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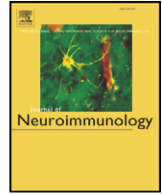

The contribution of the sympathetic nervous system to the immunopathology of experimental pulmonary tuberculosis

Jorge Barrios-Payán [a,1], Alberto Revuelta [a,1], Dulce Mata-Espinosa [a], Brenda Marquina-Castillo [a], Enrique Becerril Villanueva [b], María Eugenia Hernández Gutiérrez [b], Gilberto Pérez-Sánchez [b], Lenin Pavón [b], Rogelio Hernandez-Pando [a,*]

[a] *Experimental Pathology Section, Pathology Department, National Institute of Medical Sciences and Nutrition Salvador Zubiran, Vasco de Quiroga 15, Colonia Belisario Dominguez Seccion XVI, 14080, Deleg. Tlalpan, México City, Mexico*

[b] *Department of Psychoimmunology, National Institute of Psychiatry, "Ramón de la Fuente", Calzada México-Xochimilco 101, Colonia San Lorenzo Huipulco, Deleg. Tlalpan, 14370 México City, Mexico*

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A B S T R A C T

The role of norepinephrine (NE) in the immunopathology of experimental tuberculosis (TB) was studied by measuring pulmonary NE and determining its cellular sources and targets. Functional studies were performed administrating adrenergic and anti-adrenergic drugs at different TB phases. Results showed high production of NE during early infection by adrenergic nerve terminals and lymphocytes located in the lungs and mediastinal lymph nodes, these cells highly expressed β2 adreno-receptors (β2AR) which by an autocrine mechanism promote Th-1 cell differentiation favoring protection. During advanced infection, the production of NE and β2AR sharply decreased, suggesting that adrenergic activity is less important during late TB.

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1. Introduction

The brain and the immune system are the major adaptive systems of the body (Elenkov et al., 2000). Both systems are constantly in communication, overlapping their biochemical language: neurons produce cytokines and express cytokine receptors, while immune cells produce neurotransmitters and express their receptors (Rosas-Ballina and Tracey, 2009). In fact, cytokines can alter neural activity in the brain and neural activity can alter immunological processes, which is highly relevant to maintain homeostasis (Tracey, 2009). Together with the hypothalamic–pituitary–adrenal (HPA) axis, the sympathetic nervous system (SNS) represents the major pathway involved in the cross-talk between the brain and the immune system (Elenkov et al., 2000). The SNS is the first division of the autonomous nervous system (ANS), whose principal function is the control of visceral activity, such as heart rate, hormone secretion, gastrointestinal peristalsis, digestion, as well as the regulation of inflammation and immune function.

In all mammals, the lungs are densely innervated; nerve fibres are diffusely distributed throughout the parenchyma of the airways in association with the epithelia, airway smooth muscle, vasculature, glands and bronchus-associated lymphoid tissue (BALT) (Cavallotti et al., 2004; (McGovern and Mazzone, 2014; Kummer et al., 1992). These nerve fibres are derived from either autonomic motor neurons or sensory neurons originating from vagal or spinal nerves (Mcgovern et al., 2010; Kummer et al., 1992). Autonomic motor neurons and their nerve projections drive the basal activity of bronchomotor tone, vasomotor tone and mucus secretion, which is critical for normal lung function, and also can conduct afferent peripheral stimulus such as lung inflammation or infections resulting in reflex activation of autonomic outflow (Kesler and Canning, 1999). In fact, these autonomic nerve fibres serves as a sentinel for the immune system, sending alerting signals when exposed to pathogen-associated molecular patterns (PAMPs) and cytokines activating the sympathetic centers in the brain (locus coeruleus, hippocampus, hypothalamus among others). Sympathetic neurotransmission from the brain to the periphery occurs via projections extending from the paraventricular nucleus (PVN) of the hypothalamus, rostral ventrolateral/ventromedial medulla and caudal raphe nucleus to preganglionic neurons of the spinal cord (Nance and Sanders, 2007; Sawchenko and Swanson, 1982). The preganglionic cell bodies of sympathetic nerves reside in the intermediolateral cell column of the lateral horn of the spinal cord at T1-L2, which send myelinated projections that exit the spinal cord via the ventral roots to synapse

* Corresponding author.
E-mail addresses: qcjbp77@yahoo.com.mx (J. Barrios-Payán), betomedx@hotmail.com (A. Revuelta), dulmat@yahoo.com.mx (D. Mata-Espinosa), b_marquina@yahoo.com (B. Marquina-Castillo), lusenbeve@yahoo.com (E.B. Villanueva), drosoph2001@yahoo.com.mx (M.E.H. Gutiérrez), gipersanchez@hotmail.com (G. Pérez-Sánchez), lkuriaki@imp.edu.mx (L. Pavón), rhdezpando@hotmail.com (R. Hernandez-Pando).

[1] Both authors contributed equally to this work.

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primarily on neurons located in both the superior cervical and stellate sympathetic ganglia (Kummer et al., 1992). From these ganglia, a second projection follows the vasculature to innervate the airways. These sympathetic nerves form terminals from which the neurotransmitter norepinephrine (NE) is released to bind to adrenergic receptors (ARs) expressed by various cell populations. NE is produced via multiple enzymatic changes of tyrosine, of which the hydroxylation of tyrosine by tyrosine hydroxylase (TH) is the rate-limiting step (Zigmond et al., 1989).

The effects of NE are mediated by ARs, 7-transmembrane G protein-coupled receptors expressed in the central nervous system and in virtually all peripheral tissues including all the immune cells. ARs include three major types: α1, α2 and β, each further divided into three subtypes (Bylund et al., 2010). In both T cell clones and newly generated Th cell populations was demonstrated that Th1 cells but not Th2 cells preferentially express the β-2AR. Freshly isolated naive CD4T cells express a functional β-2AR and its expression is retained in newly generated Th1 cells, while β-2AR mRNA expression was repressed in newly generated Th2 cells (Swanson et al., 2001). The majority of the early studies about the effect of NE on the immune cells indicated an anti-inflammatory/immunosuppressing effect (Kohm and Sanders, 2001), but previous in-vitro studies suggested that NE may exert different effects on T cells cytokine production depending on whether the naive or effector cell is exposed to this neurotransmitter (Swanson et al., 2001). Naive CD4T cells exposed to NE during the process of differentiation generated progeny Th1 cells that produced higher levels of IFN-γ upon re-stimulation with antigen, in comparison to progeny Th1 cells generated in the absence of NE (Kin and Sanders, 2006; Swanson et al., 2001), while less IFN-γ is produced if NE is added before T cell receptor (TCR) stimulation and no change occurred on IFN-γ production if NE is added at the time of TCR stimulation. In contrast, NE has not effect on Th2 cells because this cell subtype does not express the β-2AR (Kin and Sanders, 2006).

Findings from in-vivo studies suggest that NE depletion may differentially affect the level of T cell cytokine production depending on both the specific cytokines measured and the target organ (Alaniz et al., 1999). Thus, it seems that NE modulates immune function in a context-dependent manner and several factors such as the concentration of NE, which depends on nerve terminal proximity, the pattern of AR expression and the activation state of immune cells are significant determinants to induce the NE pro- or anti-inflammatory effects (Kin and Sanders, 2006; Li et al., 2003; Lorton et al., 2003). Considering that the lung is a highly innervated organ, and the fact that the infection by *M. tuberculosis* induces chronic inflammation with significant abnormalities of cell mediated immunity, the aim of the present study was first, measure the pulmonary concentration of NE during progressive tuberculosis (TB) in BALB/c mice, determining its cellular source and cellular targets by immunohistochemistry detecting TH and β-2ADR respectively. Then, functional studies were performed, administrating adrenergic and anti-adrenergic drugs at the beginning of the infection, during the phase of granuloma formation (day 14 post infection) and at late infection (day 60), determining the evolution of the disease in terms of bacterial burdens, histological damage (pneumonia) and cytokines expression, with the aim to define the NE participation in the immunopathology of experimental TB.

2. Materials and methods

2.1. Experimental model of pulmonary TB

The experimental model of progressive pulmonary TB has been described elsewhere (Hernandez-Pando et al., 1997). Briefly, the reference *Mycobacterium tuberculosis* (Mtb) strain H37Rv was grown in 7H9 medium with OADC enrichment. Mid log-phase cultures were used. Male BALB/c mice, 6–8 weeks old, were anesthetized in a gas chamber using sevoflurane and infected through endotracheal instillation with 2.5×10^5 live bacilli. All the animal work was done according to the guidelines of the Mexican Constitution law NOM 062–200-1999, and approval of the Ethical Committee for Experimentation in Animals of the National Institute of Medical Sciences and Nutrition in Mexico, permit number: 224.

2.2. Quantification of norepinephrine (NE) by HPLC and determination of tyrosine-hydroxylase and β-2 adrenoreceptor by immunohistochemistry

Groups of four animals infected as described below were euthanized by exsanguination after anesthesia with intraperitoneal Pentothal at days 1, 3, 7, 14, 21, 28, 60 and 120 post-infection; the right lungs were immediately frozen by immersion in liquid nitrogen and used for NE quantification using HPLC. The controls were non-infected animals (Morales-Montor et al., 2014). Lungs were placed in a 1.5-ml microcentrifuge tube on dry ice. Ice-chilled 0.1 M perchloric acid (PCA) containing an internal standard was added to the tissue tube (10 mg tissue in 300 μl PCA). Samples were sonicated with a microprobe, fitting in the sample tube (6–7 s, duty cycle 80%, output control) until the tissue was completely homogenized. Tubes were kept on crushed ice or at 4 °C for 10 min. Samples were then centrifuged at 14,000 rpm (18,000 g) for 15 min, at 4 °C. The supernatant was transferred into another clean 1.5-ml microcentrifuge tube and centrifuged again as above. Pellets were washed and used to determine protein concentrations. Supernatants were used to determine NE by HPLC assay. Specific standards to each peak in the mixture by its retention time and voltammetric response were added. Ten microliters of all the standards and samples were filtered through a 0.45-μm filter and injected into the column. NE was determined by reversed-phase chromatographic analysis using a Waters Spherisorb ODS2 C18 column, (80 Å, 5 μm, 4.6 × 250 mm) installed in a chromatographic system integrated of Jasco PU-2085 pump and AS-2057 autosampler, with an Antelec Leyden Decade II electrochemical detector, controlled by Millennium 32 software. The separation of analytes was performed at 30 °C with a mobile phase consisting of 5% of acetonitrile in a buffer solution [12.16 mM citric acid, 11.60 mM $(NH_4)_2HPO_4$, 2.34 mM sodium octylsulphonate,3.32 mM dibutyl phosphate amine and 1.11 mM sodium EDTA] at isocratic conditions with a flow rate of 1 ml/min and detected in the next acquisition conditions: range 1 nA, filter 0.005 Hz, Eox 0.60 V, basal 0.001 V, Ic 2.72 nA. The order and retention time of NE was 5 min.

For immunohistochemistry, four left lungs from the same number of mice at each time point after infection in two independent experiments were fixed by perfusion with 100% ethanol via the trachea and embedded in paraffin. Sections 5 μ thick were deparaffinized, the endogenous peroxidase quenched and incubated with rabbit antibodies anti-TH or β-2ADR each diluted 1/250 in PBS (Santa Cruz Biotechnology, Santa Cruz, CA), followed by incubation with goat anti-rabbit IgG labelled with peroxidase. After extensive washings with PBS, sections were stained with hematoxylin, mounted and examined.

2.3. Administration of β-adrenergic or β-blocker drugs at the beginning, middle or late experimental tuberculosis

In order to study the contribution of NE in the evolution of pulmonary TB, groups of BALB/c mice infected with the Mtb strain H37Rv were treated from day 1, 14 or 60 post-infection with 100 mg/kg of 6-hydroxydopamine (6OH-dopamine) dissolved in 100 μl of saline solution administered by intraperitoneal route once per week. This drug efficiently destroys the noradrenergic nerve terminals without affecting the adrenergic centers in the brain (Kruszewska et al., 1995). Another group with the same number of infected animals and at the same time points were treated with α and β adrenergic antagonists phenoxybenzamine and propranolol at dose of 4 and 2 mg/kg respectively administered every day by the oral route (García, 2010). Finally, another group of infected mice was treated at the same time points with the β-2ADR adrenergic agonist formoterol (Novartis) at dose of

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