**Relationship between airway microvascular leakage, edema, and baseline airway functions**

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**Matheson, Melissa, Ann-Christine Rynell, Melissa McClean, and Norbert Berend.** Relationship between airway microvascular leakage, edema, and baseline airway functions. J. Appl. Physiol. 84(1): 77–81, 1998.—This study was designed to examine the relationship among microvascular leakage, edema, and baseline airway function. Microvascular leakage was induced in the airways of anesthetized, tracheostomized New Zealand White rabbits (n = 22) by using nebulized N-formyl-methionyl-leucyl-phenylalanine (10 mg) and was measured in the trachea by using the Evans blue dye technique. Airway wall thickness was assessed morphometrically in the right main bronchus after Formalin fixation at a pressure of 25 cmH2O. Areas calculated included the mucosal wall area, the adventitial wall area, the total wall area, and the percentage of total wall area consisting of blood vessels. A neutrophil count was also performed by analyzing numbers of cells in both the mucosal wall area and the adventitial wall area. Airway function was assessed before and 30 min after challenge with N-formyl-methionyl-leucyl-phenylalanine by determining airway resistance, functional residual capacity, specific airway resistance, and flow-volume and pressure-volume curves (after paralysis of the animals with suxamethonium). The concentration of Evans blue dye in tracheal tissue ranged from 31.3 to 131.2 µg. There was a significant correlation between this concentration and either blood vessel area or changes in respiratory physiology parameters before and after challenge. There was no significant difference between Evans blue dye concentration and other blood vessel area or changes in respiratory physiology measurements before and after challenge. We conclude that an increase in microvascular leakage correlates with airway edema in the adventitia; however, these airway changes have no significant effect on airway elastic or resistive properties.

N-formyl-methionyl-leucyl-phenylalanine; respiratory physiology; neutrophils

There is now considerable literature supporting a pivotal role for airway inflammation in the pathogenesis of asthma, with a wide range of inflammatory mediators being implicated (1). Two cardinal signs of an inflammatory response are an increase in microvascular leakage and tissue edema. Evidence suggests that microvascular leakage is present in asthmatic subjects, with plasma protein detected in the sputum and bronchoalveolar lavage fluid (1). Many of the inflammatory mediators found in increased levels in the airways of asthmatic subjects have been shown in experimental animal models to increase microvascular leakage (4–6). Theoretically, mucosal edema, with subsequent thickening of the airway wall, can be shown to produce a leftward shift of a bronchoconstrictor agonist dose-response curve and an increase in maximal airway narrowing, as observed in asthmatic subjects, despite having no pronounced effect on baseline airway function (7). Adventitial edema causing a decrease in interdependence, hence decreasing the load against which the smooth muscle contracts, would also exaggerate airway narrowing.

There is, however, a paucity of experimental evidence showing a direct relationship between the levels of microvascular leakage induced by the proposed inflammatory mediators of asthma and airway wall edema. A subsequent effect of these inflammatory responses on baseline airway function has not been thoroughly experimentally explored. This study examined the relationships between these parameters by using an animal model. We induced microvascular leakage by using the peptide N-formyl-methionyl-leucyl-phenylalanine (FMLP), as a tool not as a proposed mediator of asthma, by utilizing our knowledge of the kinetics of the microvascular leakage responses to this peptide. Microvascular leakage was assessed by using the Evans blue dye technique, bronchial wall edema was assessed by using morphometric measurements according to the methods of JAMES et al. (7), and baseline airway function was assessed by measuring and calculating pulmonary resistance (RL), lung volume, specific resistance, maximal expiratory flow-volume curves, pressure-volume curves, and maximal flow-static recoil pressure curves.

**METHODS**

Animals. All studies were approved by the Animal Ethics Committee of Royal North Shore Hospital. Studies were carried out on New Zealand White male rabbits weighing 2.5–3.5 kg, which had been individually caged and freely fed and watered before study.

Rabbits were anesthetized with ketamine (40 mg/kg im) and xylazine (8 mg/kg im), and surgical anesthesia was maintained throughout the experiment with further doses as required. The rabbits had a 4-mm-inner-diameter endotracheal tube inserted via a tracheostomy and an esophageal balloon catheter inserted orally for measurements of pleural pressure. A 21-gauge catheter was inserted into the superficial ear vein for intravenous administrations. The rabbits breathed spontaneously except where specified in the experimental protocol when skeletal muscle paralysis was induced by using suxamethonium (2.5 mg in saline) and mechanical ventilation was utilized.

Chemicals and drugs. The following chemicals were used: Evans blue, dimethyl sulfoxide (DMSO; Sigma Chemical, St. Louis, MO), and FMLP (Auspep, Melbourne, Australia). All solutions of these chemicals were prepared fresh each day of the experiment.

The following drugs were used: xylazine (Bayer, NSW Australia), ketamine (Troy, SW, Australia), pentobarbital...
sodium (Boehringer Ingelheim, NSW, Australia), and suxamethonium (David Bull Laboratories).

Experimental protocol. The rabbits (n = 22) were placed supine, under a heat lamp, in a volume-displacement plethysmograph. Rl was determined by the electrical subtraction technique of Mead and Whittenberg (10). Functional residual capacity (FRC) was measured by forcing the rabbits to pant against a shutter, and thoracic gas volume was calculated by the relationship between the change in total gas volume and change in alveolar pressure by using Boyle’s law. Specific resistance was calculated from Rl and FRC. After administration of the skeletal muscle relaxant suxamethonium (iv), rabbits were connected to a Harvard ventilator and ventilated at a rate of 25 breaths/min and a tidal volume of 15 ml, which maintained normal arterial blood-gas tensions. For the recording of pressure-volume curves, the lungs were inflated to a transpulmonary pressure (P_l) of 25 cmH_2O (defined as total lung capacity) followed by interrupted deflation with recordings of 10–15 pressure-volume points per deflation. Three deflations were performed in each rabbit. Flow-volume curves were recorded by inflating the lungs to a P_l of 25 cmH_2O. The endotracheal tube was attached to a negative-pressure reservoir at -150 cmH_2O. The valve connecting the endotracheal tube and reservoir was opened, the lung was deflated, and lung volume and expiratory flow were measured on a cathode-ray oscilloscope by taking a photo record of each measurement. Two curves were recorded per rabbit. Both pressure-volume and flow-volume curves were preceded by a constant volume history. By this time the paralyzing effect of suxamethonium had worn off, and (if Rl was within 20% of baseline) all rabbits were given a slow intravenous injection of Evans blue (50 mg/kg), which had been dissolved in 4 ml of sterile saline. Immediately after Evans blue infusion, nebulization of either FMLP, dissolved in 2 ml DMSO-saline, or the vehicle only was performed for 2 min by using a Devilbiss 646 nebulizer driven by air at 8 l/min via the endotracheal tube. Thirty minutes later, when, as we know from previous studies (2), FMLP-induced microvascular leakage is maximal, airway function was assessed as previously described by recording Rl, FRC, pressure-volume curves, and flow-volume curves. While the rabbits were kept paralyzed with an infusion of suxamethonium, a thoracotomy was performed. The trachea 1–1.5 cm in length just proximal to the major carina for incubation overnight in formamide to extract extravascular Evans blue and by measuring the concentration of dye spectrophotometrically. The amount of dye (µg/g wet wt) was calculated by interpolation on a standard curve of Evans blue dye concentrations.

Morphometric analysis. Airway tissue was Formalin fixed, sectioned, and stained with Masson’s trichrome for morphometry and with hematoxylin and eosin for neutrophil counts and assessment of blood vessel area as a percentage of wall area. Morphometry was performed on three sections per rabbit (blind to treatment), and means ± SE were determined. The following calculations were made.

1) Three wall areas were calculated by measuring the internal perimeter (epithelial border), the perimeter tracing outside the muscle layer (the mucosal perimeter), and the perimeter tracing the adventitial perimeter (the adventitial perimeter); the mucosal wall area, being the area between the mucosal perimeter and the internal perimeter; the adventitial wall area, being the area between the adventitial perimeter and the internal perimeter; these perimeters were measured by projection of the microscopic image, tracing the appropriate perimeters, and subsequent use of a digitizer using the Phoenix Enhanced Video BIOS (version 1.07) software. The square root of each wall area was taken and divided by the internal perimeter to enable comparisons between airways of different sizes.

2) To determine the effect of blood vessels on total wall area, blood vessel area was measured and expressed as total area and as a percentage of total wall area. Methodology was identical to that used for airway perimeters.

3) A neutrophil count was performed on both the mucosal wall area and adventitial wall area and expressed as total neutrophils per mucosal or adventitial wall areas. These counts were performed on a subset of rabbits (n = 10).

Statistics. Means ± SE were calculated. Lung function measurements were analyzed by using paired t-tests to compare lung function before and after challenge with FMLP or the control (DMSO-saline). Unpaired t-tests were performed between the two groups challenged with FMLP or DMSO-saline for Evans blue concentration, the square root of wall area divided by the perimeter, the square root of the inner wall area divided by the perimeter, the square root of the outer wall area divided by the perimeter, and the percentage of total area represented by inner wall and outer wall. Correlations were performed between Evans blue concentration and the previously mentioned four wall areas and between Evans blue concentration and the neutrophil counts. P ≤ 0.05 was considered significant.

RESULTS

Lung function measurements. There was no significant difference between measurements of resistance, FRC, and specific resistance made before and after challenge with either DMSO or FMLP as shown in Figs. 1, 2, and 3, respectively (expressed as means ± SE).

Pressure-volume curves before and after challenge were compared by determining pressure at a range of specific lung volumes of 60, 70, 80, 90, and 100% of total lung capacity. No significant change was induced by challenge as shown in Fig. 4.

Flow-volume curves were assessed by determining the flow at the volume at which P_l was 7 cmH_2O. No significant change was induced by challenge as shown in Fig. 5.

Microvascular leakage and morphometry. The concentration of Evans blue in tracheal tissue ranged from 31.3 to 131.2 µg Evans blue/g trachea with a large range in values for rabbits challenged with both FMLP and the control DMSO. Therefore, rather than making comparisons between these two groups, we chose to perform correlations between Evans blue concentrations and the various measured wall areas. Correlations were performed between the concentration of Evans blue and changes in the above-mentioned lung
function parameters pre- and postchallenge. No significant correlation was found.

The percentage of total wall area consisting of blood vessel ranged from 0.08 to 1.7%. There was no significant correlation between this area and the concentration of Evans blue dye.

The range of wall areas measured expressed as square root divided by the perimeter was 0.065 to 0.102 cm² for the inner wall area and 0.081 to 0.220 cm² for the outer wall area. There was a positive correlation (P < 0.01, r² = 0.416) between the concentration of Evans blue dye and the adventitial wall area (the square root of outer wall area divided by the internal perimeter) as shown in Fig. 6 but not between mucosal wall area and Evans blue dye or internal perimeter and outer wall area.

Neutrophil counts were done in a subset of rabbits. Comparisons were made between Evans blue concentration and the number of mucosal and adventitial neutrophils. There was no correlation between dye and adventitial neutrophils; however, mucosal neutrophils positively correlated with Evans blue dye (P < 0.005, r² = 0.650) as shown in Fig. 7.

**DISCUSSION**

The aim of this study was to examine the relationship between microvascular leakage and edema and to further determine whether a change in either of these parameters alters baseline airway function.

The results suggest that, within a fourfold range of airway microvascular leakage, changes in airway edema occur between the cartilage-smooth muscle and the alveolar border. Although microvascular leakage was
assessed in the trachea and morphometric measurements were made on the bronchi, we are confident that changes in tracheal microvascular leakage reflect changes in the bronchi because previous studies examining the kinetics of FMLP-induced microvascular leakage showed a good correlation between tracheal and bronchial microvascular leakage (9). This result mirrors findings by Kimura et al. (8), who, using bradykinin, also illustrated edema outside the airway smooth muscle in airways with a perimeter >3 mm, with approximately a fourfold increase in microvascular leakage. The method of assessment of microvascular leakage by using Evans blue concentration was based on previous experience in our laboratory (2, 9), with the latter study showing microvascular leakage to be maximal at 30 min when FMLP-induced increases in airway resistance are fully reversed (3).

We chose to standardize the pressure of fixation of the airways to approximate inflation to total lung capacity to try to minimize variation in fluid distribution. The original design was to inflate the lungs to FRC because this is the lung volume at which the measurements of airway resistance were made. However, this would mean individualizing inflating pressures for each lung, which may have changed fluid levels in the different airway compartments. Errors due to Formalin fixation may have occurred that are uncontrolled for in this experiment; however, we have found Formalin to be the best method of fixation.

We hypothesize that the site of the microvascular leakage may be on the mucosal side of airway wall smooth muscle. Mucosal neutrophils, which were significantly increased in the submucosa, are a likely source of inflammatory mediators responsible for increasing microvascular leakage. This suggestion is supported first by a previous finding that depletion of neutrophils with nitrogen mustard in rabbits reduces microvascular leakage induced by FMLP (9) and second by the correlation between neutrophils and microvascular leakage seen in this experiment. Thus the hypothesized chain of events is as follows. FMLP attracts neutrophils to the submucosa, which then release inflammatory mediators that induce increases in microvascular leakage. Transmural pressure gradients may then pull the edematous fluid peripherally. This chain of events could be initiated by any substance attracting cells that release mediators responsible for increases in airway microvascular leakage. Morphometric analysis of airway wall blood vessel size, which, if changed, could itself alter airway wall thickness, revealed no evidence of vascular hyperemia, indicating that extravascular fluid was responsible for the changes in airway wall thickness.

Despite an increase in microvascular leakage and the subsequent induction of airway wall edema, there was no significant change in measured lung function. It was important to rule out a change in elastic recoil pressure, which would affect flow without necessarily affecting the caliber of the conducting airways. There was no major effect on central airway caliber, as shown by no change in resistance, and no airway obstruction in...
smaller airways, as illustrated by the lack of change in the flow-volume curves.

In conclusion, no change in elastic or resistive forces occurred within a fourfold range in airway microvascular leakage and subsequent airway wall edema.

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