Role of macrophages in virus-induced airway hyperresponsiveness and neuronal M₂ muscarinic receptor dysfunction


Role of macrophages in virus-induced airway hyperresponsiveness and neuronal M₂ muscarinic receptor dysfunction. *Am J Physiol Lung Cell Mol Physiol* 286: L1255–L1259, 2004; 10.1152/ajplung.00451.2003.—Viral infections exacerbate asthma. One of the pathways by which viruses trigger bronchoconstriction and hyperresponsiveness is by causing dysfunction of inhibitory M₂ muscarinic receptors on the airway parasympathetic nerves. These receptors normally limit acetylcholine (ACh) release from the parasympathetic nerves. Loss of M₂ receptor function increases ACh release, thereby increasing vagally mediated bronchoconstriction. Because viral infection causes an influx of macrophages into the lungs, we tested the role of macrophages in virus-induced airway hyperresponsiveness and M₂ receptor dysfunction. Guinea pigs infected with parainfluenza virus were hyperresponsive to electrical stimulation of the vagus nerves but not to intravenous ACh, indicating that hyperresponsiveness was due to increased release of ACh from the nerves. In addition, the muscarinic agonist pilocarpine no longer inhibited vagally induced bronchoconstriction, indicating M₂ receptor dysfunction. Treating animals with liposome-encapsulated dichloromethylene-diphosphonate (Cl₂ MDP) depleted macrophages as assessed histologically. In these animals, viral infection did not cause airway hyperresponsiveness or M₂ receptor dysfunction. These data suggest that macrophages mediate virus-induced M₂ receptor dysfunction and airway hyperresponsiveness.

IN THE LUNGS, parasympathetic nerves cause bronchoconstriction by releasing acetylcholine (ACh) onto M₂ muscarinic receptors on the smooth muscle (21). ACh also feeds back onto inhibitory M₂ muscarinic receptors on the nerves to decrease further ACh release in animal (7) and human (19) airways. Thus neuronal M₂ receptors limit vagally induced bronchoconstriction.

Viruses cause 80–85% of asthma exacerbations in children (18) and are associated with the majority of asthma exacerbations in adults (2). Virus-induced airway hyperresponsiveness in humans is vagally mediated (5). In guinea pigs, viruses cause M₂ receptor dysfunction (6) leading to vagally mediated hyperresponsiveness. Viral infection causes M₂ receptor dysfunction by multiple mechanisms, some of which require leukocytes and are prevented when leukocytes are depleted with cyclophosphamide (8). Lung macrophages may be abnormal in asthma. Bronchial macrophages from patients with mild asthma exhibit decreased phagocytosis, and the degree of phagocytic dysfunction correlates with increased airway hyperresponsiveness and worse baseline spirometric measurements (1). Macrophage chemotactic protein and monocyte activating/monocyte chemoattractant protein are increased in atopic asthmatics and correlate with increased bronchial responsiveness (23).

Treatment with intratracheal liposome-encapsulated dichloromethylene-diphosphonate (Cl₂ MDP; clodronate) depletes alveolar macrophages in mice (25) and in rats (13) within hours, causing apoptosis of the macrophages that ingest the liposomes (28). Liposomes not containing Cl₂ MDP do not affect macrophage viability, phagocytic and killing ability, surface adherence, or respiratory burst (10). Cl₂ MDP does not directly alter neutrophil number, and Cl₂ MDP-exposed neutrophils still demonstrate normal activation phenotype and phagocytic activity (20).

To test the role of macrophages in virus-induced hyperresponsiveness and M₂ receptor dysfunction, we depleted macrophages using liposome-encapsulated Cl₂ MDP.

**METHODS**

**Animals.** Pathogen-free female Dunkin-Hartley guinea pigs (300–350 g; Hilltop Animal Farms, Scottdale, PA) were shipped in filtered crates and housed in high-efficiency particulate-filtered air. All animals were handled in accordance with the standards established by the U.S. Animal Welfare Acts set forth in the National Institutes of Health guidelines and the Policy and Procedures Manual published by the Johns Hopkins University Animal Care and Use Committee.

**Viral infection.** Parainfluenza type 1 (Sendai virus, VR-105; ATCC) was grown in rhesus monkey kidney cell monolayers as previously described (17). At the end of each experiment, viral content of the lungs was determined as the amount required to produce infection in 50% of rhesus monkey kidney monolayers [the 50% tissue culture infection dose (TCID₅₀)] and is expressed as TCID₅₀/g lung wet wt.

**Macrophage depletion.** On day 0, guinea pigs were treated with 0.3 ml of liposome-encapsulated Cl₂ MDP intranasally (in) and 1 ml of liposome-encapsulated Cl₂ MDP intraperitoneally (ip). The purpose of the ip dose was to decrease the monocytes bone marrow response to macrophage depletion. On day 3, guinea pigs received another dose of 0.3 ml of liposome-encapsulated Cl₂ MDP in. On day 5, guinea pigs were anesthetized with ketamine (45 mg/kg im) and xylazine (8 mg/kg im) and infected with 5 × 10⁵ TCID₅₀/ml Sendai virus in. To control for nonspecific effects of the liposome carrier, another group of guinea pigs was treated with liposome-encapsulated phosphate...
buffered saline (PBS), and some of those animals were infected with virus.

Vagal reactivity and \( M_2 \) receptor function. Four days after infection, guinea pigs were anesthetized with urethane (1.8 g/kg ip). Heart rate and blood pressure were measured via a carotid artery cannula. Both jugular veins were cannulated for the administration of drugs. Both vagus nerves were cut, and the distal ends were placed on shielded electrodes immersed in a pool of mineral oil. Body temperature was maintained at 37°C by use of a heating blanket. The animals were paralyzed with succinylcholine (10 \( \mu \)g/kg/min) and ventilated via a tracheal cannula (tidal volume 1 ml/100 g body wt at 100 breaths per min; Harvard Apparatus, South Natick, MA).

All animals were treated with guanethidine (20 mg/kg iv) and propranolol (1 mg/kg iv) 15 min before physiological measurements to block sympathetic nerves.

Bronchoconstriction was measured as an increase in pulmonary inflation pressure (Ppip) via a pressure transducer on a sidearm of the tracheal cannula as previously described (17). We induced bronchoconstriction by stimulating the vagi at 2–25 Hz, 0.2-ms pulse duration, 10 V, and 5-s pulse train at 2-min intervals. We tested the function of postjunctional \( M_3 \) receptors on smooth muscle by measuring bronchoconstriction in response to exogenous ACh (1–10 \( \mu \)g/kg iv) in vagotomized animals.

Neuronal \( M_2 \) receptor function was measured by the ability of the muscarinic agonist pilocarpine (0.001–100 \( \mu \)g/kg iv) to inhibit vagally induced bronchoconstriction in a dose-dependent manner. For these experiments, the vagi were electrically stimulated at 2 Hz, 0.2-ms pulse duration, 5–15 V, and 44 pulses per train. The voltage was chosen in the absence of pilocarpine to induce an increase in Ppip of 15–25 mmH2O above baseline. The effect of pilocarpine on vagally induced bronchoconstriction was measured as a ratio of bronchoconstriction in the presence of pilocarpine to bronchoconstriction in the absence of pilocarpine. Voltages were not different between groups.

Histological examination for tissue macrophages. Frozen sections of paraformaldehyde-fixed tissues were stained for acid phosphatase by the method of Burstone (4). Macrophages per high-power field were counted independently by two investigators who were blinded to the treatment and infection status of the animals.

Lung lavage. At the end of the experiment, guinea pigs were killed and the lungs were lavaged five times with 10-ml aliquots of PBS via the tracheal cannula. Differential cell counts were obtained with cytopsin slides stained with Diff-Quick (Scientific Products, MacGaw Park, IL).

To quantify the adherent macrophages, we counted macrophages from the bronchoalveolar lavage (BAL) before and after allowing them to adhere to Corning polystyrene dishes at 37°C for 1 h in Dulbecco’s modified Eagle’s medium (DMEM) with 10% bovine serum albumin. Medium containing the nonadherent cells, i.e., nonadherent macrophages, eosinophils, and lymphocytes, was collected, and a differential cell count was obtained. We obtained the number of adherent macrophages by subtracting the nonadherent macrophage count from the total macrophage count. Trypan blue staining was used to assess cell viability.

Drugs and reagents. Acetylcholine chloride, atropine, bovine serum albumin, guanethidine, propranolol, succinylcholine chloride, pilocarpine, DMEM, and urethane were purchased from Sigma (St. Louis, MO). Rhesus monkey kidney cells were purchased from Viromed (Minneapolis, MN). Cl2 MDP was a gift of Roche Diagnostics (Mannheim, Germany). Liposome solutions were prepared as previously described (27).

Statistical analysis. All data were expressed as means ± SE. Pilocarpine, frequency, and ACh responses were analyzed by two-way ANOVAs for repeated measures (30). Histological examination for tissue macrophages were analyzed by two-way ANOVA. A \( P \) value of <0.05 was considered significant. All statistical analyses were made with the software package Statview 4.5 (Abacus Concepts, Berkeley, CA).

RESULTS

Effect of macrophage depletion on vagal reactivity. Electrical stimulation of the vagi caused a frequency-dependent bronchoconstriction (measured as an increase in Ppip) and bradycardia. Vagally induced bronchoconstriction was potentiated in virus-infected guinea pigs, demonstrating airway hyperresponsiveness. Neither Cl2 MDP liposomes (n = 2) nor PBS liposomes (n = 2) affected vagally induced bronchoconstriction or bradycardia in uninfected animals. Pretreatment with liposome-encapsulated Cl2 MDP before viral infection not only prevented hyperresponsiveness but depressed vagally induced bronchoconstrictions below control (Fig. 1). Virus-induced hyperresponsiveness was not inhibited by treatment with PBS liposomes (n = 3, data not shown). Vagally induced bradycardia was not different among groups.

Effect of macrophage depletion on neuronal \( M_2 \) receptor function. In uninfected guinea pigs, pilocarpine inhibited vagally induced bronchoconstriction in a dose-dependent manner, demonstrating that neuronal \( M_2 \) muscarinic receptors respond to muscarinic agonists by decreasing ACh release. In virus-infected guinea pigs, pilocarpine did not inhibit vagally induced bronchoconstriction, demonstrating loss of \( M_2 \) receptor function (Fig. 2). Cl2 MDP liposome treatment had no effect on the inhibition of vagally induced bronchoconstriction by pilocarpine in uninfected guinea pigs. Pretreatment with liposome-encapsulated Cl2 MDP not only prevented loss of \( M_2 \) receptor function in virus-infected guinea pigs but caused a further leftward shift in the dose-response curve to pilocarpine past control, suggesting increased \( M_2 \) receptor function (Fig. 2). Treatment with PBS liposomes did not preserve \( M_2 \) receptor function in virus-infected animals.

Effects of macrophage depletion on \( M_3 \) receptor function. Intravenous ACh induced dose-dependent bronchoconstriction in uninfected guinea pigs. The bronchoconstrictor response to

![Fig. 1. Depleting macrophages with dichloromethylene-diphosphonate (Cl2MDP)-liposomes prevents hyperresponsiveness to vagal stimulation in virus-infected guinea pigs. Stimulation of the vagi (1–25 Hz, 0.2-ms pulse duration, 10 V, 44 pulses per train) causes a frequency-dependent bronchoconstriction measured as an increase in pulmonary inflation pressure in uninfected controls (○, n = 6). This response is potentiated in virus-infected animals (●, n = 5). In animals that were depleted of macrophages, hyperresponsiveness to vagal stimulation was prevented (●, n = 5; means ± SE). Significantly different from control.](http://ajplung.physiology.org/issue)
ACh was not potentiated by viral infection. Rather, it was slightly decreased \( (P / H_{11005} 0.058) \) compared with control (Fig. 3). Thus virus-induced hyperresponsiveness to vagal stimulation was not due to effects of the virus on airway smooth muscle M3 receptors. Pretreatment with liposome-encapsulated Cl2 MDP did not affect the ACh response in infected or uninfected animals.

**Tissue histology.** Histological examination of tissues for macrophages was done independently by two investigators who were blinded to the treatment and infection status of the animals. The macrophage counts of the two investigators agreed closely \( (r^2 = 0.91) \).

Tissue macrophage counts are shown in Fig. 4, and representative histology is shown in Fig. 5. Viral infection increased macrophage number in untreated animals but not in liposome-
encapsulated Cl2MDP-treated animals. The virus-induced increase in tissue macrophages was not prevented by treatment with empty liposomes.

**Lung lavage.** The number of lymphocytes in lavage was similar in control (6.8 ± 2.2 × 10^5 cells/ml), virus-infected (8.1 ± 2.9 × 10^5 cells/ml), macrophage-depleted uninfected (1.0 ± 0.21 × 10^6 cells/ml), and macrophage-depleted virus-infected (5.2 ± 1.1 × 10^5 cells/ml) animals. Empty liposomes significantly increased lymphocytes in both uninfected (2.5 ± 0.31 × 10^6 cells/ml) and virus-infected (1.8 ± 0.34 × 10^6 cells/ml) animals, but this was not accompanied by changes in airway responsiveness or M2 receptor function. Viral infection increased neutrophils in untreated animals (2.5 ± 1.3 × 10^6 cells/ml) and in animals treated with empty liposomes (2.1 ± 0.30 × 10^6 cells/ml) compared with uninfected controls (2.1 ± 0.91 × 10^6 cells/ml). This increase was partially attenuated by liposome-encapsulated Cl2MDP (1.3 ± 0.19 × 10^6 cells/ml). Liposome-encapsulated Cl2MDP increased eosinophils in both uninfected (3.8 ± 0.86 × 10^6 cells/ml) and virus-infected animals (2.6 ± 0.51 × 10^6 cells/ml) when compared with untreated uninfected (6.0 ± 2.1 × 10^6 cells/ml) and virus-infected (9.5 ± 0.20 × 10^6 cells/ml) animals. Despite the increase in eosinophils, airway responsiveness and M2 receptor function were normal in these animals.

Despite substantial depletion of macrophages as demonstrated histologically, macrophages in lung lavage were only mildly decreased. However, in virus-infected animals, whereas Cl2MDP treatment decreased lavaged macrophages by only 25% (6.8 ± 0.9 × 10^6 cells/ml in untreated, 5.1 ± 0.8 × 10^6 cells/ml in treated animals), the macrophages in treated animals appeared markedly enlarged and vacuolated. The macrophages in lavage fluid from these animals, although alive as assessed by trypan blue exclusion, were markedly abnormal in their ability to adhere to plastic (only 13.6% of these macrophages adhered, compared with 85.9% in control animals). Thus in addition to depletion of >90% of macrophages in the tissues (histologically), the remaining macrophages appear functionally abnormal.

**Viral titers.** Viral titers in guinea pigs pretreated with liposomes, PBS (n = 16), or Cl2MDP (n = 14) were no different from virus-infected animals (n = 29). The viral titer for virus-infected controls was 6.75 × 10^5 ± 2.01 × 10^2 TCID50, for the PBS liposome-pretreated group was 7.24 × 10^5 ± 4.95 × 10^3 TCID50, and for the Cl2MDP liposome-pretreated group was 15.1 × 10^5 ± 6.71 × 10^2 TCID50 (P = 0.32). Thus the severity of viral infection among treatment groups did not differ at day 4 after infection and could not account for the difference in the physiological measurements (data not shown).

**DISCUSSION**

Viral infection causes airway hyperresponsiveness in guinea pigs that is mediated by loss of neuronal M2 muscarinic receptor function (6). Depleting macrophages by pretreating with liposome-encapsulated Cl2MDP prevented both vagally mediated airway hyperresponsiveness and M2 receptor dysfunction in virus-infected guinea pigs.

Neither viral infection nor Cl2MDP liposome treatment changed ACh-induced bronchoconstriction, demonstrating that muscarinic receptor function on airway smooth muscle was not altered. Thus neither virus-induced hyperresponsiveness nor the prevention of airway hyperresponsiveness in Cl2MDP-treated animals was due to any change in airway smooth muscle function. Virus-induced hyperresponsiveness and the inhibition of hyperresponsiveness by Cl2MDP were mediated at the level of the vagus nerves.

Treatment with Cl2MDP liposomes markedly depleted macrophages as demonstrated on histological analysis (Figs. 4 and 5). Macrophages in lung lavage were abnormal morphologically and displayed markedly deficient adhesion to plastic plates.

An association between abnormal macrophage function and asthma has recently been described. Increased levels of both monocyte chemotactic protein and activating factor/monocyte chemoattractant protein in BAL fluid were measured in atopic asthmatics and were found to correlate with increased baseline airway resistance and airway hyperresponsiveness, suggesting macrophage activation in asthma (23). The data presented here would support the conclusion that macrophages can contribute to airway hyperresponsiveness by decreasing M2 receptor function after viral infection.

Potential mechanisms of macrophage-mediated airway hyperresponsiveness and loss of M2 receptor function in viral infection include release of cytokines from virus-activated macrophages or from other inflammatory cells that are stimulated by macrophages. Transforming growth factor-β1, tumor necrosis factor-α, interleukin-1β, fibroblast growth factor, and platelet-derived growth factor are macrophage-derived cytokines that have been shown to alter the expression or function of muscarinic receptors in vitro (11, 12, 15, 22, 24). Macrophages produce interferons (IFNs) in response to parainfluenza virus infection (14). IFN-γ treatment of parasympathetic nerve cell cultures decreases M2 receptor gene expression by more than an order of magnitude (16). Macrophages produce primarily type 1 IFNs (α and β), whereas T lymphocytes produce both type 1 and type 2 (γ) IFNs (29). Thus IFNs from either macrophages or from macrophage-stimulated T lymphocytes may cause M2 receptor dysfunction in viral infection. In support of this hypothesis, virus-induced loss of M2 receptor function and airway hyperresponsiveness are mimicked by systemic treatment with double-stranded RNA, a product of replicating RNA viruses that is known to stimulate interferon-α and -β production (3). Nitric oxide (NO) can cause M2 receptor dysfunction in pathogen-free guinea pigs (9). Thus high levels of NO from virus-activated macrophages (29) may contribute to M2 receptor dysfunction and airway hyperresponsiveness.

Treating uninfected animals with liposome-encapsulated Cl2MDP produced a mild neutrophilic inflammation. This neutrophilic inflammation did not cause hyperresponsiveness or M2 receptor dysfunction. There was also a small attenuation of virus-induced neutrophilic inflammation of the lungs in macrophage-depleted animals. We have previously demonstrated, using cyclophosphamide, that neutrophil depletion prevents virus-induced hyperresponsiveness and M2 receptor dysfunction in about half of animals so treated (particularly in those with milder infections as assessed by viral titers) (8). Thus a lessening of the neutrophil response to virus as a result of macrophage depletion may participate in the effects of macrophage depletion.
In noninfected animals, macrophage depletion in itself affected neither airway responsiveness nor M<sub>2</sub> receptor function. However, in the virus-infected animals, macrophage depletion not only prevented hyperresponsiveness but decreased responsiveness to vagal stimulation below that seen in uninfected controls (Fig. 1). Likewise, M<sub>2</sub> receptor dysfunction was not only prevented in virus-infected macrophage-depleted animals, but M<sub>2</sub> function was actually increased beyond normal (Fig. 2, dose-response curve shifted significantly to the left compared with controls). The mechanisms of this increased M<sub>2</sub> receptor function and the resulting airway hyperresponsiveness are not known. It is possible that compensatory mechanisms limit the degree of hyperresponsiveness and M<sub>2</sub> dysfunction in virus-infected animals, and that the effects of these compensatory mechanisms are seen only when the macrophage-mediated hyperresponsiveness and M<sub>2</sub> receptor dysfunction are eliminated.

The data presented here confirm that virus-induced loss of M<sub>2</sub> receptor function causes airway hyperresponsiveness and show that virus-activated macrophages mediate airway hyperresponsiveness and loss of M<sub>2</sub> receptor function in guinea pigs. Potential mechanisms of macrophage-mediated airway hyperresponsiveness and loss of M<sub>2</sub> receptor function in viral infection include release of cytokines or NO from virus-activated macrophages or from other inflammatory cells stimulated by macrophages. Elucidation of the mechanism of this macrophage-dependent effect may further demonstrate the tenuous balance between proinflammatory, antipathogen duties as the first line of defense against airborne insults and side effects that are deleterious to the whole organism.

ACKNOWLEDGMENTS

We thank Bethany L. Yost and Brian Schofield for technical assistance.

GRANTS

This work was supported by National Institutes of Health Grants AI-10587, ROI HL-55543, ROI HL-10342, ROI HL-54659, and ROI HL-61013.

REFERENCES


