

Immunohistochemical demonstration of substance P in the lower respiratory tract of the rabbit and not of man

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ABSTRACT Substance P (SP)-immunoreactive nerve fibres were searched for at all levels of both fetal and adult human lower respiratory tract. Because the demonstrability of substance P immunoreactivity varies between different animal species, rabbit pulmonary tissue was also subjected to SP immunohistochemistry. Human irises and corneas served as positive human controls. The specimens were taken from 10 human lungs during pulmonary operations. Tracheal tissue was obtained from three patients during bronchoscopy. Five fetal human lungs were examined. Human specimens examined included the trachea, main bronchi, segmental bronchi, and peripheral pulmonary tissue. In addition, the tracheobronchial tissues of four rabbits were studied. SP immunoreaction was demonstrated in formaldehyde-fixed cryostat sections by either the indirect immunofluorescence technique or the peroxidase-antiperoxidase procedure. Both monoclonal and conventional antibodies to SP were tested. In the rabbit SP-immunoreactive nerves were found in both the submucosa and the smooth muscle layer of the main bronchi and trachea. Specimens from human trachea, bronchi, and bronchioli were all negative. Since the SP immunoreaction was easily demonstrated in both human cornea and human iris, it was concluded that there are no SP-immunoreactive nerves in the human pulmonary tissues or that their SP content is very low and below the sensitivity of all the techniques used.

Published reports describe four types of innervation around the lower respiratory tract: cholinergic (parasympathetic bronchoconstrictive), adrenergic (bronchodilator), and non-adrenergic-non-cholinergic (probably "purinergic" bronchodilator) nerves and afferent sensory fibres located in both the epithelium and the deeper structures of the airway.¹

It has been suggested that the human bronchial smooth muscle lacks true adrenergic innervation. Thus the sympathetic bronchodilator effect would be produced by circulating catecholamines.² We have, however, recently demonstrated adrenergic nerves around the human bronchial glands and also in human bronchial smooth muscle (M Partanen

et al, 1982, papers presented to the International Conference on Bronchial Hyperreactivity and the Sixth European Neuroscience Congress). Both cholinergic and non-cholinergic-non-adrenergic nerve endings have been found in the bronchial smooth muscle of man.¹ The transmitter of the latter type of nerve is still unknown. These nerves resemble morphologically both the so-called purinergic nerves³ and P-type peptide-containing nerve endings.⁴ Thus there are at least three possible transmitter candidates: ATP, vasoactive intestinal polypeptide (VIP), and substance P (SP). Both VIP and SP are neuropeptides and they have recently been found in nerve fibres in tracheobronchial tissues of several species.^{5,7} In man SP has been reported only in the nasal epithelium,⁸ whereas VIP has also been demonstrated within the walls of pulmonary and bronchial vessels, in the smooth muscle layers, and around the glands of the airways^{5,9} (see also M Partanen *et al* as above).

We undertook this study to determine whether

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there are SP-positive nerve fibres at any level of either fetal or adult human lower respiratory tract. Because there seems to be species variation in the demonstrability of substance P immunoreactivity, corresponding pulmonary tissue from rabbits was also subjected to SP immunohistochemistry.

Material and methods

The human material consisted of specimens taken from ten lungs at pulmonary operations in the third department of surgery of the Helsinki University Central Hospital. The tracheal tissues were taken from three patients in the course of direct bronchoscopy at Laakso Hospital, Helsinki, and Tiuru Hospital, Imatra. Five fetal lungs were provided by the State Maternity Hospital. In addition, tracheobronchial tissues from four rabbits was studied.

Human specimens were taken from different levels of the respiratory tract, including the trachea, main bronchi, segmental bronchi, and peripheral pulmonary tissue. Two human corneas and irises, obtained at either corneal surgery or sector iridectomy at the department of ophthalmology, University of Helsinki, served as SP-positive human control tissues. They were treated and processed in the same way as the pulmonary specimens. The distribution of SP in these tissues has been described elsewhere.¹⁰

FIXATION PROCEDURES

The rabbits were anaesthetised with an intraperitoneal injection of sodium pentobarbitone (Nembutal, 50 mg/kg) and perfused with cold (4°C) 4% (w/v) formaldehyde solution in 0.1 mol/l phosphate buffer, pH 7.4, through the right ventricle of the heart. The left auricle was opened with scissors so that the fixative passed only through the pulmonary circulation. After perfusion for 15 minutes, trachea, main bronchi, and peripheral pulmonary tissue were prepared and immersed in the same fixative for two to four hours at 4°C. Then they were rinsed overnight in 0.1 mol/l phosphate buffer, containing 25% sucrose (w/v). The human specimens were fixed by immersing them for 3 h in 4% formaldehyde solution in 0.1 mol/l phosphate buffer, pH 7.4, at 4°C. Then they were rinsed as described above.

The specimens were either frozen on metal chucks and cut at 15 µm in a cryostat at -20°C or dehydrated in a graded ethanol series and embedded in paraffin, after which they were sectioned at 8 µm with a sliding microtome.

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IMMUNOHISTOCHEMICAL TECHNIQUES

SP immunoreactivity was demonstrated with either monoclonal SP antibody or conventional rabbit SP antiserum. The former was used only with the indirect fluorescence technique¹¹ and the latter with both fluorescence and light microscopy.

For the demonstration of SP immunoreactivity with the monoclonal antibody technique, the sections were attached to coverslips and preincubated at 22°C in 0.1 mol/l phosphate buffer, pH 7.4, containing 0.9% sodium chloride and 0.3% Triton X-100 (phosphate-buffered saline (PBS)/Triton X-100), for 15 minutes. The coverslips were then wiped dry around the sections, placed in a petri dish, covered with a few drops of the SP antibody diluted 1:200 in PBS/Triton X-100, and left overnight at 0°C. The monoclonal antibody was produced and characterised in the laboratory of Dr AC Cuello. Details of the technique and controls used in the production of the antibody as well as its properties have already been published.¹² After incubation with the primary antibody, the coverslips were rinsed three times in PBS/Triton X-100 and incubated for 40 minutes at 37°C with fluorescein-isothiocyanate-conjugated rabbit anti-rat IgG (Wellcome) diluted 1:40 in PBS/Triton X-100. The sections were then rinsed again several times and mounted in a mixture (3:1 v/v) of glycerol and PBS/Triton X-100.

In the immunohistochemical control experiments the specimens were treated identically except that either the primary or the fluorescent secondary antibody was left out or 40 µg/ml of SP (Sigma) was added to both the preincubation and the incubation solutions to absorb the SP antibody.

A conventional rabbit antiserum against substance P was also used to demonstrate immunoreactivity by either the indirect immunofluorescence method of Coons¹¹ as described elsewhere¹³ or the peroxidase-antiperoxidase (PAP) method of Sternberger *et al.*¹⁴ The antiserum was produced by injecting BSA-conjugated synthetic SP (Beckman, Geneva) in complete Freund's adjuvant into rabbits according to the method of Kanazawa and Jessell.¹⁵ This antiserum recognises the carboxyterminal sequence of substance P and does not recognise the aminoterminal sequence or fragments smaller than hexapeptide substance P (6-11).

The sections were first incubated with normal swine serum diluted 1:5 with PBS, pH 7.4, to diminish non-specific binding of antibodies to the tissue for 20 minutes at room temperature. SP antiserum diluted 1:200 or 1:400 for the PAP-method or 1:40 for the immunofluorescence method was then applied. All antisera were diluted in PBS containing 0.25% Triton X-100 at pH 7.4. The sections were

incubated with SP antiserum at room temperature for two hours. After being washed twice with PBS for 20 minutes the sections were incubated with swine anti-rabbit serum IgG (DAKO, Copenhagen) diluted 1:20 for the PAP-method or with fluorescein-isothiocyanate-conjugated swine anti-rabbit IgG (DAKO) diluted 1:20 for the immunofluorescence method for three minutes at room temperature. They were then washed again with PBS-containing Triton X-100, and fluorescent samples were embedded in glycerol-PBS (1:2) and examined under a fluorescence microscope, while the PAP samples were further incubated with a soluble complex of horseradish peroxidase/rabbit anti-horseradish peroxidase (DAKO) diluted 1:100 or 1:200 for 30 minutes at room temperature. This was followed by washing with 0.05 mol/l tris-HCl buffer (pH 7.6) for 20 minutes. Finally, the PAP sections were treated with 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma, St Louis, USA), 50 mg/100 ml in 0.05 mol/l tris-HCl buffer (pH 7.6) containing 0.003% hydrogen peroxidase, for eight minutes. These samples were embedded in Entellan (Merck, Darmstadt, Germany) and viewed under bright-field illumination.

Both the immunofluorescence and the PAP procedure gave good staining in control tissue (formalin-fixed rat spinal cord posterior horn). There was considerable non-specific staining of both fetal and adult human lung when the PAP procedure was used. This was shown to be due to endogenous peroxidase activity in the bronchial walls since the reaction was present even after incubation without the specific antibody. The reaction was completely abolished by pretreatment of the samples with hydrogen peroxidase (0.5%) in methanol (100%). Other controls included absorption of the antiserum with synthetic SP (50 μ g/ml diluted antiserum), which completely abolished the staining considered specific for SP, and omission of each step of the incubation procedure.

Results

In the rabbit SP-immunoreactive nerves were found in both the submucosa (fig 1) and the smooth muscle layer (fig 2) of the main bronchi and trachea. The basement membrane of the respiratory epithelium was highly autofluorescent (fig 1). This was, however, easily distinguished from the specific SP immunofluorescence located in varicose nerve fibres, since it also appeared after incubation with the secondary antibody only. Absorption of the primary antibody with synthetic SP also abolished the staining of SP-immunoreactive nerves.

SP-immunoreactive nerves seemed to be

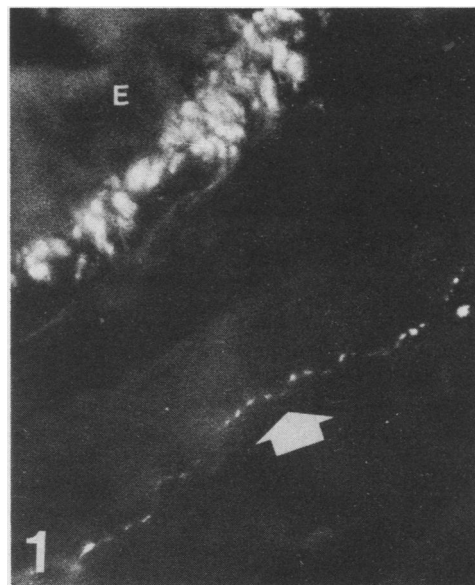


Fig 1 Substance P (SP)-immunoreactive nerve fibre (arrow) in the submucosa of the right main bronchus of rabbit. The basal lamina of the respiratory epithelium (E) is highly autofluorescent. ($\times 500$.)

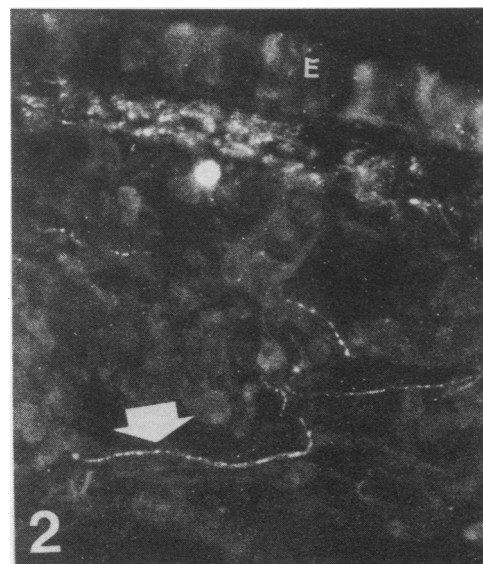


Fig 2 SP-immunoreactive nerve fibres (arrow) in the smooth muscle layer of the rabbit main bronchus. E—respiratory epithelium. ($\times 320$.)

restricted to the proximal part of the respiratory tract and were not detected more distally than in the lobar bronchi.

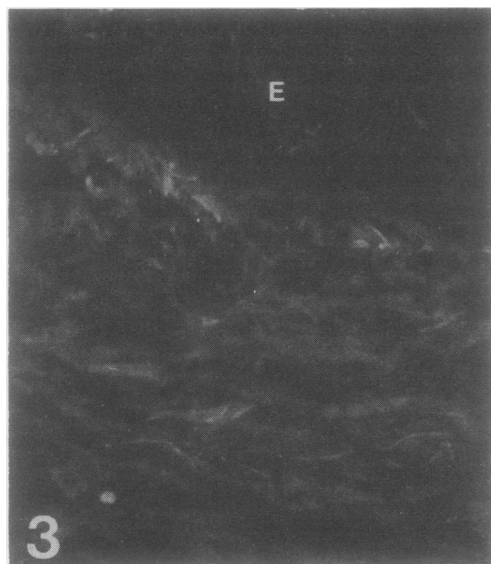


Fig 3 Human main bronchus, with no SP-immunoreactive nerves detectable. The epithelium (E) is less autofluorescent than the muscle layer. ($\times 320$.)

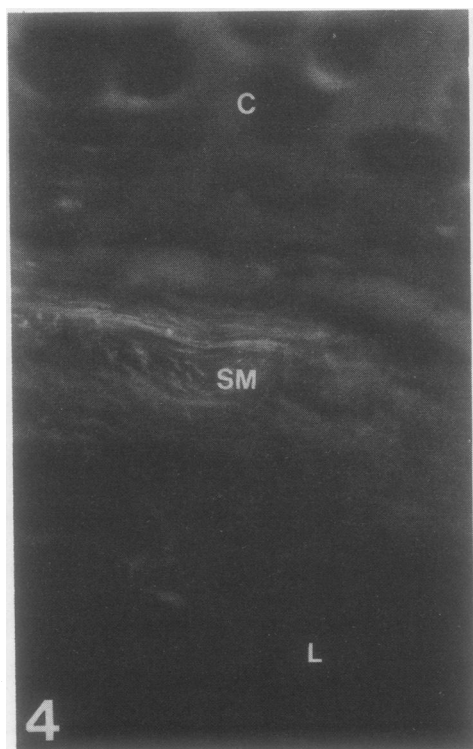


Fig 4 Human trachea (epithelium partly separated), with no SP-immunoreactive nerves visible in the submucosa (SM). C—cartilage; L—lumen of the trachea. ($\times 320$.)

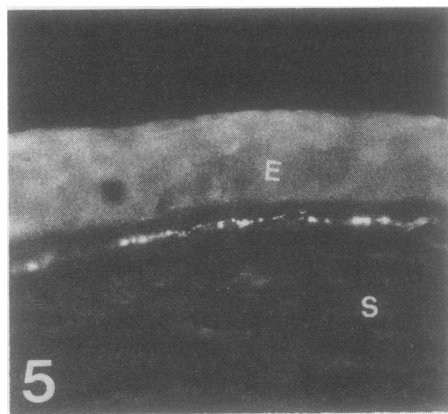


Fig 5 SP-immunoreactive nerve fibre in the stroma (S) of the human cornea. The nerve fibre is located just beneath the epithelium (E). ($\times 320$.)

Specimens from human trachea, bronchi, and bronchioli were all devoid of SP immunofluorescence (fig 3 and 4). The general tissue fluorescence was somewhat greater than in rabbits and blood vessels showed non-specific fluorescence due to blood cells that bound the secondary antisera. This was not present in the rabbit tissues, which were fixed by perfusion. To ensure that the negative result with the human material was not an artefact, we also incubated in parallel sections from human corneas (fig 5) and irises as well as rat spinal cord, all of which exhibited SP immunoreaction in nerve fibres.

Discussion

von Euler and Gaddum discovered in 1931 a substance which had the effect of causing contraction of the intestine.¹⁶ They gave it the provisional name of substance P and later showed it to be a peptide.¹⁷ Lembeck suggested that substance P might be a sensory transmitter.¹⁸ Later studies have provided indirect support for his idea.^{19–22} Substance P has been found in those fibres of the dorsal horn which transmit nociceptive stimuli, the vagal nerve in the intestine, the urinary tract, and the airways.^{19–20–23} In both the intestine and the bladder the cells which show SP immunoreactivity build up neural networks where the cell bodies are located in the target organ. It has been suggested that a similar neural system also exists in the lung, but its nature is unknown.^{6–24}

The results of the present study indicate that the lower respiratory tract of the rabbit but not of man is innervated by SP-immunoreactive nerve fibres. The failure to demonstrate SP immunofluorescence in the human specimens is probably not due to the method used, since SP immunofluorescence was eas-

ily demonstrable in human control tissues (cornea and iris), treated identically, and all methods gave the same negative results for both fetal and adult pulmonary specimens. This finding may be due to species differences either in the density of SP-immunoreactive nerves or in the SP concentration in them. The results also indicate that there are fewer SP-immunoreactive nerves in the rabbit lung than in guinea pigs.⁶

In man VIP-immunoreactive nerves have been found in the airways and around the pulmonary vessels⁹ (see also papers by M Partanen *et al* referred to in the introduction). The presence of a non-adrenergic-non-cholinergic inhibitory (relaxant) system of innervation in the airway smooth muscle has been demonstrated by observing relaxation of segments of the tracheobronchial tree in the presence of both atropine and β -adrenergic blocking agents after electrical field stimulation.² VIP is released during this relaxation of guinea-pig tracheal segments, and the magnitude of the relaxation is reduced if the tracheal segments have previously been incubated with antiserum to VIP.²⁵ The physiological effects of VIP and its localisation in the smooth muscle of the lower respiratory tract support the idea that VIP is one of the transmitters concerned in the non-adrenergic-non-cholinergic relaxation of bronchi.

Substance P does not seem to be located in nerve fibres innervating human bronchial smooth muscle. Negative results in immunohistochemistry must, however, be interpreted with caution and it remains possible that all the methods we have used have failed to demonstrate SP or an SP-like peptide which is nevertheless present in human lung. Several factors relating to the manner of obtaining the human pulmonary tissues may have led to negative results. Although the specimens were obtained from healthy locations in 10 lungs during operations for carcinoma, many of the patients were old, which probably made it more difficult to demonstrate SP-immunoreactive nerves. This appeared to be true of ocular tissues in our earlier studies. Perfusion fixation could not be used to improve the tissue preservation and to eliminate the non-specific intravascular fluorescence. This and the higher collagen autofluorescence in primates as well as the pulmonary endoperoxidase activity in the PAP procedure contributed to the relatively poor "signal-to-noise ratio." Finally, the pulmonary tissues may have been crushed or devascularised, or both, during operation. The specimens were considerably larger than the ocular specimens, which could have led to poor penetration of fixative.

On the other hand, SP might still—if transported to lungs from other organs—have a similar non-

adrenergic-non-cholinergic contracting effect on bronchial smooth muscle, as in the iridic sphincter.²⁶ It has been reported that SP causes release of histamine from cutaneous and peritoneal mast cells,²⁷ which might secondarily lead to constriction of the bronchi. Nilsson *et al* described constriction of isolated guinea pig bronchi by SP.⁶ Guinea pigs are widely used as experimental animals in studying mechanisms which may be important in bronchial asthma. It is now evident, however, that both the quality of innervation and the density of mast cells vary so much between animal species that results of animal experiments should be interpreted with caution.

The potential physiological importance of SP and some other neuropeptides acting on smooth muscle cells emphasises the need for attempts to identify new neuronal systems regulating the tracheobronchial tree.

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