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DISTRIBUTION OF PEPTIDERGIC NERVE FIBERS IN RAT BRONCHUS-ASSOCIATED LYMPHOID TISSUE: LIGHT MICROSCOPIC OBSERVATIONS

N. Inoue, S. Magari, M. Sakanaka

Department of Anatomy, Osaka Medical College, Osaka, Japan

ABSTRACT

The localization of neuropeptide Y (NPY), substance P (SP), calcitonin gene-related peptide (CGRP) and vasoactive intestinal polypeptide (VIP) in the nerve fibers of rat bronchus-associated lymphoid tissue (BALT) was investigated by light microscopic immunohistochemistry. Nerve fiber bundles revealing NPY-like immunoreactivity were shown to enter the BALT together with pulmonary artery branches. They frequently reached the central zone of the BALT to give rise to fine, tortuous fibers. On the other hand, nerve fibers immunoreactive for SP and CGRP seemed to distribute in the subepithelial zone of the BALT after dissociating from fiber networks in the walls of bronchi, although small numbers of SP and CGRP fibers were also seen in the BALT central zone. CGRP fibers formed a more intense network than SP fibers in the BALT. Scattered VIP fibers were found only in the subepithelial zone of the BALT. These findings not only suggest that the four kinds of peptidergic fibers act on BALT in multiple ways, but also that these neuropeptides may be involved in the control of mucosal immunity, lymphocyte migration and proliferation within the BALT.

Bronchus-associated lymphoid tissue (BALT) is a peripheral lymphoid organ present in the lungs of most mammals (1-8). The morphological characteristics of BALT have been described by Bienenstock et al (2,4) who showed that it is usually situated between a bronchus and an artery, or located close to bronchial bifurcations. A close association with bronchial epithelium suggests that BALT plays an important role in mucosal immunity (1,9). Activation of the mucosal immune system by exogenous antigens arising from the respiratory tract frequently results in movement, differentiation and proliferation of lymphocytes in the BALT (7,10,11). There are still many points to be clarified as to the mechanisms by which lymphocytes in the BALT migrate to and fro between blood vessels and lymphatics and proliferate in response to certain stimuli. A tentative candidate for regulation of such lymphocyte migration or proliferation is neuronal components in the BALT, since Plesch et al (1) demonstrated a significant number of nerve fibers in rat BALT. Few studies have so far dealt with neurotransmitters or neuromodulators present in nerve fibers of the BALT. In this immunohistochemical study we investigated the distribution of peptidergic nerve fibers in rat BALT using rabbit antisera against neuropeptide Y (NPY), substance P (SP), calcitonin gene-related peptide (CGRP) and vasoactive intestinal polypeptide (VIP).

MATERIALS AND METHODS

Seven Wistar male rats weighing 100-120g were housed at constant temperature (20°C) in a 12:12 hour light: dark

cycle, and given food and water ad libitum. Each rat was deeply anesthetized with pentobarbital and perfused via the right ventricle, first with 100ml of heparinized saline, then with 300ml of Zamboni's solution (12). After perfusion, the lung was quickly removed and fixed in the same fixative for 2h at 4°C, then transferred to 0.1M phosphate buffer containing 30% sucrose for at least 48h. The whole lung was frozen by dry ice and serially cut into 30μm sections on a cryostat. The sections were kept floating in glass vials containing phosphate buffered saline (PBS) for 2h, then treated with ethanol (50% for 10 min, 70% for 30 min, 50% for 10 min) to facilitate the penetration of antibodies, and rinsed again in PBS for 30 min. The sections were kept in a PBS solution containing 0.1% gelatin and 0.005% hydrogen peroxide for 20 min to suppress endogenous peroxidase activity in the tissue. Following a brief rinse in PBS, the floating sections were treated with PBS containing 1% normal goat serum (NGS) for 30 min and processed for the unlabeled antibody peroxidase-antiperoxidase (PAP) method (13) using rabbit antisera against synthetic NPY (Amersham), CGRP (Amersham), VIP (Cambridge Research Laboratory) and SP (kindly supplied by Dr. S. Shiosaka) (14) which showed no significant cross-reactivity with other related peptides. Briefly, 1) the sections were incubated with one of the above antisera for 48h to 72h, which were diluted with PBS containing 1% NGS, 1:4000, 3000, 500 and 1000, respectively; 2) washed three times with PBS containing 1% NGS (15 min for each washing); 3) incubated for 2h with anti-rabbit IgG (Sigma, goat) diluted 1:50 with PBS containing 1% NGS; 4) washed three times with PBS containing 1% NGS (15 min for each washing); 5) incubated for 2h with a PAP solution (DAKO) diluted 1:80 with PBS; 6) washed once with PBS and twice with 0.05M Tris-HCl buffer (pH 7.4) (15 min for each washing); 7) exposed to 0.4% diaminobenzidine and 0.002% hydrogen peroxide in 0.05M Tris-HCl buffer (pH 7.4) for 15 min; 8) washed three times with Tris-HCl buffer (pH 7.4); 9) mounted on gelatin-coated slides, dried and coverslipped. After immunostaining, one-third of the sections were counterstained with hematoxylin and eosin for the location of immunoreactive structures in BALT.

Control sections were first incubated with the antisera absorbed with an excess of the homologous antigens, and then they were processed as described above.

Terminology used was based on the study of Vai et al (15).

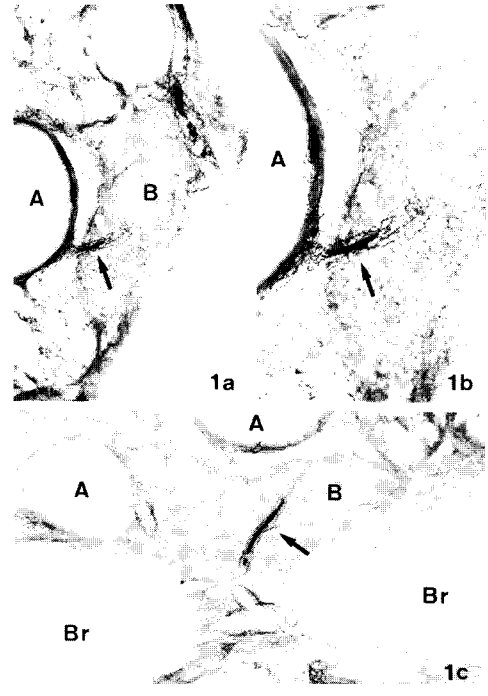

*Fig. 1. Bright-field photomicrographs showing nerve fiber bundles with NPY (arrow) which enter the BALT together with a pulmonary artery branch (A), at low magnification (**a**) and at high magnification (**b**). Note spindly and tortuous NPYI fibers forming a neuronal network in the central zone of the BALT. A = pulmonary artery branch; B = BALT; (**a**), x150; (**b**), x300. (**c**) Bright-field photomicrograph showing NPYI nerve fibers in the central (arrow) and peripheral zones of BALT. BR = bronchus; x150.*

RESULTS

Nerve fiber bundles with neuropeptide Y-like immunoreactivity (NPYI)

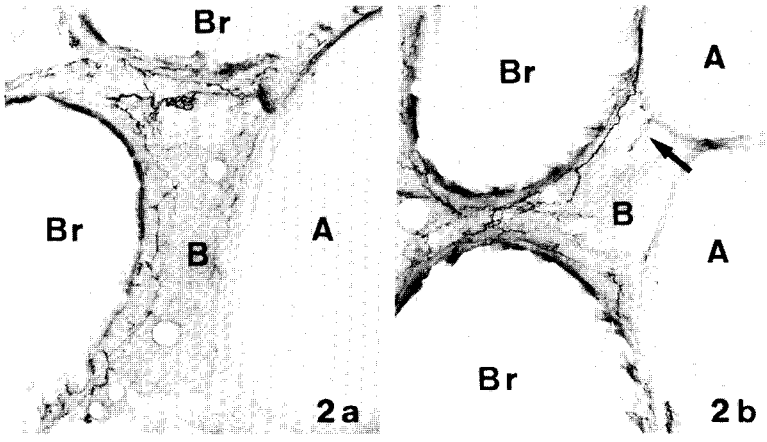

*Fig. 2. (**a**): Bright-field photomicrograph of SPI nerve fibers in the walls of bronchi (BR), which reach the subepithelial zone of BALT (B). A =pulmonary artery branch. x150. (**b**): Bright-field photomicrograph of SPI nerve fibers in the subepithelial zone of BALT (B). Note that single SPI fibers (arrow) run toward the BALT from an adjacent pulmonary artery branch (A). x150.*

were frequently observed in the walls of pulmonary artery branches that were closely apposed to the BALT of rats *(Fig. 1a)*. Some NPYI fibers dissociated from the nerve fiber bundles to reach the central zone of the BALT together with pulmonary artery branches *(Fig. 1a,b)*. In the central zone, the NPYI fibers around the branches of the pulmonary artery further gave rise to fine, tortuous fibers forming local neuronal networks *(Fig. 1b)*. There was another NPYI fiber system which contributed mainly to the innervation of the peripheral zone of BALT *(Fig. 1c)*. These immunoreactive fibers as well as those in the central zone of the BALT seemed to derive mainly from nerve fiber bundles in the walls of pulmonary artery branches *(Fig. 1a,c)*.

Substance P-like immunoreactive (SPI) fibers were associated more closely with bronchial walls than with the pulmonary artery in the lung. The SP nerve fiber plexus around the bronchial walls appeared to be continuous with that in the subepithelial zone of BALT *(Fig. 2a)*. Scattered SPI nerve fibers ran toward the BALT from adjacent pulmonary artery branches *(Fig. 2b)*.

Nerve fibers exhibiting calcitonin gene-related peptide-like immunoreactivity (CGRPI), located in and close to bronchial walls and adjacent BALT, showed more intense staining than SPI nerve fibers *(Figs. 2a and 3a)*. They were mainly seen at the subepithelial zone of the BALT with occasional occurrence in the central zone *(Fig. 3a)*. Nerve fibers labeled weakly with CGRP, which presumably dissociated from the walls of pulmonary artery branches, were also scattered in both the peripheral and central zones of BALT *(Fig. 3b)*. Generally, CGRPI nerve fibers exhibited a distribution pattern similar to that of SPI nerve fibers in the BALT, leaving open the possibility of partial co-localization of the two peptides in this tissue.

Nerve fibers with vasoactive intestinal polypeptide-like immunoreactivity (VIPI) were occasionally seen in bronchial walls close to the BALT. A number of fine VIPI fibers ran beyond the boundary between the bronchial walls and BALT to terminate possibly within the subepithelial zone of the BALT *(Fig. 4)*.

Pretreatment of NPY, SP, CGRP and VIP antisera with an excess of the homologous antigens abolished all immunoreactions.

DISCUSSION

The present study demonstrates that NPY fibers in rat BALT dissociate from

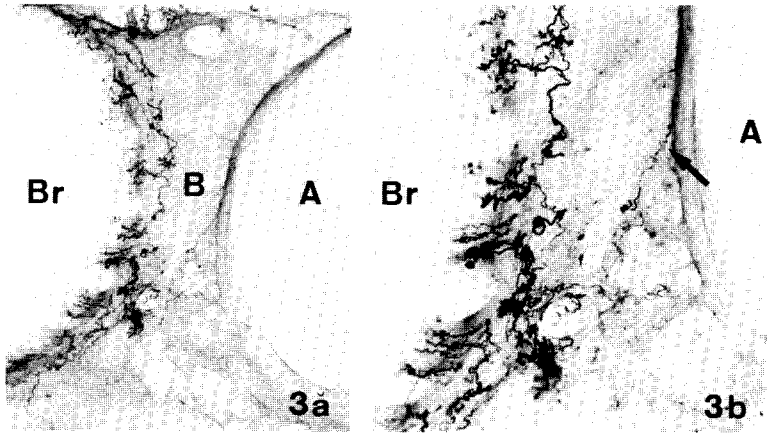

Fig. 3. Bright-field photomicrograph showing CGRPI nerve fibers in the wall of a bronchus (BR), which reach the subepithelial zone of BALT (B), at low magnification (a) and at high magnification (b). Note that a few CGRPI nerve fibers (arrow) dissociate from the nerve fiber bundle within a pulmonary artery branch (A) to project into the BALT. (a) x150; (b) x300.

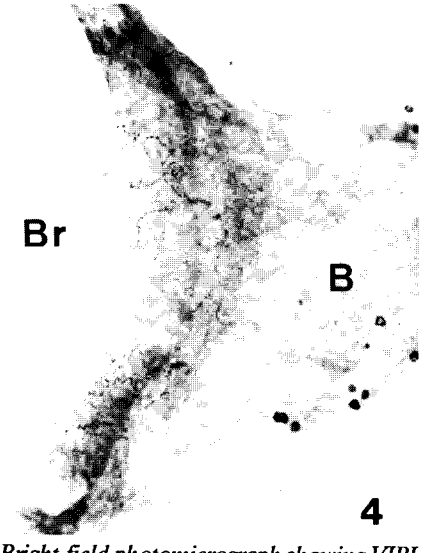

Fig. 4. Bright-field photomicrograph showing VIPI nerve fibers in the subepithelial zone of BALT. B=BALT; Br=bronchus. x150.

nerve plexuses in the walls of pulmonary artery branches, and SP, CGRP and VIP fibers dissociate mainly from those in bronchial walls. Within the BALT the four peptides exhibit different distribution patterns, although SP and CGRP seem to be localized, at least in part, to the same neuronal elements on the basis of previous studies (16-20). These findings suggest that the peptidergic nerve fibers subserve different functions in BALT. Since NPY as a potent vasoconstrictor is closely associated with blood vessels throughout the BALT (21,22), it may participate in microcirculatory control in the BALT by changing blood vessel diameter, possibly together with catecholaminergic fibers (22,23). It is also tempting to speculate that NPY indirectly affects the number of lymphocytes reaching high endothelial venules or postcapillary venules which are the sites of lymphocyte recirculation in the BALT (24,25). Although SP and CGRP neuronal elements in the walls of bronchi, but not those in motor end plates of the larynx or in some neuroendocrine cells of the trachea and stem bronchi, appear to be of sensory origin (26,27), these peptides may be liberated from peripheral nerve terminals in response to exogenous stimuli (28-30). Indeed, SP is hypersecreted from sensory nerve endings of the inflamed knee joint (31-34). It is likely that SP in BALT, which presumably originates in sensory ganglia (35,36), is actively released from nerve terminals in patients with bronchitis. If so, the released SP might act on local lymphocytes with SP receptors on

their surfaces to affect their migration and proliferation in the BALT (37). In support of this concept, our preliminary immunoelectron microscopic study demonstrates SP fibers in proximity to numerous lymphoblast-like cells within the BALT (38). CGRP fibers, especially when co-releasing SP into the BALT, might be involved in similar pathophysiological events. The functional significance of scattered VIP fibers at the subepithelial zone of BALT remains to be determined.

This study raises the possibility that peptidergic nerve fibers exert effects on the mucosal immune system within the lung in complex ways as well as participating in the regulation of blood vessel diameter, bronchial wall contractility, and secretory processes in the respiratory tract (39).

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Naomi Inoue, M.D.
Department of Anatomy
Osaka Medical College
2-7, Daigakumachi, Takatsuki
Osaka (569), JAPAN