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

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Lee, Ann M., Allison D. Fryer, Nico van Rooijen, and David B. Jacoby. 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. 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. Thus neuronal M2 receptors limit vagally induced bronchoconstriction.

Virus infections exacerbate asthma 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).

Macrophage depletion. On day 0, guinea pigs were treated with 0.3 ml of liposome-encapsulated Cl2MDP intranasally (in) and 1 ml of liposome-encapsulated Cl2MDP intraperitoneally (ip). The purpose of the ip dose was to decrease the monocytic bone marrow response to macrophage depletion. On day 3, guinea pigs received another dose of 0.3 ml of liposome-encapsulated Cl2MDP in. On day 5, guinea pigs were anesthetized with ketamine (45 mg/kg im) and xylazine (8 mg/kg im) and infected with 5 × 103 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 M2 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 μg/kg−1 min−1 iv) 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 (Ppi) 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 M3 receptors on smooth muscle by measuring bronchoconstriction in response to exogenous ACh (1–10 μg/kg iv) in vagotomized animals.

Neuronal M2 receptor function was measured by the ability of the muscarinic agonist pilocarpine (0.001–100 μg/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 Ppi 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 cytospin slides stained with Diff-Quik (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 Ppi) 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 M2 receptor function. In uninfected guinea pigs, pilocarpine inhibited vagally induced bronchoconstriction in a dose-dependent manner, demonstrating that neuronal M2 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 M2 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 M2 receptor function in virus-infected guinea pigs but caused a further leftward shift in the dose-response curve to pilocarpine past control, suggesting increased M2 receptor function (Fig. 2). Treatment with PBS liposomes did not preserve M2 receptor function in virus-infected animals.

Effects of macrophage depletion on M3 receptor function. Intravenous ACh induced dose-dependent bronchoconstriction in uninfected guinea pigs. The bronchoconstrictor response to
ACh was not potentiated by viral infection. Rather, it was slightly decreased (P = 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 M<sub>3</sub> receptors. Pretreatment with liposome-encapsulated Cl<sub>2</sub>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<sup>2</sup> = 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-
Mildly decreased. However, in virus-infected animals, whereas demonstrated histologically, macrophages in lung lavage were only animals.

Lung lavage. The number of lymphocytes in lavage was similar in control (6.8 ± 2.2 × 10⁵ cells/ml), virus-infected (8.1 ± 2.9 × 10⁵ cells/ml), macrophage-depleted uninfected (1.0 ± 0.21 × 10⁶ cells/ml), and macrophage-depleted virus-infected (5.2 ± 1.1 × 10⁵ cells/ml) animals. Empty liposomes significantly increased lymphocytes in both uninfected (2.5 ± 0.31 × 10⁶ cells/ml) and virus-infected (1.8 ± 0.34 × 10⁶ 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 × 10⁶ cells/ml) and in animals treated with empty liposomes (2.1 ± 0.30 × 10⁶ cells/ml) compared with uninfected controls (2.1 ± 0.91 × 10⁶ cells/ml). This increase was partially attenuated by liposome-encapsulated Cl₂ MDP (1.3 ± 0.19 × 10⁶ cells/ml). Liposome-encapsulated Cl₂ MDP increased eosinophils in both uninfected (3.8 ± 0.86 × 10⁶ cells/ml) and virus-infected animals (2.6 ± 0.51 × 10⁶ cells/ml) when compared with untreated uninfected (6.0 ± 2.1 × 10⁶ cells/ml) and virus-infected (9.5 ± 0.20 × 10⁶ 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 Cl₂ MDP treatment decreased lavaged macrophages by only 25% (6.8 ± 0.9 × 10⁶ cells/ml in untreated, 5.1 ± 0.8 × 10⁶ 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.

Viruses. Viral titers in guinea pigs pretreated with liposomes, PBS (n = 16), or Cl₂ MDP (n = 14) were no different from virus-infected animals (n = 29). The viral titer for virus-infected controls was 6.75 ± 2.01 × 10⁷ TCID₅₀, for the PBS liposome-pretreated group was 7.24 × 10⁵ ± 4.95 × 10⁵ TCID₅₀, and for the Cl₂ MDP liposome-pretreated group was 15.1 ± 6.71 × 10⁵ 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 Cl₂ MDP prevented both vagally mediated airway hyperresponsiveness and M₂ receptor dysfunction in virus-infected guinea pigs.

Neither viral infection nor Cl₂ MDP liposome treatment altered. Thus neither virus-induced hyperresponsiveness nor the prevention of airway hyperresponsiveness in Cl₂ MDP-treated animals was due to any change in airway smooth muscle function. Virus-induced hyperresponsiveness and the inhibition of hyperresponsiveness by Cl₂ MDP were mediated at the level of the vagus nerves.

Treatment with Cl₂ MDP 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 (15). Macrophages produce primarily type I 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 lipid encapsulated Cl₂ MDP 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 M2 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, M2 receptor dysfunction was not only prevented in virus-infected macrophage-depleted animals, but M2 function was actually increased beyond normal (Fig. 2, dose-response curve shifted significantly to the left compared with controls). The mechanisms of this increased M2 receptor function and the resulting airway hyperresponsiveness are not known. It is possible that compensatory mechanisms limit the degree of hyperresponsiveness and M2 dysfunction in virus-infected animals, and that the effects of these compensatory mechanisms are seen only when the macrophage-mediated hyperresponsiveness and M2 receptor dysfunction are eliminated.

The data presented here confirm that virus-induced loss of M2 receptor function causes airway hyperresponsiveness and show that virus-activated macrophages mediate airway hyperresponsiveness and loss of M2 receptor function in guinea pigs. Potential mechanisms of macrophage-mediated airway hyperresponsiveness and loss of M2 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.

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REFERENCES