Acetylcholinesterase-positive nerves of the rhesus monkey bronchial tree

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El-Bermani, A-W. I. and Grant, Margaret (1975). Thorax, 30, 162–170. Acetylcholinesterase-positive nerves of the rhesus monkey bronchial tree. The rhesus monkey lung was stained both by histological methods and histochemically for specific acetylcholinesterase (AChE). AChE-containing nerves in bundles were demonstrated in connective tissue of the hilum and in association with clusters of ganglion cells. These bundles became associated with the bronchial tree as they enter the lung parenchyma, and their numbers of myelinated fibres diminish as they pass scattered ganglion cells along the bronchial system. Extradanchroidal and subchondral plexuses of nerves were found to be interconnected and to contribute to the perimucosal varicose nerve plexus of the bronchi and bronchioles. These nerve plexuses were found to extend as far as the respiratory bronchioles.

In the bronchial submucosa there are AChE-positive nerve plexuses which arise from three sources: (1) the adventitial plexus in bronchioles, or the subchondral plexus in bronchi, (2) the perimucosal nerve plexus, and (3) ACh-containing nerves associated with the bronchial artery. The submucosal plexus appears to innervate the acinar submucosal glands in bronchi as well as continuing as central nerves in the mucosal folds. In the bronchioles the nerves in the mucosal fold are in close relationship with the mucosa.

The pulmonary innervation is derived mainly from the autonomic nervous system (Kuntz, 1953; von Hayek, 1960). Pulmonary nerves arise from large plexuses found at the arch of the aorta and about the bifurcation of the trachea (Mitchell, 1956) and were classified by Larsell (1921; 1922) as bronchial or vascular nerves.

Most previous anatomical studies have concentrated on rat, cat, and dog lungs (Larsell, 1921; Elftman, 1943; Honjin, 1956a and b; El-Bermani, McNary, and Bradley, 1970) with little attention to the innervation of the primate lung. Until recently much confusion has arisen from the idea that one mammalian species is very much like any other in terms of its pulmonary innervation. In addition, conflicting reports have arisen because of the staining artifacts of various histological techniques for neural tissue (Stirling, 1876; Larsell, 1921; Honjin, 1956a and b; Hirsch et al., 1968).

The present studies were undertaken to demonstrate the pattern and distribution of the bronchial innervation in an erect primate. Histological staining methods and a method specific for acetylcholinesterase were used. The results indicated that acetylcholinesterase-containing nerves innervate both muscular and glandular cells of submucosal glands in the bronchi and the mucosa of bronchioles. They further show that any classification of pulmonary nerves as 'vascular' or 'bronchial' is an artificial one since a significant proportion of the bronchial innervation arises from that of the bronchial artery. Finally, they demonstrate marked differences between this primate and other 'lower' mammalian species used more frequently for physiological study.

METHODS

Twelve adult Macaca rhesus monkeys were used for these studies. Lungs were judged to be non-diseased at the time of necropsy. The lungs were dissected and one cubic inch blocks of tissue...
representing the hilum, the ‘body’, and the peripheral (subpleural) areas were selected randomly for histological and histochemical processing. A total of at least 24 such blocks for each animal was processed and examined. Histological staining by supravital methylene blue and two methods for silver impregnation were used to visualize the general distribution of nerves. Alternate sections were processed for histochemical study of acetylcholinesterase. The following methods were used for histological and histochemical staining:

1. METHYLENE BLUE STAINING According to the method described by Hillarp (1946) with modifications by El-Bermani et al. (1970), anaesthetized animals were injected intratracheally with sufficient amounts of 0.04% methylene blue in physiological saline containing glucose and magnesium bromide. The solution remained in the lungs for 12–15 minutes and was then withdrawn and the animal was placed on an artificial respirator for 30 minutes. The lungs were then removed and fixed in 8% ammonium molybdate containing 3 drops of 2% osmium tetroxide per 100 ml for 24 hours at 4°C; 20 μ frozen sections were placed on slides, air dried, and mounted with permount.

2. SILVER IMPREGNATION Silver impregnation was performed by two techniques. Paraffin sections of Bouin's fixed inflated lung were stained by the method of Fitzgerald (1964), while frozen sections of formol-cadmium chloride fixed lungs were impregnated by the method of Namba et al. (1967).

3. ACETYLCHOLINESTERASE (AChE) 25 μ frozen sections were fixed in cold (4°C) formol-saline and stained by a modification of Karnovsky and Roots' (1964) technique, as described by El-Badawi and Schenk (1966). Tetra isopropyl phosphoramid (iso OMPA) in a final concentration of 8×10⁻⁴ M was used to inhibit non-specific cholinesterase. Adjacent sections were simultaneously incubated in butryliothiocholine as a substrate for cholinesterase, and acetylthiocholine iodine was used as substrate for specific acetylcholinesterase.

Histological staining by both silver impregnation and supravital methylene blue has an advantage over silver impregnation alone, firstly because of the higher specificity of methylene blue for neural tissue, as demonstrated by neurochemical methods (acetylcholinesterase, nor-

epinephrine), and secondly because of the higher resolving power of methylene blue. Most silver methods leave large silver grains which mask the outline of nerve cells and fibres as well as tending to stain connective tissue.

The Namba, Nakamura, and Grob (1967) technique of all the silver methods appears to be the most selective as it gives fairly high resolution of nervous structures. Combination methylene blue and silver staining with the histochemical method for specific acetylcholinesterase gives one a good picture of the general pattern of innervation and an indication of which proportion of that innervation contains significant amounts of acetylcholinesterase.

RESULTS

Bundles of large numbers of nerve fibres were demonstrated entering the hilum of the lung in connective tissue between blood vessels and bronchi with no association with any particular structure. After entering approximately one-third of the depth of the hilum the bundles become intimately associated with the adventitia of the bronchial system (Fig. 1). In large bronchi these bundles run outside the cartilagenous rings and in the trigonal area between the blood vessels and bronchi. These bundles are in closer relation to the cartilages than the centre of trigonal connective tissue. At the bronchiolar level the bundles are concentrated at the periphery of the adventitia. Both types of bundles are of mixed fibre populations with thick and thin fibres present (Fig. 1).

The number of thick nerve fibres differs from one bundle to the other. As sections proceed deep into the lung parenchyma a reduction in the number of thick fibres in each bundle becomes evident. The greater reduction is encountered when the nerve bundles contact ganglion cells along their course.

The ganglion cells are round or ovoid with an eccentric nucleus and granular cytoplasm, especially near the origins of axons. At the hilum (Fig. 2) the ganglion cells are grouped in large clusters in connective tissue. Both at the hilum and in the parenchyma of the lung most of the ganglion cells are concentrated at the bifurcations of the airways. In the bronchi, they are external to the cartilagenous plates.

The bronchiolar nerve bundles contribute nerve fibres to the musculature of the bronchiolar wall (Fig. 3). The fibres reaching the muscular coat of a bronchiole have a closer relation to the
muscle cells. These nerves form an intricate, intermeshed plexus of beaded fibres (Fig. 3). They are of the thin non-myelinated type and surround the surface of the bronchiolar muscle bands. The plexuses can be seen in any orientation of sectioning through the muscular coat of the bronchioles but are orientated about the circumference of the muscular bands generally, with interconnections along the radii of the bronchioles (Fig. 4). The complexity and density of these plexuses increase at the bifurcations of the bronchiolar system where they receive additional direct contributions from the scattered ganglion cells.

A number of nerve fibres have been identified in the submucosal and mucosal area. Most of these fibres originate in the muscular nerve plexus while some come directly from the adventitial plexus in the bronchiolar wall (Fig. 5). Both sources lead to a heavy innervation in the submucosal area. A few fibres were observed to proceed toward the lumen in the connective tissue of the mucosal fold. Some fibres were also found to reach the basal layer of mucosal cells and extended between the mucosal cells. They are a continuation of the innermost component of the submucosal nerves (Fig. 6).

AChE was identified in bundles of nerve fibres in the hilar area and in those fibres associated with the bronchial system. The latter were located in the connective tissue external and internal to the cartilagenous plates (Fig. 7). In bronchioles where cartilagenous tissue is absent, the nerve bundles are based in adventitia. All the above-mentioned nerve bundles were composed of thick myelinated and thin non-myelinated fibres. The adventital nerves will be called so at the bronchiolar level, but at the bronchial level where they divide on either side of the cartilagenous plates they will be termed extrachondral and subchondral nerve plexuses.

The nerve plexuses of the muscular coat in the bronchial system were found to contain AChE. These AChE-containing nerves form the characteristic network of irregularly outlined nerve fibres seen with the histological methods (Fig. 8). They surround the smooth muscle bundles in every plane of section and criss-cross each other. The density of the AChE-positive nerve plexuses in
higher at the bifurcation points of the bronchiolar system.

In the submucosa of bronchioles, a large number of AChE-positive nerve fibres were identified. These submucosal fibres arise from both the AChE-positive perimuscular nerve plexus and as direct continuations of AChE-containing subchondral or adventitial nerve bundles. These submucosal fibres can be followed in the direction of the mucosa or circumferentially in submucosal tissue. In large bronchi where glands are present, the acinar cells are surrounded by AChE-containing nerve fibres (Fig. 7). The nerves of the muscular coat as well as those of the submucosa were present as far as the respiratory bronchioles.

Some of the bronchial arteries demonstrated AChE-positive nerves while other bronchial arteries had nerves negative for AChE. The AChE-containing nerves from bronchial arteries were observed to interconnect with perimuscular and submucosal nerve plexus of the bronchi (Fig. 7). This arrangement is prominent mainly in the hilar area, and no ganglion cells have been identified in association with bronchial artery.

The ganglion cell clusters associated with the bundles of nerves (at the hilar area) are AChE-positive. Both thick and thin fibres were observed near and between the ganglion cells. A close inspection of these ganglion cells revealed fine plexuses of AChE-positive fibres dispersed over the surface of each ganglion. The plexuses can be seen to arise from large myelinated nerves (Fig. 9). In the periphery of the lung parenchyma the ganglion cells are scattered along the peribronchiolar nerve bundle and contribute AChE-positive nerves to the muscular nerve plexus and submucosal plexus.

**DISCUSSION**

Little work has been done on the anatomy of the innervation of primate lung despite the fact that the physiology of the erect primates approximates human physiology more closely than does that of lower species. The present results show marked differences between the innervation of the Rhesus monkey lung and that of other mammalian non-primate species.

The major features of the innervation of the Rhesus bronchial system which differ from those...
of the other mammals involve the distribution of nerve types in the bronchial arteries, a pronounced AChE-containing innervation to acinar glands, and the pattern by which nerves enter the lung at the hilum.

At the hilar area in the rhesus monkey lung, there are large nerve bundles which have no definite association with any specific structure. This is in contrast to our findings in the rat lung (El-Bermani et al., 1970; El-Bermani, 1973a and b) and to the findings of others (Stirling, 1876; Dgien, 1898; Hirsch et al., 1968) in other non-primate species, that all entering structures, blood vessels, and airways had associated pulmonary nerves. In the macaca, organization of the pulmonary nerve bundles into groups associated with the bronchial system, bronchial arterial system, and pulmonary vein occurs only after a certain depth in the hilar parenchyma.

The peribronchial nerve bundles are situated in the connective tissue around the bronchi and bronchioles. These bundles have a mixed population of myelinated and non-myelinated fibres. The mixed nature of these nerves has been described similarly in other species (Berkley, 1893; Elftman, 1943; Fisher, 1965; El-Bermani et al., 1970; El-Bermani, 1973a and b). Both components of the peribronchial bundles are AChE-positive, and we have found none demonstrating fluorescence for the presence of catecholamines (El-Bermani, 1973a and b). Thus these nerves must be cholinergic. There is a decrease in the ratio of myelinated to non-myelinated nerves as the bundle proceeds deep into the lung parenchyma, the most marked reduction occurring in the presence of large numbers of ganglion cells. This finding is in accord with our previous observations in the monkey lung (El-Bermani, 1973a and b) and in the rat lung (El-Bermani et al., 1970). Since the ganglion cells innervated by these myelinated fibres also react positively to AChE, it is likely that these fibres represent preganglionic parasympathetic nerves. The ganglion cells are secondary cholinergic neurons of the vagal parasympathetic system to the lung (Larsell, 1922; Nadel, 1974). The preganglionic nerves end on the ganglion cells in a basket-like ending (Figs 2 and 9). The basket-like arrangement of the preganglionic axon terminals were boutons around the soma.
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FIG. 7. Acetylcholinesterase-containing nerves in bundles associated with the bronchial system (single arrow) and investing acinar cells of the submucosa (double arrow). The cartilagenous plates of the bronchial wall are labelled C. A bronchial artery (ba) with acetylcholinesterase-positive nerves penetrating its walls contributes nerves to the bronchial wall (L=lumen; sm=smooth muscle) (Acetylcholinesterase X64).

FIG. 8. Acetylcholinesterase-containing nerve plexus (arrow) associated with bronchiolar smooth muscle (L=lumen; mu=mucosa) (Acetylcholinesterase X126).

The bronchial nerves are distributed in the adventitio-muscular junction and in submucosal groups. This basic pattern is similar to previous findings in different species (Larsell, 1922; El-Bermani et al., 1970; El-Bermani, 1973a and b) but is in contrast to observations that the main contribution to bronchial innervation is from a perivenous source (Takino, 1933). The subchondral nerve plexus contributes AChE-containing innervation to the mucosa of the large bronchi. These fibres form a network of fine nerves around the acinar gland cells. Both the extra and subchondral nerve plexuses are associated with ganglion cells.

The perimuscular nerve plexuses are formed of a network of intermeshed varicose nerves distributed in a three-dimensional pattern throughout the muscle bands. The maximum concentration of these nerves was observed at the bifurcation of the bronchioles. At these points the plexuses receive additional nerves from nearby ganglion cells. These varicose nerves represent terminal endings to the muscles similar to those of other species (El-Bermani et al., 1970; Spencer and Leof, 1964; Honjin, 1956a and b). The perimuscular varicose terminal plexuses receive their innervation from adventitial nerve bundles and adventitial ganglion cells. The bronchi receive a contribution for nerve fibres around some of the large branches of bronchial artery. All these contributing nerves contain AChE. The origin of these perimuscular nerves and their high content of AChE suggest that they are parasympathetic postganglionic nerves. Vagal stimulation has been shown to produce constriction of the trachea and bronchioles with the maximum effect in the intermediate size bronchioles (Nadel, Cabezas, and Austin, 1971). It seems probable that the nerves observed in the present studies represent those which would mediate this type of bronchial constriction. The AChE-containing innervation of the bronchiolar muscle was traced as far as the terminal bronchioles in the present studies. In physiological studies on dogs, Nadel et al. (1971) showed that vagal stimulation has no effect on the
terminal bronchioles and alveolar ducts. Thus it appears that there is a major difference in the extent of bronchiolar innervation between the dog and the monkey. Hirsch and Kaiser (1969) described in a morphological study of the dog a continuous innervation of the bronchial system involving the alveolar wall. Our results and the physiological results of Nadel et al. (1971) are in marked contrast to those of Hirsch and Kaiser (1969), whose silver staining appears to have produced artifacts due to staining of connective tissue.

Since we have been unable to demonstrate catecholamine-containing nerves in the bronchial musculature (El-Bermani, 1973a and b) and because of the strong AChE reactions in the innervation of the bronchial musculature as well as in the contributing nerves, we believe that most, if not all, of the innervation to the airway smooth muscle in the monkey is cholinergic. This is in accord with the work of Hebb (1969). The presence of cholinergic innervation has been demonstrated physiologically by experiments which showed that the airways of dogs, cats, rabbits, and humans are tonically constricted by vagal efferent activity (Nadel and Widdicombe, 1962; Olson et al., 1965; Karczewski and Widdicombe, 1969). In reimplanted lung the airway smooth muscle is unreactive to vagal stimulation, indicating that parasympathetic vagal fibres contribute to the airway smooth muscle innervation (Edmunds, Graf, and Nadel, 1971). The presence of parasympathetic ganglia, which contribute directly to the innervation of the bronchioles and are in close proximity to the bronchioles, is reflected in the fast reinnervation of reimplanted dog lung (Nadel, 1974). The parasympathetic innervation of bronchiolar muscle is important in histamine-initiated bronchoconstriction, which is abolished by cutting the pulmonary vagi (Dekock et al., 1966) and has been proposed to be a partly reflex reaction (Mills, Sellick, and Widdicombe, 1969; Nadel, 1974).

The submucosal nerves were AChE-positive and were derived from either the adventitial, subchondral plexuses or, in larger bronchi, from contributions from the bronchial artery cholinergic nerves. The submucosal plexus consists of nonmyelinated fibres present in the connective tissue and in close relation to the mucosa itself. The presence of such nerves agrees with results demonstrated in other animal species (Larsell, 1922; Honjin, 1956a and b; Spencer and Leaf, 1964), although we have found no intracellular or intra-epithelial endings as described by Hirsch et al. (1968). Our finding is in agreement with that of Jefferey and Reid (1973) who, in an extensive electron microscopic study found intra-epithelial nerves in the trachea and extrapulmonary bronchi and none in the intrapulmonary airways. We have not differentiated sensory endings in the bronchial system using histological or histochemical methods. No knob-like sensory endings as described by McLelland (1972) in birds have been found. Both previously mentioned authors used the silver impregnation method of Bielschowsky (1961), which produces a coarse non-specific silver precipitate, a characteristic artifact seen by us with this method. A few myelinated nerves which showed no AChE reaction were observed in the centre of the submucosal fold. The presence of myelination to the position of these nerves near the mucosal cell layer and the lack of AChE suggest that these nerves are sensory. Mills et al. (1969), based on physiological evidence, have postulated that irritant receptors are present in the airway epithelium. These receptors are important in the production of asthma, as aerosols of acetylcholine induce an increase in airway resistance of 250% which remains for more than

FIG. 9. Acetylcholinesterase-positive reaction of ganglion cells (G) near the hilum. Acetylcholinesterase-positive nerves can be seen to end on these cells (N=nerve bundle) (Acetylcholinesterase ×260).
10 minutes (Gayrard et al., 1971; Svedmyr and Thiringer, 1971). The response has been shown to be a reflex one mediated through the vagus and blocked by atropine (Widdicombe, 1974). Application of novocaine to the vagi blocks the bronchoconstriction (Ulmer and Islam, 1974). The sensory fibres as at present described may be those involved in the reflex increase in pulmonary resistance in response to irritants (Lawther et al., 1973).

The cholinergic nerves seen in the submucosa and in direct relation to the mucosal cells probably represent parasympathetic innervation to the glandular cells in this tissue.

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