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Vascular permeability and airway narrowing during late asthmatic response in dogs treated with Metopirone

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Recently, we have developed an animal model of late asthmatic response (LAR) by treating naturally sensitized dogs to Ascaris suum *antigen with the cortisol-synthesizing inhibitor, Metopirone. By using this animal model, we examined the contribution of edema in the airway wall to the development of LAR. To study whether airway microvascular leakage is increased in association with LAR, we performed antigen challenge in dogs treated with Metopirone. We measured the amount of extravasated Evans blue (EB) dye from the esophagus, trachea, and large and small bronchi 8 hours after the antigen challenge in dogs demonstrating immediate asthmatic response alone (IAR) and in dogs demonstrating both IAR and LAR. Airway responses to* A. suum *antigen were assessed by changes in respiratory resistance measured with the force oscillation technique at 3 Hz. EB dye extravasation did not increase significantly from that of control in any tissues in IAR ($p > 0.10$), but in LAR, it increased significantly from that of control ($p < 0.01$) and IAR ($p < 0.05$) in large and small bronchi. Histologic assessment of vascular permeability revealed that Monastral blue–labeled leaking vessels were only in sections from LAR, and leaking vessels were limited to small vessels (10 to 25 μm) in the trachea, large (diameter, >5 mm) and small bronchi (2 to 4 mm in diameter), and bronchiole. The permeability index defined as the ratio of area of small vessels labeled with Monastral blue to that of the total small vessels in the walls was highest in the small bronchi. LAR significantly increased submucosal thickness of the small bronchi ($p < 0.05$) compared with that in IAR. Both EB dye extravasation and permeability index in large and small bronchi also significantly increased during IAR within 3 minutes after the antigen challenge ($p < 0.05$), but IAR did not alter the submucosal thickness of the small bronchi. These results imply that the increase in vascular permeability and submucosal thickness, especially in small bronchi, may be an important factor in the pathogenesis of LAR. (J ALLERGY CLIN IMMUNOL 1992;89:933-43.)*

Key words: *Asthma, bronchial provocation tests, airway obstruction, bronchi, edema, adrenalectomy, steroid*

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A number of studies have demonstrated tht the airways of patients with asthma reveal signs of chronic inflammation.[1-7] In the postmortem studies, there is loss of surface-lining epithelium, thickening of the reticular layer beneath the basal lamina of the epithelium, dilation of bronchial blood vessels, and mucosal edema and hypertrophy of both submucosal glands and bronchial smooth muscle. A dense infiltration of inflammatory cells is also present in the airway mucosa.[8-10] Many of the inflammatory and structural changes that are observed in asthma deaths are also a feature of mild asthma. An increase in the number of eosinophils and activated lymphocytes are found in bronchial biopsy specimens from subjects with mild asthma.[11] It has been also demonstrated that subjects with asthma shed significantly higher numbers of epithelial cells,[12] mast cells,[12, 13] and eosinophils[3-5, 12, 14] into the bronchoalveolar lavage fluid than do control subjects. Likewise, asthma exacerbations in other types of asthma may be accompanied by an acute inflammatory response, such as in the late airway response after exposure to allergen[1, 15, 16] or toluene diisocyanate.[17] Inflammation causes vascular dilatation and increased vascular permeability with the formation of an exudate consisting of both plasma protein and migrating inflammatory cells.[18]

Recently, we have developed an animal model of LAR by treating naturally sensitized dogs to *Ascaris suum* antigen with the cortisol-synthesizing inhibitor, metyrapone (Metopirone; Ciba-Geigy Pharmaceutical Co., Tokyo, Japan).[19] Antigen challenge–induced IAR is reported to be associated with an increase in vascular permeability in guinea pigs[20, 21] and atopic subjects with asthma.[22] Likewise, depletion of endogenous corticosteroid by bilateral adrenectomy has been demonstrated to potentiate platelet activating factor–induced increases in vascular permeability.[23] Therefore, in our dog model treated with Metopirone, antigen challenge may cause an increase in vascular permeability as the result of two separate and possibly related conditions, such as depletion of endogenous corticosteroid and allergic reactions. In this study, to examine whether or not airway microvascular permeability increased in association with LAR, we measured the amount of extravasated EB dye and determined abnormally permeable vessels with Monastral blue, histologically, in dogs demonstrating both IAR and LAR and in dogs demonstrating IAR alone. Furthermore, to determine possible sites of airway vascular leakage, we studied vascular permeability in four different-sized airways: trachea, large and small bronchi, and bronchiole.

Abbreviations used

LAR:	Late asthmatic response
IAR:	Immediate asthmatic response
EB:	Evans blue
Rrs:	Respiratory resistance
PNU:	Protein nitrogen unit
PI:	Permeability index
BM:	Basement membrane
CV:	Coefficient of variation

METHODS

We studied adult mongrel dogs, weighing 6 to 12 kg, free of cough and rhinorrhea and with a skin reactivity to 1:100 dilution of *A. suum* extract (Greer Diagnostics, Lenor, N.C.). The dogs were anesthetized with intravenously administered thiopenthal sodium (20 mg/kg). After an endotracheal tube (8 to 10 mm inside diameter) was inserted, a catheter was placed in the femoral vein for drug injection and measurement of serum-cortisol level. The endotracheal tube was inserted approximately 5 cm in depth from the vocal folds, and care was taken to avoid the use of tracheal segments that may have been mechanically manipulated by the endotracheal tube. Dogs were then paralyzed with intravenous pancronium bromide (0.2 mg/kg), supplemented every 60 minutes, and ventilated with a constant-volume ventilator (model 607, Harvard Apparatus, South Natick, Mass.) set at a tidal volume of 15 ml/kg and a frequency of 25 breath per minute with humidified 40% oxygen. The animals were warmed by a heated pad to maintain a rectal temperature of 37° C. To maintain an adequate anesthesia level, we intravenously administered diazepam (0.3 mg/kg) (Yamanouchi Pharmaceutical Co., Tokyo, Japan) every 60 minutes.

Rrs was determined during mechanical ventilation with the forced oscillation technique. The oscillatory pressure was produced by a loudspeaker system driven by a 3 Hz sine wave generator. Tracheal airflow was measured with a heated pneumotachometer (Fleish No. 2, Hewlett-Packard Co., Cupertino, Calif.) and a differential pressure transducer (model MP-45, Validyne Corp., Northridge, Calif.). Airway-opening pressure was measured with another differential pressure transducer (model MP-45, Validyne Corp.). Calculation of Rrs was performed with an analog computer, previously described,[19] and by Hyatt et al.[24] Rrs was calculated as the mean value of resistance during 20 tidal breaths, corrected for the resistance of the endotracheal tube. The CV, calculated from the measured Rrs, ranged from 3.1% to 5.0% in all experiments. Tidal volume was derived by integrating the flow signal. Mouth flow, airway-opening pressure, tidal volume, and Rrs were displayed on a pen recorder (8S, Sanei, Tokyo, Japan).

Skin test

Three weeks before the study, we performed intracutaneous skin tests 30 minutes after intravenous administration of EB dye (10 mg in 2 ml of saline). Subsequently, 0.1 ml of either *A. suum* extract (1:100, Greer Diagnostics) or ragweed extract (1:200, Torii Pharmaceutical Co., Tokyo, Japan) was injected intracutaneously at three points on the shaved chest wall. The skin test reactions were scored positive when a blue wheal of at least 1 cm in average diameter

appeared within 30 minutes. We chose, for the following experiments, dogs that demonstrated a positive skin test to *A. suum* but not to ragweed extract.[19]

Antigen challenge

A. suum aerosol was generated by a jet nebulizer (Vaponefrin; USV-Grossman-Armour Pharmaceutical, Mexico) and delivered via an endotracheal tube at a constant airflow of 5 L/min. The nebulizer output was 0.15 ml/min, consisting of particles whose aerodynamic mass mean diameter was 2.5 μm (manufacturer's specifications). The stock solution of *A. suum* extract (1:10 wt/vol) contained 10^5 PNU/ml and was serially diluted with saline providing a series of *A. suum*-antigen concentrations (1, 10, 10^2, 10^3, and 10^4 PNU/ml). After saline inhalation, the dogs sequentially inhaled the antigen aerosol, beginning with the lowest concentration, until a clear response of more than double the baseline Rrs was observed. Saline or antigen aerosols at each concentration were inhaled for 5 minutes, with an interval of 5 minutes between each challenge.

Experimental protocol 1

The response to *A. suum* antigen was determined in sensitized dogs pretreated with Metopirone (n = 15) or with a vehicle of Metopirone (n = 5). Ten other dogs were pretreated with a vehicle of Metopirone (n = 5) or Metopirone (n = 5), and we performed saline inhalation instead of *A. suum*-antigen inhalation. Metopirone (120 mg/kg), an inhibitor of cortisol synthesis, was injected intravenously 3 hours before the antigen challenge. Half the initial dose of Metopirone was again injected 3 hours after the antigen challenge. The serum-cortisol level was measured before and 8 hours after the antigen challenge with a cortisol RIA kit (CA-529J; Otsuka Assay Laboratory, Tokushima, Japan). All experiments began between 8 and 9 AM, and the examination of vascular permeability with EB dye began 8 hours after the antigen challenge.

Measurement of plasma extravasation

The magnitude of vascular permeability was quantified with a modification of the method according to Saria and Lundberg,[25] which correlates well with extravasation of radiolabeled albumin.[26] EB dye (20 mg/kg), dissolved in saline (10 mg/ml) and filtered with a 5 μm Millipore filter (Millipore Corp., Bedford, Mass.), was injected into a femoral vein. Five minutes later, the animal was killed by an overdose of intravenous pentobarbital sodium. After the chest was opened wide in both the right and left fifth or sixth intercostal spaces, both right and left ventriculotomies were performed. A blunt-ended polyethylene tube was passed via the right ventriculotomy into the pulmonary artery; then, that portion of the incision was clamped. The animal was perfused with saline at a 30 torr pressure to expel intravascular dye from the pulmonary circulation. Then, the same tube was passed via the left ventriculotomy into the aorta, and the animal was perfused with saline at 120 torr pressure to expel intravascular dye from the systemic circulation. After all blood was washed out (about 7 minutes), the trachea, lungs, and esophagus were removed. The trachea (1 cm above the bifurcation to the proximal; 1 cm long) and large bronchi (diameter, >5 mm; 1 cm long) were carefully dissected free from the connective tissues. Small bronchi (2 mm in diameter; 4 mm long) were dissected free from the vessels and surrounding parenchymal tissues under observation with a light microscope. We randomly selected three pieces of large and small bronchi from the 10 pieces from each animal and measured the dye content in each piece of tissue separately. Wet weight of all tissues was measured after gently removing fluid with a blotter. We did not observe removal of the epithelium and submucosal tissue. To quantify the amount of extravasated EB dye, each tissue was immersed in 2 ml of 100% formamide for 24 hours at 50° C to extract the dye, and the concentrations of dye in the solutions were determined spectrophotometrically by measuring the absorbance at 620 nm (220A spectrophotometer, Hitachi Ltd., Tokyo, Japan). Calculations were based on the standard curve obtained by EB dye standards in formamide. The standard curve was linear between EB dye concentrations of 0.5 and 10 μg/ml. To test the reliability and reproducibility, EB dye (2 μg) was added exogenously to small bronchi sample (100 mg) in 2 ml of 100% formamide and incubated for 24 hours at 50° C. The average recovery of EB dye from 10 samples was 1.95 ± 0.03 μg (mean ± SE), and the CV was 4.9%. The EB-dye content of each tissue was expressed as nanograms of dye per milligram of wet weight tissue. To determine whether pressure perfusing the blood vessels altered EB-dye extravasation, we perfused with saline at 40 torr for systemic circulation and at 10 torr for pulmonary circulation in other animals pretreated with Metopirone (n = 3).

Experimental protocol 2

We studied seven dogs treated with Metopirone (n = 7) and three dogs treated with the vehicle of Metopirone (n = 3) for histologic assessment of vascular permeability. All experiments began between 8 and 9 AM, and the examination of vascular permeability with Monastral blue dye began 8 hours after the antigen challenge.

Histologic assessment of vascular permeability and airway narrowing

To visualize vessels developing increased vascular permeability in the airways, animals were injected intravenously with a macromolecular tracer, Monastral blue (30 mg/kg; 3% suspension of copper phthalocyanine blue pigment in 0.85% NaCl; Sigma Chemical Co., St. Louis, Mo.) 8 hours after the antigen challenge (n = 10). Five minutes later, the animals were killed by an overdose of intravenous pentobarbital sodium. Monastral blue remaining in the vessels was then washed out by perfusion of the pulmonary circulation at 30 torr and systemic circulation at 120 torr with 1% paraformaldehyde in 0.05 mol/L of phosphate-buffered saline solution (pH 7.4). After all blood was washed out (about 8 minutes), trachea and lungs were removed and immersed in 1% paraformaldehyde for 3 days, cut into tissue blocks, and fixed again in 1% paraformaldehyde for 1 week. After fixation, the tissue blocks were sliced, dehydrated through a graded series of ethanol, and embedded in paraffin. Tissue blocks were then cut into 4 μm thick sections and stained with hematoxyin-eosin.

More than three pieces of trachea from the upper, middle, and lower portions, more than five large bronchi (diameter,

>5 mm) and >20 small bronchi (2 to 4 mm in diameter) and bronchioles (airways without cartilage of approximately 1 mm in diameter) were selected from each animal. All morphometric measurements described below were performed with a digitizer tablet coupled to a computer, with an image on a television monitor obtained directly from the microscope (PC-9801; NEC Corp., Tokyo, Japan, and LA-500; PIAS Co., Tokyo, Japan). We measured the length of the BM and submucosal area. Airway diameter was estimated by assuming a round airway from the circumference of BM.[27] Because the whole aspects of the trachea and large bronchi could not be observed in a microscopic field, the airway wall was magnified with a photographic enlarger (model V-14, Nikon Profile projector, Tokyo, Japan), and the tracings from the enlarged images were measured.

To evaluate the vascular permeability, we measured the area of vessels in which Monastral blue dye was trapped in the vascular wall and the total area of vessels in the wall. Because Monastral blue was observed solely in the walls of vessels from 10 to 25 μm in diameter (small vessels that are likely postcapillary venules inferred from their size[18] and structure without having smooth muscle) in all sized airways, we measured only these sized vessels. Vessel areas in 10 visual fields for trachea and bronchi, and that of five for bronchiole, were randomly chosen and were summed for each animal. We defined PI as the ratio of the area of vessels with Monastral blue to that of the total vessels in each animal. Because the area of vessels are influenced by the cut of vessels, we also calculated the ratio of the number of vessels in which Monastral blue dye was trapped in the vascular wall to the number of the total vessels. Furthermore, it is possible that recruitment of new vessels during an acute inflammatory process might have increased the denominators, both in terms of area and in terms of the number of vessels. Therefore, we also calculated the mean number of Monastral blue-labeled vessels per square millimeter from seven randomly selected places in one sample.

In preliminary studies, PI was higher in the large and small bronchi than in the trachea and bronchiole; therefore, we determined the submucosal thickness in large and small bronchi from the length of BM and area of submucosa. We chose airways that had a similar length of BM from animals with IAR and LAR. We calculated submucosal thickness by a formula[27] as follows:

$$\text{Submucosal thickness} = \sqrt{BM^2 + 4\pi\,Sm} - BM/2\pi$$

We also measured the thickness of the epithelial layer in large and small bronchi.

Experimental protocol 3

To compare the magnitude of vascular permeability during IAR with that during LAR, we quantified extravasation of EB dye and performed histologic assessment of vascular permeability during IAR. Another 10 dogs pretreated with Metopirone were challenged with *A. suum* antigen (n = 10). When the increase in Rrs by antigen inhalation reached a plateau (within 3 minutes after antigen inhalation), either EB dye (20 mg/kg; n = 5) or Monastral blue (30 mg/kg; n = 5) was injected into a femoral vein. After an additional 5 minutes, the animals were killed by an overdose of intravenous pentobarbital sodium. We measured extravasation of EB dye and performed histologic examination with Monastral blue, as described above. All experiments began between 8 and 9 AM, and the examination of vascular permeability with EB and Monastral blue dyes began within 3 minutes after the antigen challenge.

Data analysis

Results are reported as mean ± SE. For statistical analysis, we used one-way analysis of variance and Duncan's multiple-range test. Significance was accepted at $p < 0.05$. We studied 53 dogs, 30 dogs for EB-dye experiments, and 10 dogs for Monastral blue–dye experiments 8 hours after the antigen challenge, 10 dogs for either EB-dye or Monastral blue–dye experiments during IAR within 3 minutes after the antigen challenge, and three dogs for the study on the influence of perfusion pressure, respectively.

RESULTS

Changes in Rrs after antigen challenge

Dogs with and without Metopirone demonstrated no changes in Rrs throughout the experiment after saline inhalation (control and control with Metopirone, n = 5 each). *A. suum*-antigen aerosols increased Rrs to more than double the baseline values immediately after challenge at a concentration of $(1.2 \pm 0.6) \times 10^3$ PNU/ml in dogs without Metopirone (n = 5) and at a concentration of $(0.9 \pm 0.5) \times 10^3$ PNU/ml in dogs with Metopirone (n = 15). Increased Rrs returned to the baseline values within 3 hours after the antigen challenge, and Rrs did not change thereafter in dogs without Metopirone (IAR, n = 5). However, Rrs increased again between 6 to 8 hours after the antigen challenge in 10 of the 15 dogs treated with Metopirone (LAR, n = 10). The remaining five dogs treated with Metopirone demonstrated IAR alone (IAR with Metopirone, n = 5). We did not observe differences in serum-cortisol levels, skin test results, and provocation concentrations of *A. suum* antigen between dogs treated with Metopirone demonstrating IAR alone and LAR ($p > 0.20$). Changes in Rrs during IAR and 8 hours after the antigen challenge, and the serum-cortisol levels before and after the challenge, are summarized in Table I. The IARs were of similar magnitude among the three groups consisting of IAR, IAR with Metopirone, and LAR. However, the percentage changes in Rrs from baseline values 8 hours after the antigen challenge were significantly higher in the group with LAR than in the groups with IAR and IAR with Metopirone because the dogs of the group with LAR were selected from the delayed increase in the resistance after the antigen challenge. Serum-cortisol levels were significantly higher in the controls and groups with IARs than in the control group with Me-

TABLE I. Changes in Rrs during IAR and 8 hours after antigen challenge, and serum cortisol

	Baseline Rrs (cm H_2O/L/sec)	Rrs during IAR (% baseline value)	Rrs 8 hours after antigen challenge (% baseline value)	Serum cortisol (μg/dl)	
				Before	8 Hours after antigen challenge
C (n = 5)	3.0 ± 0.3	99.6 ± 1.4	101.2 ± 0.8	3.3 ± 0.3	5.0 ± 1.2
C + M (n = 5)	2.5 ± 0.3	102.0 ± 2.1	102.3 ± 2.3	3.0 ± 0.2	<1.0*
IAR (n = 5)	3.0 ± 0.2	398.6 ± 83.2	101.5 ± 0.9	3.1 ± 0.2	5.6 ± 1.6
IAR + M (n = 5)	2.6 ± 0.2	287.3 ± 23.7	114.7 ± 1.7	3.3 ± 0.4	<1.0*
LAR (n = 10)	2.8 ± 0.3	367.2 ± 75.9	190.0 ± 11.0†	2.9 ± 0.2	<1.0*

C, Control; *M*, Metopirone.
Data are reported as mean ± SE.
Comparison of serum cortisol before and 8 hours after antigen challenge was done by adding 1 μg/dl of serum cortisol when level was <1.0 μg/dl 8 hours after antigen challenge.
*$p < 0.01$, significant differences between, before, and 8 hours after antigen challenges.
†$p < 0.01$, significant differences among C, IAR, and LAR.

topirone, IARs with Metopirone, and groups with LAR (Table I).

Vascular permeability to EB dye

Vascular permeability to EB dye did not differ significantly between control dogs with and without Metopirone ($p > 0.50$; n = 5) (Fig. 1). Likewise, the increase in vascular permeability was not significant in any of the tissues in the groups with IAR and IAR with Metopirone when increases were compared with those of control groups with or without Metopirone ($p > 0.10$). Vascular permeability to EB dye was significantly increased in the large bronchi and small bronchi of the group with LAR compared with that of the control groups with and without Metopirone ($p < 0.01$) and compared with that of the groups with IAR and IAR with Metopirone ($p < 0.05$ in large bronchi and $p < 0.01$ in small bronchi). However, EB-dye extravasation in the trachea and esophagus from the group with LAR did not differ significantly from that of the control groups ($p > 0.20$) (Fig. 1). Changes in perfusion pressure did not alter EB-dye extravasation in any of the tissues from the group with LAR (5.9 ± 2.1 ng/ml of tissue in the esophagus, 14.1 ± 6.8 ng/mg of tissue in the trachea, 37.1 ± 7.6 ng/mg of tissue in the large bronchi, and 49.6 ± 6.5 ng/mg of tissue in the small bronchi, respectively; n = 3; $p > 0.50$).

Histologic assessment of vascular permeability

Five of seven dogs treated with Metopirone demonstrated LAR (n = 5) after the *A. suum*-antigen challenge at a concentration of (2.0 ± 0.8) × 10^3 PNU/ml. The remaining two dogs treated with Metopirone and the three dogs without Metopirone demonstrated IAR alone (n = 5) after the antigen challenge at a concentration of (1.7 ± 0.6) × 10^3 PNU/ml. Changes in Rrs during IAR were 274% ± 44% (percent of baseline value) in the group with IAR and 235% ± 27% (percent of baseline value) in the group with LAR and did not differ significantly between groups ($p > 0.20$). However, changes in Rrs 8 hours after the antigen challenge were significantly higher in the group with LAR than in the group with IAR (202% ± 10% of baseline value versus 105% ± 5% of baseline value; $p < 0.01$).

In dogs with IAR alone, no Monastral blue–labeled blood vessels were visible in the airway wall (Fig. 2, *A* and *B*), whereas in the dogs with LAR, a number of such labeled blood vessels were visible (Fig. 2, *C*). The particles were trapped against the BM in the wall of the vessels (Fig. 2, *D*). The distribution of small vessel area (10 to 25 μm in diameter) in dogs with IAR alone and in dogs with LAR are presented in Table II. There were no significant differences between areas in all sized airways. The percentage of small vessel area to total wall area from the highest to the lowest value was the small bronchi > large bronchi > bronchiole > trachea, in that order. The PI was significantly higher in the group with LAR than in the group with IAR in all sized airways ($p < 0.01$) (Fig. 3). Similarly, as in the distribution of small vessel area, the rank order of PI was the small bronchi > large bronchi > bronchiole > trachea. The ratios of the number of Monastral blue–labeled vessels to the number of total vessels were 6.5% ± 0.6% in trachea, 21.6% ± 2.3% in large bronchi, 37.8% ± 5.6% in small bronchi, and 18.4% ± 2.9% in bronchiole, respectively. The number of Monastral blue–labeled vessels per square millimeter were 6.9 ± 0.5/mm^2 in trachea, 78.2 ± 6.1/mm^2 in large bronchi, 128.3 ± 7.1/mm^2 in small bronchi,

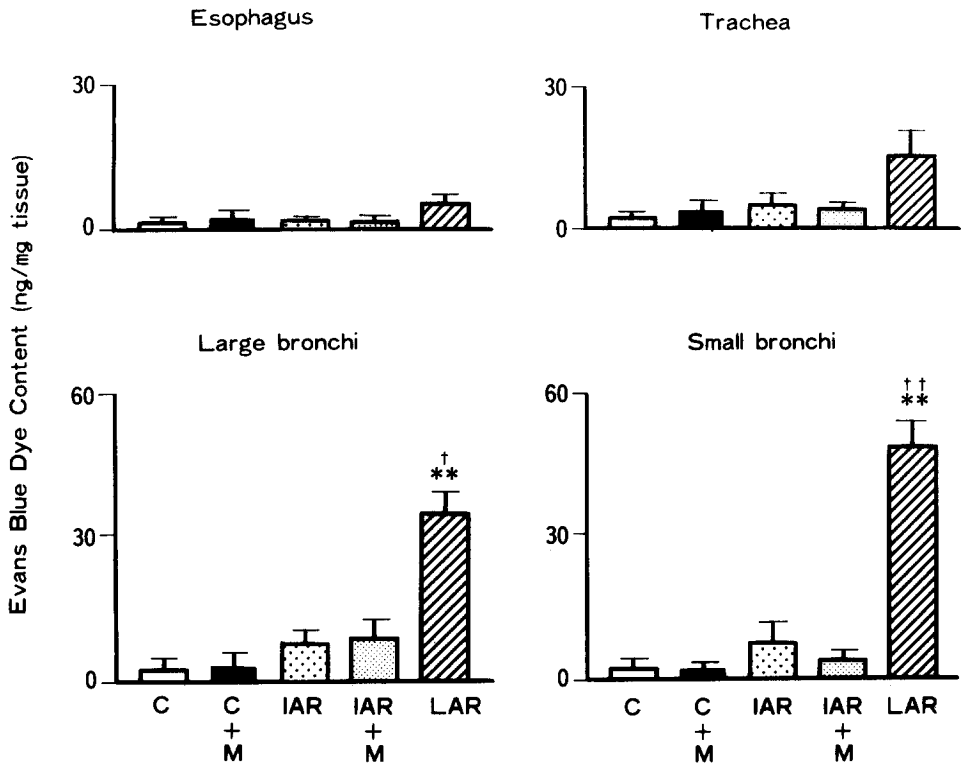

FIG. 1. Amount of extravasation of EB dye from esophagus, trachea, and large and small bronchi 8 hours after antigen challenge; *C,* control dogs not treated with Metopirone and without antigen challenge; *C + M,* control dogs with Metopirone and without antigen challenge; *IAR,* dogs without Metopirone demonstrating IAR alone; *IAR + M,* dogs with Metopirone demonstrating IAR alone; *LAR,* dogs with Metopirone demonstrating both IAR and LAR. Results are reported as mean ± SE; $^{**}p < 0.01$, significant differences from *C* and *C + M*; $^{+}p < 0.05$ and $^{++}p < 0.01$, significant differences between IAR and LAR.

and 48.9 ± 5.2/mm² in bronchiole, respectively. Therefore, results were similar when the PI was determined by either the area or the number of Monastral blue–labeled vessels and when the degree of vascular permeability was inferred from the actual number of Monastral blue–labeled vessels per square millimeter.

Lengths of the BMs used for calculation of the submucosal thickness in the groups with IAR and LAR were 22.5 ± 1.3 mm, and 20.8 ± 0.5 mm for the large bronchi, and 8.4 ± 0.4 mm and 8.5 ± 0.5 mm for the small bronchi. There were no significant differences in length of the BM between the groups with IAR and LAR ($p > 0.20$). However, calculated submucosal thickness was significantly higher in the group with LAR than in the group with IAR only in the small bronchi ($p < 0.01$) (Fig. 4). The thickness of the epithelial layer in the groups with IAR and LAR was 40.0 ± 3.7 μm and 38.8 ± 6.6 μm for the large bronchi, and 28.0 ± 3.1 μm and 26.5 ± 1.7 μm for the small bronchi. There were no significant differences in the thickness of the epithelial layer between the groups with IAR and LAR ($p > 0.20$).

IAR

A. suum-antigen aerosols increased Rrs by 298.6% ± 30.0% (percent of baseline value) in dogs receiving EB dye and by 344.0% ± 78.2% (percent of baseline value) in dogs receiving Monastral blue. Data obtained during IARs within 3 minutes after the antigen challenge are summarized in Table III. EB-dye extravasation in the large and small bronchi during IAR was significantly higher than that from the control group and from the group with IAR alone 8 hours after the antigen challenge ($p < 0.05$) and significantly lower than that from the group with LAR ($p < 0.01$). Likewise, the PI in large and small bronchi during IAR was significantly higher than that from the group with IAR alone 8 hours after the antigen challenge ($p < 0.05$) and significantly lower than that from the group with LAR ($p < 0.01$). However, both parameters in the trachea during IAR did not differ

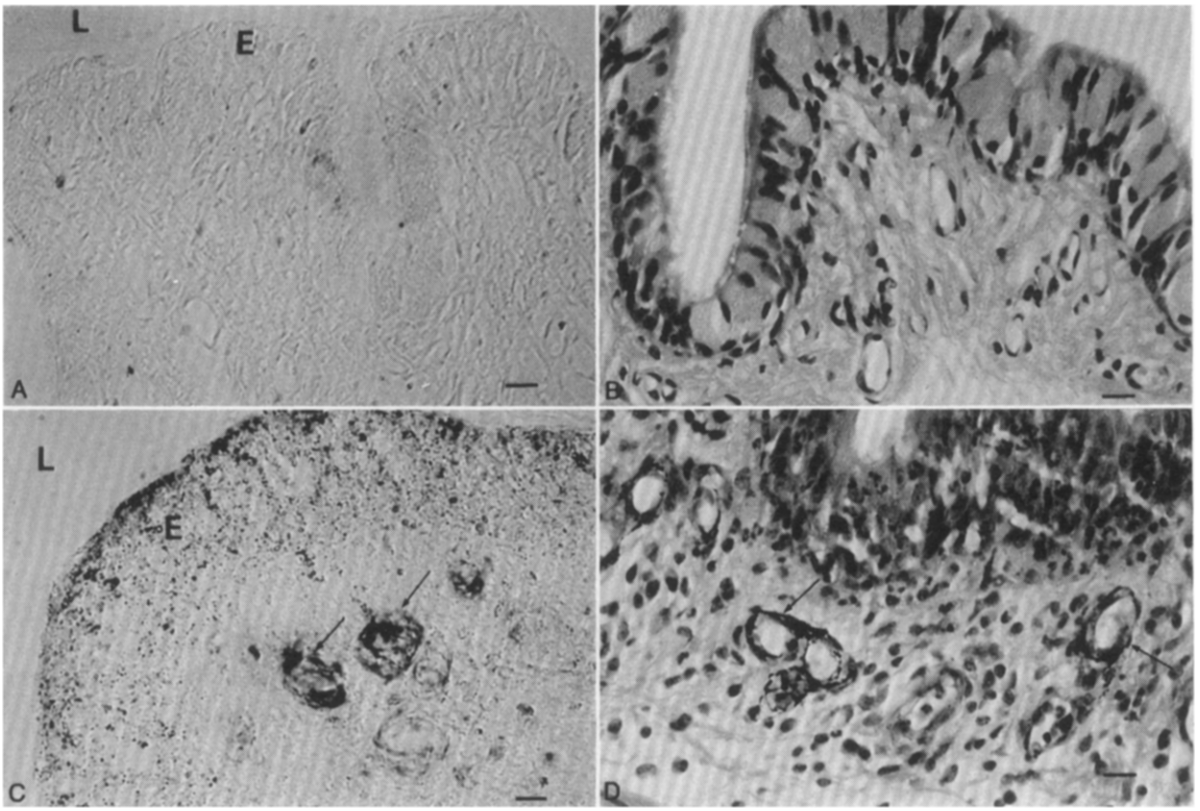

FIG. 2. Light micrograph of sections of small bronchi from dogs. **A** and **B**, With IAR alone. **C** and **D**, With LAR. **A** and **C**, Not stained. (Original magnification ×400.) **B** and **D**, Stained with hematoxylin-eosin indicate small vessels in submucosal layer. (Original magnification ×400.) *Arrows*, Monastral blue–labeled leaking vessels; *L*, lumen of the airways; *E*, epithelium; *Bar*, 25 μm. **A** and **B**, No Monastral blue deposits in walls of vessels in IAR. **C**, However, many Monastral blue–labeled vessels visible in mucosa in LAR and also appeared to be between epithelial cells. **D**, Monastral blue particles trapped against BM in wall of vessels.

significantly from that of the group with IAR alone 8 hours after the antigen challenge ($p > 0.30$). The submucosal thickness during IAR did not differ significantly from that of the group with IAR alone 8 hours after the antigen challenge ($p > 0.20$).

Interobserver and intraobserver reproducibility

To evaluate the results, we examined the interobserver and intraobserver reproducibility for morphometric measurements. Because we did not observe Monastral blue deposits in the wall of vessels in any tissues from dogs with IAR alone 8 hours after the antigen challenge, three parameters of PI, the ratio of the number of Monastral blue–labeled vessels to the number of total vessels, and the number of Monastral blue–labeled vessels per square millimeter did not differ between interobserver and intraobserver. Correlations of interobserver ranged from 0.92 to 0.98 in the PI, from 0.90 to 0.99 in the ratio of the number of Monastral blue–labeled vessels to total vessels, and from 0.93 to 0.99 in the number of Monastral blue–labeled vessels per square millimeter in LAR 8 hours after the antigen challenge and during IAR within 3 minutes after the antigen challenge, and ranged from 0.93 to 0.98 in the submucoal thickness in IAR and LAR 8 hours after the antigen challenge and during IAR within 3 minutes after the antigen challenge, respectively. The CV obtained from a tenfold determination of the same sample by the same observer (intraobserver reproducibility) ranged from 1.6% to 3.3% in the PI, from 1.8% to 3.9% in the ratio of the number of Monastral blue–labeled vessels to total vessels, and from 1% to 2.9% in the number of Monastral blue–labeled vessels per square millimeter in LAR 8 hours after the antigen challenge and during IAR within 3 minutes after the antigen challenge, and ranged from 1.7% to 4.1% in the submucosal thickness in IAR and LAR 8 hours after the antigen challenge and during IAR within 3 minutes after the an-

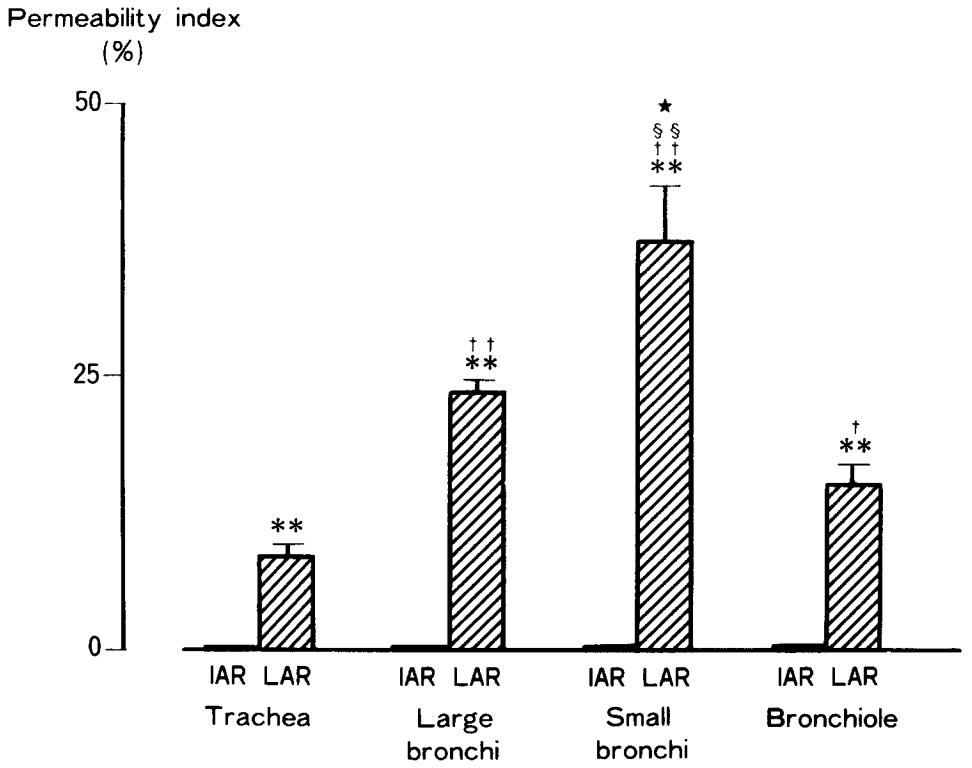

FIG. 3. PI defined as ratio of area of small vessels labeled with Monastral blue to that of total small vessels (10 to 25 μm in diameter) in trachea, large and small bronchi, and bronchiole in IAR (■) and LAR (▨). Results, mean ± SE. PI, nearly zero in all sized airways in IAR; $^{**}p < 0.01$, significant differences from IAR; $^{+}p < 0.05$ and $^{++}p < 0.01$, significant differences from trachea; $^{*}p < 0.05$, significant difference from large bronchi; $^{\S\S}p < 0.01$, significant difference from bronchiole.

TABLE II. Percentage of small vessel area to whole wall area (percent)

	Trachea	Large bronchi	Small bronchi	Bronchioles
IAR (n = 5)	2.7 ± 0.5	6.5 ± 0.5*	8.4 ± 0.9*†‡	6.1 ± 0.4*
LAR (n = 5)	3.0 ± 0.9	7.5 ± 0.4*	10.2 ± 0.6*†‡	7.0 ± 1.1*

Data are reported as mean ± SE.
*$p < 0.01$ compared with that of trachea.
†$p < 0.05$ compared with that of large bronchi.
‡$p < 0.05$ compared with that of bronchioles.

tigen challenge, respectively. Furthermore, the CV obtained from five different slides of the same dog (intradog reproducibility) was <4.8% in all parameters in IAR and LAR 8 hours after the antigen challenge and during IAR within 3 minutes after the antigen challenge. Therefore, the present morphometric data were generally reproducible.

DISCUSSION

Present studies provide evidence that vascular leakage is increased, especially in small bronchi, in association with the occurrence of LAR in dogs when the serum-cortisol level is significantly reduced. However, similarly to the previous studies in sheep,[28] dogs,[19] and guinea pigs,[29] late-phase bronchoconstriction did not occur after the immediate response in all dogs treated with Metopirone in the present study.

Antigen challenge is demonstrated to cause inflammatory changes in both pulmonary airways and vessels.[18, 30, 31] Our observation that an antigen challenge increased vascular permeability during IAR is in agreement with previous studies.[20, 21, 32, 33] The present results thus suggest that the allergen-induced bron-

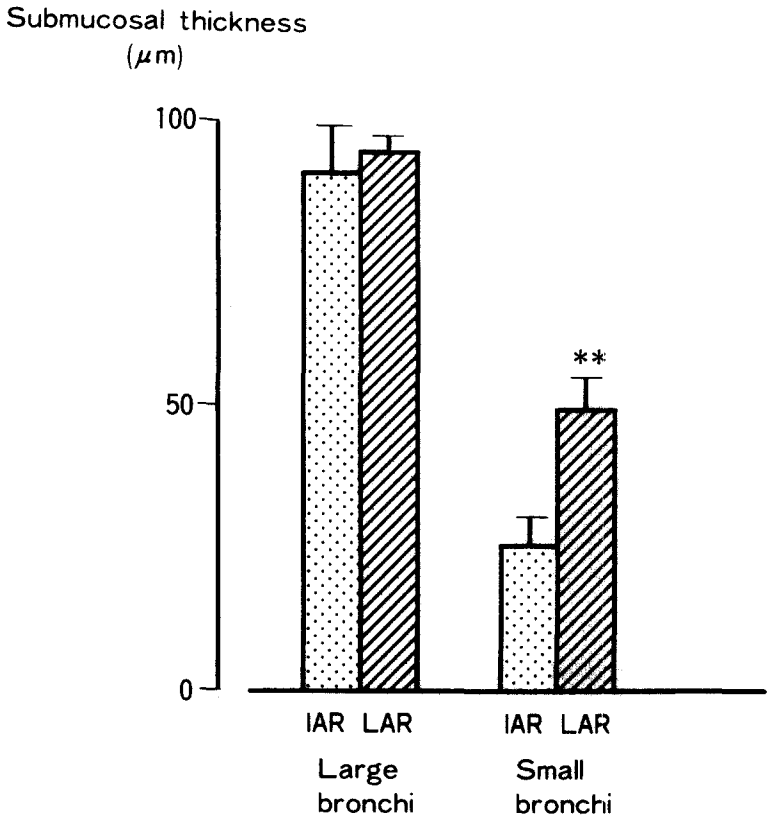

FIG. 4. Submucosal thickness in large and small bronchi in dogs demonstrating IAR (▩) and in dogs demonstrating LAR (▨) 8 hours after antigen challenge. Submucosal thickness, calculated from length of BM and area of submucosa. Constricted segment, standardized into circular state without changing perimeter length of BM, described by James et al.[27] Results, reported as mean ± SE; $**p < 0.01$, significant difference from IAR.

TABLE III. Vascular permeability during IAR

	EB-dye content (ng/mg tissue)	PI (%)	Submucosal thickness (μm)
Trachea	4.6 ± 0.9	0	ND
Large bronchi	16.1 ± 1.1*	9.6 ± 0.5†	78.5 ± 9.0
Small bronchi	26.7 ± 2.5†‡	16.0 ± 0.8†‡	30.5 ± 2.7

ND, Not determined.
Data are reported as mean ± SE for five dogs.
*$p < 0.05$, compared with that of trachea.
†$p < 0.01$, compared with that of trachea.
‡$p < 0.05$, compared with that of large bronchi.

choconstriction might be associated with an acutely increased permeability to macromolecules in the lower airways. However, vascular permeability was not increased in any of the tissues obtained from dogs demonstrating IAR alone at 8 hours after the antigen challenge. Evans et al.[20] performed measurements of vascular permeability 5 and 10 minutes after antigen exposure and found that, after 20 minutes, almost all EB dye had cleared from the tissues. If the time course of edema clearance after IAR is minutes in duration and if edema occurred during IAR in the present study, 8 hours is a sufficient time for clearance.

Bilateral adrenectomy has been demonstrated to increase the microvascular leakage induced by the platelet-activating factor in several organs, including airways in rats.[23] However, vascular permeability did not

differ between control dogs with and without Metopirone, and it did not increase in dogs with Metopirone demonstrating IAR alone. Depletion of endogenous glucocorticoid may not increase vascular permeability, and it is not the mechanism responsible for the increase in vascular permeability observed during LAR in the present study. However, the present study could not point to a precise role of endogenous glucocorticoid in microvascular leakage during IARs and LARs because we did not know the level of serum cortisol during IAR and did not have control dogs with LAR without Metopirone. However, depletion of glucocorticoid by Metopirone induced the LAR in this dog model, as reported previously.[19] Therefore, endogenous glucocorticoid may suppress the initiation of the process causing LAR without influence on the vascular permeability. Alternatively, an increase in vascular permeability may be a consequent phenomenon, after development of LAR.

Increased vascular permeability to EB dye during LAR may not be due to either an increased tendency of airway microvessels to leak albumin in response to perfusion or increased tissue blood pool after washout, because we obtained similar results when perfusion pressure was lowered to approximately one third that of the initial pressure. Second, we normalized the EB dye content by tissue weight. Therefore, EB dye extravasation would be underestimated in trachea and large bronchi as a result of nonvascular tissue, such as cartilage. However, we also assessed vascular permeability during LAR, qualitatively, with a macromolecular tracer, Monastral blue pigment, and obtained similar results, as in the case of EB-dye experiments. Monastral blue is useful in detecting abnormally permeable blood vessels because this pigment does not cross the endothelium of normal blood vessels, but when vascular permeability is increased, the pigment passes through gaps in the endothelium and becomes trapped by the basal lamina surrounding the endothelium.[34] As a result of this trapped Monastral blue, the abnormally permeable blood vessels become labeled and thus can be observed in subsequent morphologic studies.

The increase in vascular permeability during LAR was observed in the large and small bronchi, the latter being more prominent. Pietra and Magno[35] reported that, in dogs, the venular subepithelial plexuses in the large bronchi are located mainly between the cartilage ring structure, and in the smaller bronchi, the plexuses occupy the entire surface of the submucosal layer. Therefore, the abundance of venular plexuses might cause more EB dye extravasation in the small bronchi than in other parts of the airways. The fact that the small blood vessels (10 to 25 μm in diameter) responsible for Monastral blue–labeled leaking vessels during LAR were most abundant in the small bronchi supports the hypothesis that the distribution of blood vessels in which exudation occurs is an important factor in the airway extravasation. Evans et al.,[20] using several receptor antagonists, suggested that different inflammatory mediators acted at different anatomic sites to cause increased microvascular leakage of the airways in guinea pigs. Thus, the small bronchi might be susceptible to unidentified mediators released from either inflammatory cells or the lung tissues during LAR.

The reason for an increase in submucosal thickness observed only in the small bronchi is uncertain. However, the increase in the vascular permeability was highest in the small bronchi compared with that of other parts of the airways. Therefore, excessive plasma extravasation may lead the submucosal layer of the small bronchi to increase in thickness. Alternatively, it is possible that this might be explained by better clearance from the larger airways in which the bronchial veins drain into the azygos and hemiazygos system than in the more distal airways in which it drains into the pulmonary system. Although in IAR an increase in vascular permeability occurs in this dog model, the airway edema induced in association with LAR appears more severe and prolonged in duration and, perhaps, in initiation as well.

In conclusion, the present study suggests that increased vascular permeability and submucosal thickness in the small bronchi are important factors in the pathogenesis of LAR.

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