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Cutting Edge

Cutting Edge: Sympathetic Nervous System Increases Proinflammatory Cytokines and Exacerbates Influenza A Virus Pathogenesis

Kristie M. Grebe, Kazuyo Takeda, Heather D. Hickman, Adam M. Bailey, Alan C. Embry, Jack R. Bennink, and Jonathan W. Yewdell

Although the sympathetic nervous system innervates the lung, little is known about its participation in host immunity to pulmonary pathogens. In this study, we show that peripheral sympathectomy reduces mouse morbidity and mortality from influenza A virus-induced pneumonia due to reduced inflammatory influx of monocytes, neutrophils, and NK cells. Mortality was also delayed by treating mice with an α-adrenergic antagonist. Sympathectomy diminished the immediate innate cytokine responses, particularly IL-1, which was profoundly reduced. These findings demonstrate an unexpected role for the sympathetic nervous system in innate antiviral immunity and in exacerbating the pathology of a virus of great significance to human and animal health. The Journal of Immunology, 2010, 184: 540–544.

Influenza A virus (IAV) remains a leading domestic and worldwide cause of mortality and morbidity. Attaining a deeper understanding of host immunity to IAV is important for improving therapies and designing vaccines to cope with the rapid antigenic evolution of IAV. Immune responses to IAV have been the focus of thousands of studies. Although IAV is known to activate the sympathetic nervous system (SNS), few studies have examined the contribution of the SNS to IAV immunity and pathogenicity (1).

The SNS is the arm of the autonomic nervous system responsible for the fight-or-flight response. Sympathetic nerves track with blood vessels into nearly all tissues. Stimulation of sympathetic nerves releases granules containing neurotransmitters including norepinephrine (NE), neuropeptide Y, and ATP. Sympathetic neurotransmitters probably diffuse widely in tissues because release appears to occur non-synaptically, and NE exhibits a tissue half-life on the order of 7 h (2). Importantly, many immune cells (including macrophages, dendritic cells, and lymphocytes) express adrenergic receptors and can receive signals from the SNS (3–5).

A classic model for investigating the influence of the SNS on host immune responses is based on chemical sympathectomy mediated by the NE analog 6-hydroxydopamine (6-OHDA). 6-OHDA is actively and selectively transported via NE receptors into sympathetic nerve termini, where it is oxidized to generate free radicals that destroy the termini (6). Following i.p. administration, 6-OHDA rapidly distributes into tissues, with the exception of the CNS, which is protected by the blood–brain barrier (6). Nerve regeneration requires at least a month, providing a window to examine host antiviral immunity in the absence of a functional peripheral SNS.

It is well established that the SNS can modulate immune response to viruses (7–9). Hunzeker et al. and Hermann et al. (10–12) have demonstrated that behavioral stressors that activate the SNS and hypothalamic pituitary axis modulate numerous aspects of the mouse response to IAV. In this study, we use 6-OHDA to selectively examine the role of the SNS in the immune response to lethal pulmonary IAV infection in C57/B6 (B6) mice.

Materials and Methods

Mice

Six- to 8-wk-old female C57BL/6 mice were purchased from Taconic Farms (Germantown, NY). Mice were housed in our animal care facility at National Institute of Allergy and Infectious Diseases under specific, pathogen-free conditions and maintained on standard rodent chow, water supplied ad libitum, and a 14:10 h light:dark cycle. Mice were allowed to adapt at least 1 wk before use.

Viruses and immunizations

A/Puerto Rico/8/34 (PR8) was propagated in 10-d-old embryonated chicken eggs and used as infectious allantoic fluid. Virus titers were determined by 50% tissue culture-infective dose in Madin-Darby canine kidney cells and by LD50 in 8-wk-old B6 mice. Mice were infected intranasally (i.n.) with 1 LD50 PR8 diluted in PBS in a volume of 25 μl.

Chemical sympathectomy

Mice were treated with 100 mg/kg 6-OHDA (Sigma-Aldrich, St. Louis, MO) in 0.9% NaCl plus 10−7 M ascorbic acid on day −7 and day −5 and 200 mg/kg on day −3. Control mice received injections of 0.9% NaCl plus 10−7 M ascorbic acid. Sympathectomy was confirmed in the spleen frozen sections by sucrose-phosphate-glyoxaldehyde reaction (described in Ref. 9) to identify sympathetic nerves.

The online version of this article contains supplemental material.

Abbreviations used in this paper: BAL, bronchoalveolar lavage; IAV, influenza A virus; i.n., intranasally; NE, norepinephrine; 6-OHDA, 6-hydroxydopamine; SNS, sympathetic nervous system; TCD8+, CD8+ T cell.
Analysis of cellular infiltrates

Mice were sacrificed by avertin overdose. The airspace was lavaged three times with 1 ml PBS through the trachea to collect bronchoalveolar lavage (BAL) cells. The mouse was then perfused with PBS through the heart to remove the circulating blood from the lung tissue. Lung tissue was removed and digested with type I collagenase and DNase for 2 h at 37°C. Single-cell suspensions were blocked with Fc receptor-blocking Ab (clone 2.4G2) and stained with anti–CD11b-ACP-Cy7 (BD Biosciences, San Jose, CA), anti–CD11c-647 (BD Biosciences), anti–GR-1–pacific blue (BD Biosciences), anti–NK1.1–FITC (BD Biosciences), and anti–Mac3–PE (BD Biosciences).

Cytokine and chemokine measurement

Cytokines and chemokines were measured in BAL fluid using a bioplex cytokine bead array assay (Bio-Rad, Hercules, CA) according to the manufacturer’s specifications.

Results and Discussion

Chemical sympathectomy increases survival to pulmonary IAV infection

To examine the influence of the SNS on B6 survival to infection with mouse adapted A/Puerto Rico/8/34 (H1N1) (PR8) IAV, we treated mice with 6-OHDA three times over a 1-wk interval. Three days after the final 6-OHDA treatment, we infected mice by i.n. administration under anesthesia, resulting in an infection throughout the respiratory tract. Groups of 15 mice infected with 1L D50 PR8 were monitored daily for weight loss and other signs of morbidity. Remarkably, 6-OHDA treated mice demonstrated reduced weight loss and mortality following lethal IAV infection (Fig. 1). We previously showed that under the same experimental conditions, viral titers in the lungs are not altered by 6-OHDA treatment (13), suggesting that the difference in survival is due to a more direct alteration in the immune response.

The SNS innervates mouse lungs

Although it is clear that the mouse lung is innervated by the SNS (14), the degree of innervation is not well established. We therefore identified SNS in histological sections of normal lung by staining for tyrosine hydroxylase (Supplemental Fig. 1). This revealed that SNS nerve bundles course along with blood vessels on the main and medium bronchi and extend into bronchial smooth muscle and pulmonary glands (healthy mice do not have organized areas of lymphoid tissue). Importantly, we failed to detect SNS fibers extending into the lung parenchyma. Thus, it is likely that SNS influence of alveolar function is mediated from afar by compounds with sufficient stability to act at a distance from their sites of release.

Increased survival is not due to an enhanced CD8+ T cell response

We recently reported that 6-OHDA treatment enhances Kb- and Dβ-restricted CD8+ T cell (TC8+) responses to IAV (13). As TC8+ are known to enhance survival to IAV infection in mice (15–17), we investigated whether enhanced TC8+ responses are necessary for 6-OHDA–mediated resistance to lethal IAV infection by using mice with targeted deletion of both Kβ and Dβ genes. Despite the lack of classical class I restricted immunosurveillance, knockout mice demonstrated 6-OHDA–
mediated resistance to IAV of similar magnitude to wild-type mice (Supplemental Fig. 2). These data demonstrate that 6-OHDA can protect against lethal IAV in the absence of Kb- or Db-restricted TCD8+. Because 6-OHDA does not increase antiviral Ab responses (13), these data strongly suggest that sympathectomy protects mice against lethal infection by modulating the innate rather than the adaptive immune system.

Reduced pathology in the lungs of chemically sympathectomized mice

To understand the effect of 6-OHDA on the innate immune response, we first examined IAV-induced pulmonary histopathology. Lungs from control or 6-OHDA–treated mice were removed 5 d postinfection, fixed, sectioned, and stained with H&E (Fig. 2). As expected, IAV infection was associated with cellular infiltration, resulting in bronchitis and alveolitis in control mice. Inflammatory cells can be identified at higher magnification as lymphocytes, neutrophils, and macrophages. 6-OHDA treatment reduced inflammation (Fig. 2 and Supplemental Table I): fewer lymphocytes were present in the parenchyma and perivascular regions, bronchitis was reduced and aeration was increased relative to control-infected mice.

Reduced inflammatory cells in the lungs of chemically sympathectomized mice

To extend the histopathology finding that 6-OHDA treatment decreases immune infiltrates, we quantitated innate immune cells present in the BAL or lung parenchyma 3 d postinfection by flow cytometry. Neutrophils (identified as GR1hi CD11b+ staining) were reduced ∼3-fold in both lung tissue and the BAL of 6-OHDA–treated mice (Fig. 3). Inflammatory monocytes (Mac3hiCD11c+), likely consisting of alveolar macrophages and inflammatory dendritic cells, were similarly reduced. 6-OHDA–treated mice also exhibited decreased NK (NK1.1+) cellular infiltrates into the lung but not the BAL.

6-OHDA reduces immediate lung inflammatory cytokine production

The 6-OHDA–mediated decrease in the inflammatory response suggested that SNS ablation altered the immediate cytokine response. We therefore measured a number of cytokines and chemokines in BAL fluid collected over an 8-d period postinfection. Minute sample quantities necessitated the use of a bead-based assay to measure multiple cytokines/chemokines in individual mice for each time point (Fig. 4).

In control mice, both IL-1β and IL-6 demonstrated a biphasic response, peaking at 2 or 3 d postinfection, dropping precipitously, and then increasing for several days before dropping again by day 8 postinfection. Remarkably, 6-OHDA treatment nearly eliminated IL-1β (>30-fold reduction on day 2) and greatly reduced IL-6 production over the course of infection. 6-OHDA treatment resulted in less severe but still statistically significant decreases in IL-12p40, GMCSF, IFN-γ, and MIP-1α. However, not all cytokine and chemokine responses were changed. For example, TNF-α and RANTES production were...
similar between the 6-OHDA–treated and control groups, suggesting that the SNS does not simply reduce cytokine or chemokine production globally. These findings indicate that the pulmonary cytokine response to IAV infection is greatly blunted by the absence of SNS innervation.

\[ \text{α-blockers but not β-blockers prolong survival from IAV pneumonia} \]

Because adrenergic blockers are among the most prescribed drugs, it was of obvious interest to examine their effect on IAV induced lethality. We previously reported that β-blockers enhance mouse T_{CD8,α} responses to IAV (13). Mice were implanted with osmotic pumps that provide a constant supply of the β-blocker nadolol, or the α-blocker phentolamine. Equivalent doses were less than typically used in humans. Although nadolol had no significant effect on IAV lethality, phentolamine clearly delayed mortality by ∼5 d (Fig. 5).

These findings support the conclusion that 6-OHDA acts by modulating the SNS and not by another undefined mechanism. Further, they suggest that that there are two phases to 6-OHDA–mediated protection, with the early phase involving blocking the normal activation of innate immune mechanisms through α-adrenergic receptors.

These findings demonstrate a clear effect of the SNS on survival following lethal IAV infection. The increased survival of 6-OHDA–treated mice correlates with a decrease in inflammatory cytokines, in particular IL-1 and IL-6, and innate cellular infiltrates. We show that 6-OHDA–mediated survival does not require K_α- or D_β-restricted anti-IAV T_{CD8,α}, but we have not eliminated a possible role for CD4^+ T cells in the phenomenon. This seems unlikely, however, given the clear effects of sympathectomy on cellular infiltrates at early times postinfection when anti-IAV CD4^+ T cells are still relatively limited in number. Rather, our working hypothesis is that reduced cytokine production in the lungs of 6-OHDA–treated mice contributes to their ability to survive the lethal IAV. This would be similar to IAV-infected TLR3^−/− mice, which exhibit reduced pulmonary inflammatory cytokine production and increased survival versus wild-type mice (18).

Konstantinos and Sheridan (19) have demonstrated that during PR8-induced pneumonia, B6 mice subjected to restraint stress demonstrate decreased pulmonary levels of IL-1 and unchanged levels of IL-6 relative to unstressed mice. Because restraint stress increases SNS stimulation, this finding is the opposite of our finding that SNS ablation decreases IL-1 and IL-6 levels. This discrepancy underscores the complexity of the interaction between immune and nervous systems, where the outcome will likely depend on the precisely how the nervous system is perturbed. Restraint stress also activates the hypothalamic pituitary axis, which could profoundly influence pulmonary immune status. 6-OHDA–mediated SNS ablation probably influences immune status by whole-mouse and lung-specific adaptations.

It seems most likely that the critical event in 6-OHDA–mediated decrease in IAV pathology is the diminished immediate secretion of IL-1 and IL-6, which then influences the secretion of other cytokines/chemokines and the recruitment of inflammatory cells. This is consistent with SNS enhancement of alveolar macrophage IL-1 and IL-6 secretion (20). The decrease in IL-1 and IL-6 could be mediated either by blood-borne messengers or, more directly, by pulmonary sympathetic neurons. Although IL-1 and IL-6 can be secreted by virtually any cell type, the great preponderance of innate immune cells relative to sympathetic neurons makes it unlikely that neuronal cytokine secretion accounts for the profound 6-OHDA–mediated decrease in IL-1 and IL-6. Although SNS innervation of the lung does not appear to extend into the lung parenchyma from the bronchi, SNS neurotransmitters are known to persist for hours in tissues, so it is possible that cytokine release by alveolar cells is still controlled by the pulmonary SNS.

Finally, our findings emphasize the delicate balance that the immune system must achieve in responding to viral infections. Unchecked rapid replication of IAV and other cytopathic viruses for just a few days would overwhelm the ability of the host to replace dead cells. On the other hand, an overly robust host response can cause collateral damage sufficient to enhance lethality. We show that the SNS can play an important role in innate autoimmune immunopathology and raise the possible benefit of α-adrenergic antagonist drugs for treating highly pathogenic IAV infections.

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Disclosures
The authors have no financial conflicts of interest.

References


Corrections


The fourth author’s middle initial is incorrect. The correct name is Adam L. Bailey.

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