Labeling of vagal motoneurons and central afferents after injection of cholera toxin B into the airway lumen

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Pérez Fontán, J. Julio, and Christine R. Velloff. Labeling of vagal motoneurons and central afferents after injection of cholera toxin B into the airway lumen. Am J Physiol Lung Cell Mol Physiol 280: L152–L164, 2001.—We tested the hypothesis that application of the subunit B of cholera toxin (CTB) to the airway mucosa would produce labeling of neuronal somata and sensory fibers in the medulla oblongata. Using 125I-CTB as a tracer, we demonstrated that CTB is transported across the tracheal epithelium, but once in the airway wall, it remains confined to the subepithelial space and lamina propria. Despite the rarity of intrinsic neurons in these areas, intraluminal CTB labeled ~10–60 neurons/rat in the nucleus ambiguus and a smaller number of neurons in the dorsal motor nucleus of the vagus. Well-defined sensory fiber terminals were also labeled in the commissural, medial, and ventrolateral subnuclei of the nucleus of the tractus solitarius. Approximately 50 and 90% of the neurons labeled by intraluminal CTB were also labeled by injections of FluoroGold into the tracheal adventitia and lung parenchyma, respectively. These findings demonstrate that a substantial number of medullary vagal motoneurons innervate targets in the vicinity of the airway epithelium. These neurons do not appear to be segregated anatomically from vagal motoneurons that project to deeper layers of the airway wall or lung parenchyma.

parasympathetic system; airway ganglia; airway epithelium; retrograde neuronal markers

The airways receive the majority of their excitatory innervation through parasympathetic nerve fibers, the bodies of which are located in the nucleus ambiguus and dorsal motor nucleus of the vagus (12, 14, 15, 17, 28). These fibers are carried by the vagus nerves to the airway walls where they are thought to synapse obligatorily with an intrinsic network of airway ganglia (1). The function of the ganglia is to modify and relay central parasympathetic inputs to airway smooth muscle, glands, and blood vessels (8, 24).

The diversity of the functions carried out by these various tissues would suggest that airway parasympathetic preganglionic neurons must be segregated anatomically to reflect the location and physiological role of their ultimate effectors. Early studies that used retrograde neuronal tracers indicated that similar to the upper alimentary tract (2, 5) (with which they share a common embryological origin), the airways have a segmental representation in the organization of the vagal preganglionic neurons (17). Unfortunately, tracer methodologies cannot be applied easily to elucidate whether different tissue structures within an airway segment have a specific representation centrally. The main difficulty resides not only with localizing the tracer injections accurately in the desired tissue but also with preventing the injected material from diffusing to adjacent areas (11). As a result, the only viable experimental approach to the question of tissue-specific innervation has been to measure physiological airway responses to exogenous stimulation of specific groups of medullary neurons. Using such a technique, Haselton et al. (13) demonstrated that unlike the nucleus ambiguus, activation of neurons in the dorsal motor nucleus of the vagus with homocysteic acid does not increase total lung resistance in dogs. This observation has led to the speculation (15) that neurons projecting to the airways from the dorsal medulla innervate only airway mucus glands and blood vessels.

In the present study, we tested the hypothesis that transepithelial transport of the subunit B of cholera toxin (CTB) deposited into the tracheal lumen would label a distinctive population of preganglionic parasympathetic neurons and sensory fibers in the rat medulla. This hypothesis was based on the recent demonstration that CTB, a widely used retrograde and anterograde neuronal tracer (10, 22), undergoes transcytotic transport from the apical to the basolateral membrane of polarized intestinal epithelial cells (21). We performed three sequential experiments. First, we used radiolabeled CTB to confirm that a transport mechanism similar to that described for the intestine exists in the airway epithelium where CTB is translocated from the airway lumen to the subepithelial space and lamina propria. With such information in hand, we then analyzed the topographic distribution of the medullary neurons and sensory fibers labeled by intraluminal CTB. Finally, we examined the question of whether the neuronal population labeled with intraluminal CTB has innervation targets outside the airway...
mucosa. This was accomplished by counting the number of neurons double labeled by injections of CTB into the tracheal lumen and FluoroGold into the dorsal wall of the trachea or the lung parenchyma.

**METHODS**

All experiments were performed in male Sprague-Dawley rats (weight, 350–500 g; age, 10–16 wk; Charles River Laboratories, Wilmington, MA) following protocols approved by the Washington University (St. Louis, MO) Animal Studies Committee. The rats were kept at 23°C with access to standard rat feed and water.

**Transepithelial transport of radiolabeled CTB in the airways.** We performed two series of experiments with 125I-CTB to determine whether, as we hypothesized, CTB is transported across the airway epithelium and if so, to define its subsequent distribution in the airway wall. For both series, 125I-CTB was prepared as described previously (19) with 2 mCi of Na 125I (New England Nuclear, Boston, MA) and 2 mg of 125I-CTB suspension was then injected into the tracheal lumen. The catheter was finally removed, allowing the tracheal lumen to contact with tracheal secretions. The larynx was packed with cotton to prevent dehiscence. The adventitia was then sewn at the edges of the tracheal stoma to the subcutaneous spaces. After a small volume of air was aspirated to ascertain that the needle tip was not in contact with the tracheal wall, a 10-μl volume of 0.1% CTB suspension was injected into the tracheal lumen. The objective of this transmural technique was to avoid any potential disruption of the tracheal mucosa by the endotracheal cannula. Although the needle puncture interrupted continuity of the epithelial lining, we judged that this interruption was too small to result in substantial contact of deep tracheal tissues with the injectate.

Finally, in a third group of rats (n = 10), a 50-μl volume of 0.1% CTB was instilled directly into the tracheal lumen through a tracheostomy. The tracheostomy made it possible to occlude the larynx for a period of 24 h after the instillation, thereby preventing entry of CTB into the gastrointestinal tract. The rats were anesthetized with pentobarbital sodium. After the neck was shaved, a rhomboidal section of skin was removed to prevent obstruction of the tracheostomy stoma by redundant neck tissue. A midline incision was made through the subjacent muscle and fascia to expose the trachea. The first intercartilaginous space was incised transversely, leaving the dorsal wall of the trachea intact. The adventitia was then sewn at the edges of the tracheal stoma to the subcuticular layer of the skin with interrupted suture on a noncutting needle, carefully avoiding perforation of the tracheal mucosa. The stitches were positioned to create a small ridge in substantial contact of deep tracheal tissues with the injectate. double labeling. We used two labeling strategies.

**Double-labeling experiments.** The objective of these experiments was to determine whether the parasympathetic preganglionic neurons labeled by intraluminal CTB have concurrent innervation targets outside the airway mucosa.
In the first (n = 11 rats), we combined instillation of CTB into the tracheal lumen with injections of FluoroGold (Fluorochrome, Denver, CO) into the adventitial membrane that covers the dorsal wall of the trachea. Both the longitudinal trunk and superficial muscular neuronal plexi, which comprise the majority of the intrinsic neurons of the trachea, are embedded in this membrane (3, 9, 33). The bodies and, presumably, the short dendrites of the neurons from these plexi, however, are separated from the tracheal mucosa by several tissue barriers including the trachealis muscle. The injections were performed under a dissecting microscope while the rats were anesthetized with halothane piped into the inspiratory limb of the ventilator. CTB (100 μl of a 0.1% suspension) was injected through an endotracheal cannula as described in Intratracheal instillation of CTB. FluoroGold (0.5 μl of a 4% solution) was injected with a glass micropipette mounted on a micromanipulator (Stoelting, Wood Dale, IL). For this purpose, we first lifted the tracheal adventitia immediately anterior to the tracheoesophageal angle with the tip of the pipette. We then advanced the pipette for a short distance to an oblique dorsal direction that kept the tip of the pipette oriented away from the tracheal lumen. The injectate was divided into two to five injections distributed over the length of the extrathoracic trachea. After each injection, the site was blotted with a cotton-tipped probe; at the completion of the injections, the bed of the incision was rinsed with warm saline before the muscle and skin layers were closed with continuous sutures.

In the second strategy (n = 6 rats), we combined intratracheal injections of CTB with injections of FluoroGold into the lung parenchyma. In previous studies (12, 17, 27, 28), we and others have shown that retrograde neuronal markers injected into the lung tissue label numerous vagal motoneurons in the nucleus ambiguous and dorsal motor nucleus of the vagus. This finding is surprising because the lung periphery of mature mammals is considered to be devoid of parasympathetic ganglia (31) and therefore offers no apparent innervation targets for preganglionic neurons. The experiment described here sought to establish whether the same neurons can be labeled by parenchymal injections of FluoroGold and by intraluminal CTB (which we also found confined to an area lacking intrinsic neurons). CTB (100 μl of a 0.1% suspension) was injected through an endotracheal cannula as described in Intratracheal instillation of CTB. FluoroGold (2 μl of a 4% solution) was injected with a micropipette into the apical lobe of the right lung, which was exposed through a thoracotomy of the fourth intercostal space performed with a 1- to 2-mm depth below the visceral pleura, and lung volume was maintained constant to avoid tearing the lung tissue. After each injection, the pleural surface was blotted with a cotton-tipped probe; at the completion of all injections, the lung surface was washed thoroughly with warm normal saline and allowed to dry for 3–5 min before the thoracotomy was closed by layers. A small Silastic tube was left in the pleural space to evacuate residual air until the rat resumed spontaneous breathing. To rule out the possibility that FluoroGold entering the lung air spaces during the injections could undergo subsequent absorption, we examined the brain stems of six additional rats injected with 10 μl of 15% FluoroGold solution into the tracheal lumen. These injections were carried out through an endotracheal cannula with the rats under halothane anesthesia in a fashion similar to that described for CTB.

Identification of labeled neurons and sensory fibers in the brain. On recovery from anesthesia, the rats were returned to the holding facility where they remained for a period of 11–13 days. At the end of this period, each rat was anesthetized with pentobarbital sodium. The heart and large vessels were exposed, and heparin (1,000 U/kg) was injected directly into the right ventricle. A cannula was then inserted into the aortic root, and the systemic circulation was perfused with 0.1 M sodium phosphate-buffered saline followed by 4% buffered paraformaldehyde (pH 7.40), allowing the effluent to exit through a nick in the right atrium. Finally, the brain and spinal cord were removed, placed in 4% paraformaldehyde for 2 days, and stored in buffered 30% sucrose until sectioned.

The brain stem and cervical spinal cord were cut into transverse 50-μm sections on a freezing microtome. A 1-in-5 series of these sections (amounting to 16 sections/rat medulla) was placed for 30 min at room temperature in a blocking solution of 5% donkey serum (Sigma) in 0.3% Triton X-100 buffered with 0.02 M potassium phosphate. The section series was then incubated overnight at room temperature with primary antisera against CTB (1:10,000 dilution, goat-raised; List Biological Laboratories), FluoroGold (1:500 dilution, rabbit-raised; Chemicon International, Temecula, CA), or both (double-labeling studies). A few representative medullary sections were incubated with CTB antisera and a guinea pig- raised antisemum that recognizes residues 24–130 of the rat, β-preprotachykinin (1:500; courtesy of J. E. Krause Neurogen, Branford, CT (23)) to identify CTB-labeled sensory fibers that contained tachykinins (C fibers). After they were rinsed with buffered saline solution, the sections were placed for 3–4 h in either a 1:100 dilution of biotinylated anti-IgG antiserum (single-labeling studies) or 1:50 dilutions of fluorescent-labeled [FITC and tetramethylrhodamine isothiocyanate (TRITC)] anti- IgG antisera (double-labeling studies) at room temperature (all raised in donkey; Jackson ImmunoResearch Laboratories, West Grove, PA). After thorough washing with buffered saline, the sections exposed to biotinylated antisera were incubated for 1 h with an avidin-horseradish peroxidase conjugate (VECTASTAIN ABC kit, Vector Laboratories, Burlingame, CA) and stained with 0.05% diaminobenzidine tetrahydrochloride in potassium phosphate-buffered saline containing 0.003% hydrogen peroxide. Staining was intensified by sequential treatment with silver nitrate for 1 h at 56°C, 0.2% gold chloride for 15 min at room temperature, and 5% sodium thiosulfate for 5 min, also at room temperature. The tissue was then counterstained with 0.6% thionin in 0.2 M acetic acid buffer. The sections exposed to fluorescent antisera were simply washed with buffered saline. All the processed sections were mounted on gelatinized glass slides, covered with a buffered glycerol solution (with 0.1% p-phenylenediamine if appropriate to reduce fading during fluorescent viewing), and protected with glass coverslips.

Localization of intrinsic neurons in the rat trachea. The topographic distribution of the intrinsic neurons of the trachea has been analyzed qualitatively and quantitatively in a variety of species including rats (3, 4, 6, 7, 9, 33). The descriptions resulting from these analyses, however, have varied widely depending on the methodology used to identify the neurons and whether the investigators concentrated their attention only on cholinergic parasympathetic ganglia associated with the nerve trunks. Accordingly, to facilitate the interpretation of our findings, we obtained transverse paraffin-embedded sections of the trachea and esophagus and the lungs of six rats of age and weight analogous to those of the rats used in the preceding experiments. After deparaffinization, the sections were incubated with a rabbit-raised antisemum against rat neurofilament M (Chemicon International) followed by a FITC-labeled donkey anti-rabbit IgG antibody to stain neurons and nerve fibers.
Data analysis. Brain and cervical spinal cord sections treated with biotinylated and fluorescence-labeled secondary antisera were examined with bright-field and fluorescence microscopy, respectively. Labeled neuronal somata and nerve fibers were annotated on a computerized chart of the rat brain (25), which, after some modification, was also used to illustrate the position of the neurons in Figs. 4, 7, 9, and 10. For analysis purposes, labeled neurons were counted in medullary sections corresponding to ~0.5-mm intervals, starting 15 mm below the bregma. Some of the figures, however, contain images selected from intermediate sections for their clarity. The total neuronal counts in the nucleus ambiguus and dorsal motor nucleus of the vagus were analyzed for differences between methods of CTB injection with a Kruskal-Wallis single-factor analysis of variance by ranks. Numerical variables are presented as means ± SD or as medians and 10th and 90th percentiles, depending on whether the individual values followed a normal distribution. All the microphotographs shown in the illustrations were scanned digitally into Adobe Photoshop (Adobe Systems, San Jose, CA) for display and labeling. Whole image color and contrast were adjusted during the scanning process to reproduce the conditions viewed under the microscope.

RESULTS

Transepithelial transport of radiolabeled CTB in the trachea. The first series of experiments that used radiolabeled CTB examined the distribution of 125I-CTB in the wall of a tracheal segment bound proximally by the larynx and distally by a tracheostomy immediately proximal to the thoracic inlet. Autoradiographic anal-
ysis of tracheal sections confirmed that CTB is indeed transported across the tracheal epithelium (Fig. 1). The radioactive tracer was detected both in the epithelial cells lining the tracheal lumen and in the subepithelial space and lamina propria at all the time points selected for tracheal fixation (3–7 h after the instillation). In the lamina propria, the tracer was concentrated in cells with the morphological characteristics of macrophages. Unless bound to these cells, radioactivity was absent from the tracheal lumen, even in the rats killed 3 h after instillation of CTB. We found no radioactive tracer in the deeper layers of the submucosa, trachealis muscle, or tracheal adventitia. Small amounts of radioactivity were present in the lumen of the esophagus, especially in its cervical section, where it probably accumulated as a result of diminished swallowing activity during the prolonged anesthesia. 125I-CTB was not incorporated into the esophageal mucosa.

Distribution of radiolabeled CTB in the airways and lungs. The second series of experiments studied the localization of radioactivity in the lungs after injection of a bolus of 125I-CTB suspension into the trachea. In this instance, the radiolabeled protein was allowed to progress distally into the airways not only to obtain information about its dispersion in the lungs but also to determine whether the transepithelial absorption observed in the trachea occurred in smaller airways. Contact autoradiographs obtained at various times (3 h to 7 days) after the injection demonstrated accumulations of the radiolabeled tracer in the tracheal and bronchial epithelia and in the lumen of both conducting airways and distal air spaces. Radioactivity was distributed in a patchy pattern throughout the lung parenchyma (Fig. 2), each patch corresponding to the area subtended by an individual bronchus. At the times studied, the esophagus, duodenum, and ileum had no detectable radioactive residue.

Microscopic analysis demonstrated the concentration of 125I-CTB in the epithelium and subepithelial space of the intrapulmonary airways from main stem bronchi to small bronchioles (Fig. 3). Radioactive tracer was present in these locations at all the time points included in the experiment, from 3 h to 7 days after the injections. The signal intensity, however, decreased noticeably with time. We found no signs of radiolabeled CTB in the bronchial adventitia or in the peribronchial or perivascular tissue. Unlike the epithelium of the conducting airways, the epithelial cells lining the peripheral air spaces had no obvious uptake of radioactivity. The radiolabeled protein appeared to form deposits on the alveolar surfaces of the epithelium. Although we cannot exclude that CTB was transported into the interstitial space of the acinus, the persistence of CTB alveolar deposits 7 days after the injections contrasted sharply with the absence of radioactivity in the airway lumina, suggesting that the radiolabeled marker was not absorbed by alveolar cells and thus could not be removed from the air spaces by blood or lymphatic vessels.

Neuronal soma and sensory fiber labeling by intratracheal CTB. Injection of CTB into the tracheal lumen produced extensive labeling of both neuronal somata and sensory fibers in the medulla. Labeled neurons

![Fig. 2](image-url)
were found in all 6 rats injected through the endotracheal cannula, in 5 of 10 rats injected through the tracheal wall, and in all 6 rats that survived after being injected through a tracheostomy.

Neuronal somata were labeled bilaterally in the areas of both the nucleus ambiguus and dorsal motor nucleus of the vagus. The neurons labeled in the nucleus ambiguus were typically multipolar and were
either concentrated in the compact formation or distributed more loosely in the external formation, sometimes straying in close proximity to the anterior medullary surface (Fig. 4). Overall, CTB-labeled neurons formed a 3- to 4-mm column in the ventral medulla, extending from the area often referred to as the nucleus retroambigualis to the caudal end of the facial nucleus. The density of the column increased notably between 13 and 11.5 mm below the bregma (Fig. 5). The neurons labeled in the dorsal nucleus of the vagus were more sparse (Fig. 6) and more homogeneously distributed along the caudal-cranial axis than those labeled in the nucleus ambiguus. The method used to inject CTB into the tracheal lumen had no effect on the distribution (Fig. 5) or number (Table 1) of labeled neurons, even though the doses of CTB varied substantially with each method.

Sensory fibers were also labeled bilaterally by CTB. Labeled fibers and fiber terminals formed distinctive fields in the area of the nucleus of the tractus solitarius (Fig. 6), converging in the commissural subnucleus caudally and in the medial and ventrolateral subnuclei more rostrally. Labeled sensory fibers became rare rostral to 13 mm below the bregma. Only a small proportion of the fibers labeled by CTB were immuno-
reactive for β-preprotachykinin (Fig. 7), possibly denoting that CTB has a greater affinity for thick than for thin fibers as proposed for its horseradish peroxidase conjugate (30).

**Double-labeling experiments.** Labeling of neuronal somata by CTB injected intraluminally and FluoroGold injected into the tracheal adventitia coexisted in all of the 11 rats that underwent the double injections.

The topographic distribution of the neurons labeled by the two markers was similar (Fig. 8). The number of neurons labeled by FluoroGold, however, was considerably larger (Table 2), especially in the area of the dorsal motor nucleus of the vagus where labeled neuronal somata usually exceeded 50. Double labeling, indicating uptake of the two markers by the same neuron, was present at all levels of the medulla (Fig. 9). Approximately half the neurons labeled by CTB in the nucleus ambiguus were also labeled by FluoroGold (median proportion in all rats = 50%, 10th percentile = 15%, 90th percentile = 73%).

Neuronal soma labeling by both markers also coexisted in all six rats injected with CTB into the tracheal lumen and FluoroGold into the right apical lobe. In this case, however, double labeling was present in the majority of the CTB-labeled neurons (90 ± 18% for nucleus ambiguus neurons), indicating a surprising identity between the two neuronal populations (Figs. 8 and 10).

FluoroGold produced no detectable labeling of sensory fibers. We also found no neuronal somata labeled by FluoroGold in any of the six rats injected with this marker in the tracheal lumen.

**Localization of parasympathetic ganglia in the rat trachea.** In the trachea, the majority of the neurofilament M-immunoreactive cells were located in the dorsal adventitia either in association with the longitudinal nerve trunks that run between the trachea and the esophagus or immediately superficial to the trachealis muscle (Fig. 11). A smaller number of neuronal somata were found in the submucosa, also in the vicinity of the trachealis muscle and, more rarely, in the lamina propria, coming in close proximity to the tracheal epithelium. In the lung tissue, neuronal somata were present only in the adventitial surfaces of large bronchi and pulmonary vessels (Fig. 11).

**DISCUSSION**

Our results demonstrate that CTB is transported from the luminal to the basal surface of airway epithelial cells where it is taken up by a substantial number of vagal sensory and motor fibers. Immunohistochemical identification of the labeled motoneurons revealed the existence of an unexpectedly dense innervation of the tracheal and bronchial subepithelial space by medullary vagal motoneurons. (Our observations suggest that it is less likely that CTB was absorbed by alveolar

Table 1. Total number of CTB-labeled neurons with 3 different methods of CTB injection

<table>
<thead>
<tr>
<th>Injection Route</th>
<th>Nucleus Ambiguus</th>
<th>Dorsal Motor Nucleus</th>
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<tr>
<td>Endotracheal cannula</td>
<td>23 (11–39)</td>
<td>6 (0–9)</td>
</tr>
<tr>
<td>Transmural injection</td>
<td>24 (2–38)</td>
<td>2 (0–5)</td>
</tr>
<tr>
<td>Tracheostomy</td>
<td>27 (13–58)</td>
<td>2 (0–3)</td>
</tr>
</tbody>
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Values are medians; nos. in parentheses, 10th–90th percentiles. Cholera toxin B (CTB) was injected into the tracheal lumen. Tracheostomy was performed after occlusion of the larynx to prevent CTB from entering the gastrointestinal tract.
cells, but we cannot exclude that some labeled neurons projected to interstitial targets). Contrary to our stated hypothesis, however, these motoneurons lack a distinctive topographic distribution compared with motoneurons labeled by direct injection of retrograde tracers into the tracheal wall or the lung parenchyma (12, 14, 15, 17, 27, 28). Moreover, many of the neurons labeled by CTB via the transepithelial route appear to have concurrent innervation targets in the tracheal adventitia and in the lung tissue. These findings contradict the idea that vagal motoneurons are segregated anatomically by the functions of their effector tissues in the airways. They also raise the possibility that some medullary neurons innervate airway targets without interposition of intrinsic neurons.

Transepithelial transport of CTB and labeling of medullary neurons. The pathway of absorption and subsequent disposition of CTB in the trachea recapitulates much of what is known about the transport of cholera toxin in the intestine. When placed in contact with the luminal pole of intestinal cells, the two subunits of the toxin, A and B (CTB), undergo vesicular transport initiated by the binding of CTB to a ganglioside GM1 receptor on the cell surface (20, 21). The process, which interestingly is three times more efficient for CTB alone than for the two subunits combined (21), directs the toxin to the basolateral surface of the cell where subunit A exerts its catalytic action. In the intestinal wall, CTB binds to macrophage-like cells analogous to the ones that we observed in the trachea, a property that may facilitate the strong immunogenicity of the toxin (29).

The idea that once it is transported across the epithelium, CTB remains confined to the subepithelial space and lamina propria is crucial to our interpretation of the neuron- and fiber-labeling patterns reported here. This idea is substantiated by the absence of radioactivity in the tracheal and bronchial submucosa, muscle, and adventitia after intraluminal injections of 125I-CTB. Even when present in the tracheal lamina propria, the radioactive tracer was associated with macrophage-like cells, which may have carried it there from the subepithelial space.

Selectivity of retrograde and anterograde neuron labeling. In the classic conception of the airway parasympathetic system, vagal motoneurons innervate tissue effectors only through synapses with airway parasympathetic ganglia (1). However, ganglia are rarely found in the lamina propria and are absent altogether from the bronchial subepithelial space and the alveolar interstitium (3, 9, 33). How then could a retrograde marker such as CTB, which is not transferred transsynaptically or taken up by intact fibers of passage (22), have labeled such a large number of medullary neurons from these locations?

To answer this question, we must first consider the possibility that medullary vagal motoneurons were labeled by CTB at sites other than the subepithelial space or lamina propria (nonselective labeling) of the airway. CTB was injected into the airways at doses greater than those used by other investigators in direct tissue injections. These large doses, however, do not appear to have resulted in nonspecific labeling of neurons as shown best by the absence of labeled
neurons on the side of the medulla ipsilateral to the cervical vagotomies. Deep injury to the tracheal or laryngeal mucosa during endotracheal cannulation may have placed luminal CTB in potential contact with preganglionic fibers innervating the deeper neuronal plexi. We believe that such an event is unlikely, however, because the intensity of neuron and sensory fiber labeling was unchanged when CTB was injected through the tracheal wall with a needle or was placed directly into the tracheal lumen through a tracheostomy. Furthermore, when we injected FluoroGold intraluminally after cannulating the trachea with a similar technique, we found no labeled neurons in the medulla. Swallowing of CTB transported to the pharynx by the retrograde clearance mechanisms of the trachea may have also placed the neuronal marker within the reach of gastrointestinal parasympathetic neurons. Radiolabeled CTB was certainly not transported into the walls of the pharynx or esophagus, which are the only segments of the upper alimentary tract in which motor innervation originates primarily from the nucleus ambiguus (5). Although gastrointestinal absorption of CTB could account for labeling of some neurons in the dorsal motor nucleus of the vagus, extensive neuron labeling via the stomach or intestine seems unlikely. Laryngeal occlusion did not alter the topographic location or quantity of the neurons labeled by CTB injected through a tracheostomy. Moreover, the pattern of sensory fiber labeling did not coincide with that reported after injections of anterograde neuronal markers into upper gastrointestinal organs. Consistent with the findings of other studies of the sensory innervation of the trachea and lungs (15, 18), the highest density of labeled sensory terminals was in the commissural subnucleus of the nucleus of the solitary tract below the obex and in the medial and ventrolateral subnuclei above the obex. In contrast, Altschuler et al. (2) reported sensory fiber labeling of the central and gelatinous subnuclei after injections of anterograde tracers into the esophagus and stomach of the rat.

Fig. 8. Distribution of parasympathetic preganglionic neurons labeled in the RNA and LNA by CTB injected into the tracheal lumen and FluoroGold (FG) injected into either the dorsal aspect of the tracheal adventitia (A) or the apical lobe of the right lung (B). Counts of single (CTB or FG) and double-labeled (CTB + FG) neurons are plotted at 0.5-mm intervals from 14.5 to 11.5 mm caudal to the bregma. Each symbol represents an individual rat. The large proportion of double-labeled neurons suggests that the parasympathetic neurons labeled by intraluminal CTB also have innervation targets in the trachea and lung tissue.
Innervation targets of neurons labeled retrogradely by CTB. If we exclude nonselective labeling, we are left with only two plausible explanations for our findings. The first is that CTB labeled a population of medullary vagal motoneurons that provide innervation to the few intrinsic neurons found in the tracheal lamina propria (see Fig. 11). The function of these neurons can only be presumed from their location. In animals that, like the dog, have a well-developed system of tracheal glands, intrinsic neurons located in this layer or in the neighboring submucosa can be seen sending nerve fibers to the glandular epithelium (33). It is therefore reasonable to speculate that the lamina propria and submucosal neurons play a role in the regulation of tracheal secretions. Even if these neurons are indeed the target of the medullary neurons labeled by intraluminal CTB, it is difficult to reconcile their rarity in the trachea (they are absent altogether from the bronchial mucosa) with the abundance of CTB-labeled neuronal somata in the medulla.

This disparity prompts a second explanation, this one based on the idea that some medullary vagal motoneurons may provide direct innervation to epithelial or vascular effector organs in the airway wall. Such a proposal is not without precedent in other divisions of the parasympathetic system. Specifically, both degeneration studies after eyeball enucleation and retrograde tracer experiments indicate that the ciliary muscle receives a substantial proportion of its parasympathetic nerves directly from midbrain neurons, bypassing the ciliary ganglion (16, 32). Although further experimental evidence is needed, a similar arrangement for the vagal innervation of the airways and lungs could also explain the extensive labeling of medullary neurons by CTB, FluoroGold, and even pseudorabies virus injected into the lung parenchyma (12, 27, 28) where ganglion neurons are also rare.

Specificity of vagal innervation: double-labeling experiments. Regardless of the ultimate identity of their innervation targets, many of the individual medullary neurons labeled by intraluminal CTB were also labeled by FluoroGold injections into the tracheal adventitia or the lung parenchyma. This finding, which was highly consistent between rats, denotes a surprising lack of specificity in the distribution of parasympathetic inputs in different tissues within the airway wall.

Table 2. Total number of labeled neurons after concurrent injections of CTB and FluoroGold

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<thead>
<tr>
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<th>CTB Only</th>
<th>FluoroGold Only</th>
<th>Double Labeled</th>
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<tr>
<td><strong>CTB intratracheal, FluoroGold into tracheal adventitia</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Nucleus ambiguus</td>
<td>5 (3–15)</td>
<td>49 (27–103)</td>
<td>5 (1–9)</td>
</tr>
<tr>
<td>Dorsal motor nucleus</td>
<td>1 (0–3)</td>
<td>&gt;50</td>
<td>1 (0–9)</td>
</tr>
<tr>
<td><strong>CTB intratracheal, FluoroGold into right apical lobe</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Nucleus ambiguus</td>
<td>2 (0–7)</td>
<td>104 (54–126)</td>
<td>11 (5–20)</td>
</tr>
<tr>
<td>Dorsal motor nucleus</td>
<td>0 (0–1)</td>
<td>6 (1–23)</td>
<td>5 (0–11)</td>
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</tbody>
</table>

Values are medians; nos. in parentheses, 10th–90th percentiles. CTB was injected into the tracheal lumen; FluoroGold was injected into either the dorsal tracheal adventitia or the apical lobe of the right lung. Double labeled, CTB + FluoroGold.

Fig. 9. Double exposure demonstrating simultaneous labeling of NA neurons (bregma −12.5 mm) by CTB instilled through a tracheostomy into the tracheal lumen and FluoroGold injected into the dorsal aspect of the tracheal adventitia. Neuronal somata labeled by CTB and FluoroGold are colored in red and green, respectively, by TRITC- and FITC-conjugated antisera. Double-labeled neurons appear yellow by the superposition of the 2 colors (arrowheads). [Modified from Paxinos and Watson (25).]

The FluoroGold injections into the tracheal adventitia were designed to reach the vicinity of the longitudinal nerve and superficial muscular plexi, which contain the majority of the intrinsic neurons of the mammalian trachea (3, 9, 26, 33). Because of the existence of a natural cleavage plane between the trachea and the esophagus, the small volume of injectate, and the precautions taken to orient the pipette away from the tracheal lumen, we believe that the injected FluoroGold remained separated from the tracheal submucosa or mucosa. The extensive FluoroGold labeling of neuronal somata in the dorsal motor nucleus of the vagus suggests that the spread of these injections included endings from motor fibers innervating the...
esophagus, an unavoidable consequence of the proximity of this organ to the trachea (and also an additional proof that the injections were located on the dorsal side of the tracheal adventitia).

The FluoroGold injections into the right lung were intended to label what we believed to be a population of vagal motoneurons committed to the innervation of intrapulmonary ganglia. This interpretation needs to be revised, however, in light of the current results. Specifically, the fact that most of the neurons labeled by intraparenchymal injections of CTB were also labeled by FluoroGold has two potential implications to the contrary. First, there is clearly a subset of neurons labeled by the intraparenchymal injections, the endings of which are within the reach of CTB molecules translocated across the airway epithelium. This is, once again, unlikely to be the case in large bronchi where ganglia reside (24, 31, 33) because in those locations, the bodies

Fig. 10. Double photographic exposures demonstrating labeling of neuronal somata (bregma—13 mm) in the DMV (top) and NA (bottom) after injections of CTB into the tracheal lumen and FluoroGold into the apical lobe of the right lung. Neurons labeled by CTB and FluoroGold are colored in red and green, respectively, by TRITC- and FITC-conjugated antisera. Three neurons in the compact formation of the NA are labeled by both retrograde markers and thus appear yellow by the superposition of the 2 colors (arrowhead). [Modified from Paxinos and Watson (25).]

Fig. 11. Distribution of neurofilament M-immunoreactive cells (shown by FITC-conjugated antisera) in the dorsal tracheal wall of the trachea (top) and intrapulmonary bronchi. In the trachea, the majority of these cells are located on the adventitial side of the trachealis muscle (TM) in association with the longitudinal nerve trunks (open arrow) or the trachealis muscle itself (solid arrow). A few neurons and fibers are present in the submucosa (solid arrowhead). Two neurons are located in the lamina propria (open arrowheads). In the bronchi, neuronal somata are distributed throughout the adventitia of large bronchi, separated from the bronchial lumen by the epithelium, submucosa, and muscularis layers.

The FluoroGold injections into the right lung were intended to label what we believed to be a population of vagal motoneurons committed to the innervation of intrapulmonary ganglia. This interpretation needs to be revised, however, in light of the current results. Specifically, the fact that most of the neurons labeled by intraluminal injections of CTB were also labeled by FluoroGold has two potential implications to the contrary. First, there is clearly a subset of neurons labeled by the intraparenchymal injections, the endings of which are within the reach of CTB molecules translocated across the airway epithelium. This is, once again, unlikely to be the case in large bronchi where ganglia reside (24, 31, 33) because in those locations, the bodies
of the ganglion neurons are separated from the mucosa and submucosa by a considerable diffusion distance. In addition, the finding that FluoroGold injections into one single lobe reached most of the medullary neurons labeled transepithelially by CTB implies that many of these neurons have multilobar projections and may therefore be involved in the innervation of multiple airway segments.

Summary. Our results demonstrate a novel use of CTB as a transepithelial neuronal marker in the airways. The medullary parasympathetic neurons and fibers identified through this route, however, lack both distinctive topographic characteristics and target specificity. These findings support the notion that it is the role of the diverse populations of airway intrinsic neurons (9, 24) to impart functional selectivity to the parasympathetic outflow carried by the vagus nerves to the airways.

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