0.008 respectively.

Effect of NE on nitric oxide and TNF-α production by macrophages: Nitrite levels in supernatants of rat macrophage cultures treated with NE (10^{-5} M) were significantly (**P** < 0.01) higher (2.9 ± 0.095 nmol/well) than those of untreated control (2.17 ± 017 nmol/well) (Fig. 2a). For comparison, nitrite levels induced by LPS at concentrations of 20 ng/ml were 8.2 ± 0.2 nmol/well (data not shown). NE at 10^{-5} M was associated with 20% reduction of viability (Fig. 2a). At this concentration, NE significantly (**P** < 0.01) stimulated TNF-α production by macrophages (4 ± 0.16 pg/ml) as compared with negligible TNF-α levels observed in untreated control (Fig. 2b). For comparison, TNF-α levels induced by LPS at concentrations of 20 ng/ml were 8 ± 0.8 pg/ml (Fig. 2).

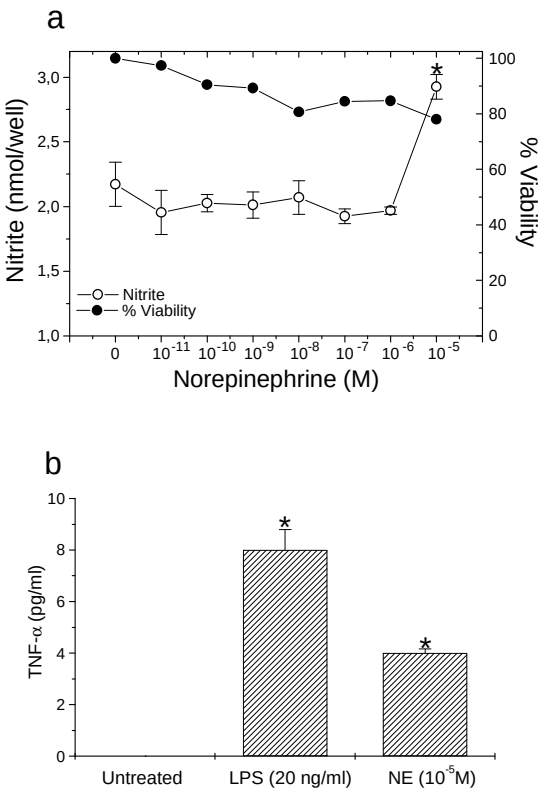

Fig. 2. Nitric oxide and TNF-α production by rat macrophages. Peritoneal cells were incubated overnight, after which adherent cells (1 X 10^6 cells/ml) were washed once and incubated for 4 h (TNF-α determination) and 72 h (nitrite determination) at 37°C, in the presence or absence of NE or LPS (20 ng/ml). Supernatants were then collected and tested for nitrite (*a*) and TNF-α (*b*) levels. *P < 0.01 compared with untreated control. LPS alone (20 ng/ml) induced the production of 8.2 ± 0.2 nmol/well by peritoneal macrophages (100% viability).

Effect of NE on inflammatory cytokine gene expression by HPBMC: In addition to measuring the effects of NE on some parameters of immune function in rat and human lymphocytes and macrophages, and because human macrophages possess limited capacity to produce nitric oxide, we evaluated the effects of NE on inflammatory cytokine gene expression and viability of HPBMC. As observed in Figure 3, NE increased the signal of TNF-α, IL-1 and IL-6 mRNA by human leukocytes, as compared with untreated and PHA-treated controls. NE at concentrations of 10^{-5} M induced 2-fold increase in TNF-α mRNA signal (Epi-Chemi Darkroom, Labworks software v. 3, Ultra-Violet Products, Upland, CA), and stimulated IL-1β and IL-6 mRNA signals, as compared with untreated control; whereas PHA treatment caused 1.33-fold increase in TNF-α mRNA signal without stimulating IL-1β and IL-6 mRNA signals, as compared with untreated control (Fig. 3) (house-keeping gene G3PDH signal was the same for all experimental conditions, including the untreated control). NE was also observed not to alter human leukocytes viability (data not shown).

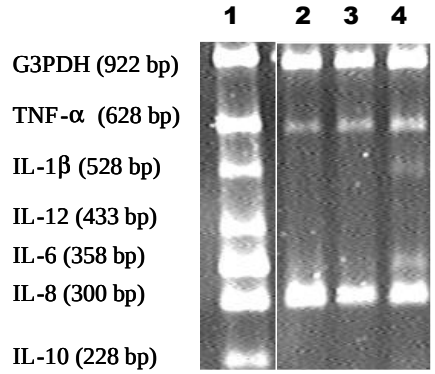

Fig. 3. Inflammatory cytokine gene expression in HPBMC induced by NE. HPBMC were adjusted to 5 X 10^6 cells/ml in AIM V medium as explained in the text. One hundred microliters of the leukocyte suspension was then incubated in the presence or absence of NE (10^{-5} M) or PHA (20 μg/ml). Cell cultures were then incubated for 5h and processed as detailed in the text. Lane 1, mRNA cytokine markers; lane 2, untreated control; lane 3, PHA treatment; and lane 4, NE treatment.

DISCUSSION

In the present study, we reported *in vitro* activation of rat thymic lymphocyte proliferation, and macrophage production of nitric oxide and TNF-α, and increased mRNA signal of the HPBMC pro-inflammatory cytokines TNF-α, IL-1 and IL-6 by NE in-vitro. NE has been shown to activate TH2 cell-dependent antibody response *in vivo*[9], and in combination with IL-12, was shown to increase the pool of TH1 cells[16]. In contrast, NE was reported to suppress proliferation of rat peripheral blood T-lymphocytes[17] (which may be related to catecholamine-induced apoptosis[18]), decrease IL-2 production by murine splenic naïve CD4+ T cells[19], and inhibit IFN-γ synthesis by PHA-stimulated splenic cells[20]. Additionally, A significant suppression of IL-1, IL-2, IL-4, and IFN-γ interferon production by splenic leukocytes, and proliferative response of splenic T lymphocytes following immunization with sheep red blood cells, was observed in the reeler (rl/rl) mice (a neurologic mutant strain with an abnormally high concentration of cerebellar NE)[21]. NE was also observed to inhibit human natural-killer (NK) cell cytotoxicity[22,23]; however, NE was shown to increase the unstimulated, the interleukin-2 and interferon-alpha stimulated NK cell activity[24].

In the macrophage, NE has been involved in suppression of IL-12 production and enhancement of IL-10 release by lipopolysaccharide- or keyhole limpet hemocyanin-stimulated bone marrow-derived dendritic cells[25]. NE was also reported to decrease TNF-α mRNA by activated macrophages[26], decrease LPS-stimulated rat splenic macrophages production of TNF-α at 10 μM[27], and inhibited IL-1 production by IFN-γ and LPS-activated murine peritoneal macrophages[28]. In addition, NE at concentrations ranging from 10^{-8} to 10^{-5} M, was shown to enhance HIV-1 replication by suppressing proinflammatory cytokine production[2]. In contrast, NE increased TNF-α[10], LPS-induced TNF-α[29], and nitric oxide production[30,31], and phagocytosis[32] by macrophages, but suppressed inducible nitric oxide synthase activity in rat astroglial cultures[33]. These conflicting reported findings, along with our results, on the *in vitro* action of NE on immune function may be related to several factors including: a) activation of different adrenoceptors[34-39], b) cell source

and NE concentration utilized, c) via of opioid administration, and d) duration of treatment.

Norepinephrine is known to act on adrenoceptors present in lymphocytes, macrophages, neutrophils and thymic epithelial cells[34,35]. Despite its high-affinity binding to α1, β1, and β3 adrenoceptors[36-39], NE inhibits the production of the pro-inflammatory cytokines IL-1, TNF-α, and IFN-γ and stimulate the production of the anti-inflammatory cytokines IL-10 and transforming growth factor-beta, through stimulation of the β2-adrenoreceptor-cAMP-protein kinase A pathway[40]; furthermore, NE at 10^{-5} M, 10^{-6} M, and 10^{-7} M was reported to suppress IFN-γ production, but at 10^{-5} M, enhanced IL-10 production through α2-adrenoceptor activation[41]. However, under certain conditions, NE may boost regional immune responses, through induction of IL-1, TNF-α, and IL-8 production[40].

The present study may add to the understanding of the effect of NE on immune function, but it is clearly necessary to elucidate the precise *in vivo* role of catecholamines on leukocyte function against infectious diseases and cancer.

ACKNOWLEDGMENTS

This work was supported by grants I-32914-N and CN-285-00 from National Council for Science and Technology of Mexico and Autonomous University of Nuevo Leon (PAICYT), México, respectively, to RGF.

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