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Distribution of Catecholaminergic Neurotransmitters and Related Receptors in Human Bronchus-Associated Lymphoid Tissue

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Key Words

Adrenergic nerve fibers · β-Adrenergic receptors · Bronchus-associated lymphoid tissue · Lung, human · Neuromodulation

Abstract

Background: The functions of the bronchus-associated lymphoid tissue (BALT) are under the control of the autonomic nervous system (sympathetic and parasympathetic nerve fibers). ***Objectives:*** The relationships between the adrenergic nerve fibers and β-adrenergic receptors were studied in the human BALT with the aim to demonstrate a probable neuromodulation. ***Methods:*** Morphological observations (staining with hematoxylin-eosin and scanning electron microscopy images) were carried out on samples of human BALT harvested during autopsies. Moreover, histochemical staining for norepinephrine (adrenaline = adrenergic nerve fibers) as well as for other catecholamines was performed. Finally, β-adrenergic receptors were stained by means of a β-blocking, radiolabeled drug (pindolol ^{125}I). All our data were submitted to morphometric analysis (quantitative analysis of images and statistical analysis of data). ***Results:*** Our results provide direct evidence of the presence and distribution of catecholaminergic nerve fibers and related β-adrenergic receptors in BALT. β-Adrenergic receptors are present above all in the most richly innervated part of the BALT, and are, therefore, in close relationship with their related adrenergic nerve fibers. ***Conclusions:*** Studies on the distribution of adrenergic neurotransmitters and related β-adrenergic receptors in the human BALT are the first step for the demonstration of a probable neuromodulation of BALT.

Introduction

The human bronchus-associated lymphoid tissue (BALT) is under both sympathetic and parasympathetic nervous control [1]. Therefore, it is possible that BALT contains the receptors of sympathetic and parasympathetic neurotransmitters [2]. Presently receptor imaging techniques are characterized by low resolution as well as technical difficulties and high costs [3]. With these limitations, we applied receptors and autoradiographic techniques on some small fragments of the human BALT obtained during autopsy. These techniques allowed us to identify the cell populations expressing catecholaminergic receptors even in such a heterogeneous and complex organ as the human lung, including BALT [4].

The circulating catecholamines in the respiratory systems play an important role in controlling airway dynamics while the sympathetic nerve supply is thought to signal to serous and mucus glands [5].

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In spite of the numerous data regarding the role of the catecholaminergic nerve supply in controlling airway dynamics [5, 6], data on the anatomical distribution of these nerve fibers in airways and in pulmonary vasculature present discrepancies.

In this study special attention was paid to the presence of β-adrenergic receptors in human BALT with the aim of identifying the possible relationship between BALT and adrenergic nerve fibers and related adrenergic receptors.

Material and Methods

Morsels of human lung (including BALT) were obtained during autopsies performed within 16–24 h after death. All procedures were in accordance with the ethical standards of the responsible committee on human experimentation and with the Declaration of Helsinki (1964) of the World Medical Association (amended in 1975 and 1983) [7].

The fresh material was fixed, dehydrated, paraffin-embedded and cut for hematoxylin-eosin staining. Other tissue samples were processed for 6–10 h in a 2% glutaraldehyde solution in 0.1 *M* cacodylate buffer at pH 7.35 and subsequently washed in tap water for 2–3 h, dehydrated by means of the critical point drying technique, gold stained by a Denton apparatus and observed and photographed by means of a scanning electron microscope JEOL JSM-35. Finally, other samples were refrigerated and cut using the freezing microtome at –20 °C. On fresh samples the catecholaminergic nerve fibers were stained by Falck's technique using the fluorescence induced by formaldehyde vapors and/or by glyoxylic acid technique (GIF) [8].

For catecholaminergic nerve fiber staining, small samples of tissue were dissected and treated with formaldehyde vapors [9]. After staining, the fluorescence microscopy observations were done by means of a Zeiss photomicroscope provided with an epi-illumination system. Samples were photographed using a black and white pan F18 DIN film. The nonspecific fluorescence was barred with special filters, furnished as an option by Zeiss. Photographs were submitted to the quantitative analysis of fluorescent structures using a Quantimet 500 image Analyzer (Leitz) equipped with specific software. The following parameters were measured: (1) total area of fluorescent structures, (2) number of nerve fibers, and (3) number of crossings or intersections of nerve fibers. All these values can be expressed in conventional units (CU) [10].

For the staining of adrenergic nerve fibers a glyoxylic acid-induced fluorescence technique was used [8]. Briefly, immediately before use the staining solution was prepared by adding a solution of 0.236 *M* potassium phosphate (pH 7.4), 0.2 *M* sucrose and 1% glyoxylic acid. This staining is named sucrose, phosphate, glyoxylic acid (SPG). The slides with samples were immediately dipped in this solution for 5 min. To assure a comparable fluorescence it is important to standardize times and temperatures without intervals.

After staining, the sections were drained, covered with nonautofluorescent immersion oil, heated at 95 °C for 5 min and observed. In fact, the sections were immediately observed and photographed to prevent any diffusion and photodecomposition of the fluorescence. The sections were examined and photographed under a Zeiss photomicroscope equipped with exciter and barrier filters and with a mercury lamp for observation of fluorescence.

β-Adrenergic Receptor Staining

The topographic localization of β-adrenergic receptors in the BALT was obtained by autoradiographic staining, using a β-blocking drug (pindolol, Sigma-Aldrich) marked with ^{125}I. The biological activity of the labeled pindolol (^{125}I-PIN) was tested in vitro and in vivo, by dosing its norepinephrine-dependent adenylate cyclase. After the binding, the pharmacological effect of ^{125}I-PIN seems to be 40% decreased.

Radiolabeled PIN was used to topographically locate β-adrenergic receptors on fresh-cut sections of human BALT. The specificity of this reaction has been evaluated by pretreating some sections with nonlabeled pindolol and thereafter exposing them to ^{125}I-PIN or treating samples with agents inhibiting the reaction. Pindolol 1-(1H-indol-4-yloxy)-3-isopropylamino-2-propanol $C_{14}H_{20}N_2O_2$ is a β_1-adrenergic receptor antagonist and putative 5-HT_{1A} serotonin receptor agonist vasodilator. This drug plays a role in the central and peripheral nervous system [11–14].

As inhibitors of pindolol we used propanolol (blocking both β_1 and β_2 receptors) [15], ICI 118 551 (blocking only β_2 receptors) [16] and CGP 20712A (blocking only β_1 receptors) [17].

The cryostatic sections were stretched on a microscope slide and treated for 120 min at room temperature with ^{125}I-PIN; subsequently, they were washed, for 10 min in an isotonic solution of 10% polyvinylpyrrolidone, thermostat dried at 37% and mounted in a water-soluble medium. The corners of the covering slide were treated with boiling paraffin and enameled.

Observations were performed under a Zeiss photomicroscope.

Quantitative Analysis of Images

In order to evaluate the amount of staining, a quantitative analysis of the intensity of the morphological and/or autoradiographic staining was performed on photographs by means of a Quantimet Analyzer (Leica®). Final values must be submitted to statistical data analysis. The values reported in our experiments represent the intensity of staining for each type of tissue and are expressed in CU ± SEM. Further details on quantitative analysis of images (QAI) and on CU are reported in the manual of the Quantimet image analyzer [10].

Statistical Analysis of Data

The statistical methods used throughout this study may be interpreted as an accurate description of the data rather than a statistical inference of such data. The preliminary studies of each value were done using basic sample statistics. Mean values, maximum and minimum limits, variations, standard deviation, SEM and correlation coefficients were calculated [18].

The relationship between each pair of variables was studied using the respective correlation coefficients. A correlative analysis of the fluorescent reaction induced by formaldehyde vapors and histofluorescent staining with GIF and/or β-adrenergic receptors was performed. Correlation coefficients denote a significant level of less than 0.001 ($p < 0.001$) while the correlation coefficient is not significant when $p > 0.05$. This correlation coefficient was calculated according to Castino and Roletto [19].

Results

Our results are shown in figures 1–6 and are summarized in tables 1 and 2.

Light microscopy: A small piece of lung obtained at autopsy was stained with hematoxylin-eosin. A little islet of BALT (arrow) is localized between two bronchiolar branches and is surrounded by lung parenchyma (fig. 1).

Scanning electron microscopy: Small fragments of the lung were treated with scanning electron microscopy, as reported in Material and Methods. A small islet of BALT (arrow; formed by about 200–300 cells) is completely surrounded by bronchial epithelial cells (fig. 2).

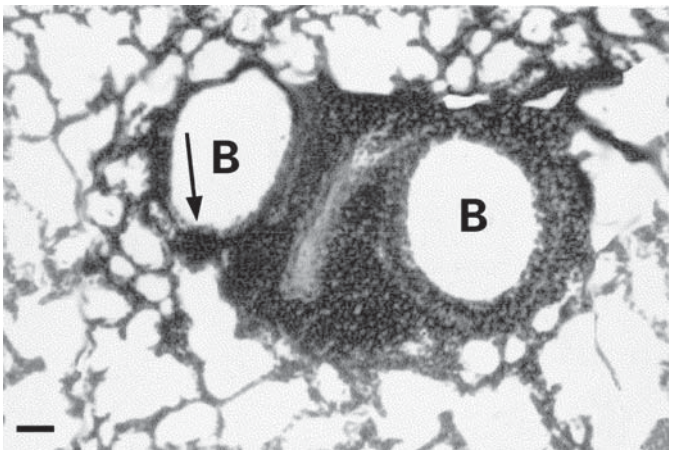
Falck staining: Many fluorescent nerve fibers, running in BALT, stained using the Falck technique as reported in Material and Methods, show crossings and varicosities along their course. Falck staining preserved monoamines in situ by means of a sophisticated freeze-drying technique inside the tissues that produce them. By using formaldehyde vapors the monoamines can be transformed in green-yellow fluorescent derivates, depending on their chemical nature. These derivates can be observed by fluorescent microscopy and are localized inside nerve fibers (arrows) (fig. 3).

Fluorescence histochemistry: Exposure of experimental sections to GIF causes the development of yellow-green fluorescent structures in BALT. The majority of fluorescent structures are nerve fibers showing varicosities and crossings along their course (fig. 4).

Overlapping autoradiography: The slice with our samples (sections of human BALT stained with hematoxylin-eosin and treated with ^{125}I-PIN) (fig. 5a) and the radiographic film exposed by radioactive PIN (as an X-ray plate without any staining), indicating the presence of radiolabeled sites and/or the presence of β-adrenoreceptors, can be seen (fig. 5b).

Light microscopy autoradiography: At a higher magnification figure 6 shows the results of light microscopy autoradiography in the human BALT. As can be seen, the dark points represent the receptor sites for the radiolabeled drug and therefore they represent the β-adrenergic receptors which bind ^{125}I-PIN. The use of radioactive chemical markers, specific for a given receptor class, became an efficient tool for identifying, mapping and quantifying neurotransmitter receptors in tissue sections. Many drugs (either agonists or antagonists) can be labeled with radioactive atoms and demonstrated abilities to bind to receptors in tissue sections with appropriate pharmacological parameters. Nevertheless, such an approach has certain limitations: it does not differentiate between receptor isotypes for the same neurotransmitter. It has limited microscopic resolution and does not allow analysis at the single cell level. Despite this, receptor radioautography is today a widely used and very efficient anatomical tool in morphological research.

We did the QAI on all our morphological images (about 500 in total) and related results are summarized in tables 1 and 2. Table 1 shows the quantitative result obtained by QAI on microscopy images of BALT (toluidine blue, Falck, GIF). As can be seen, the total area of BALT harvested from twenty different portions of the lung is 258.4 ± 1.6 CU.



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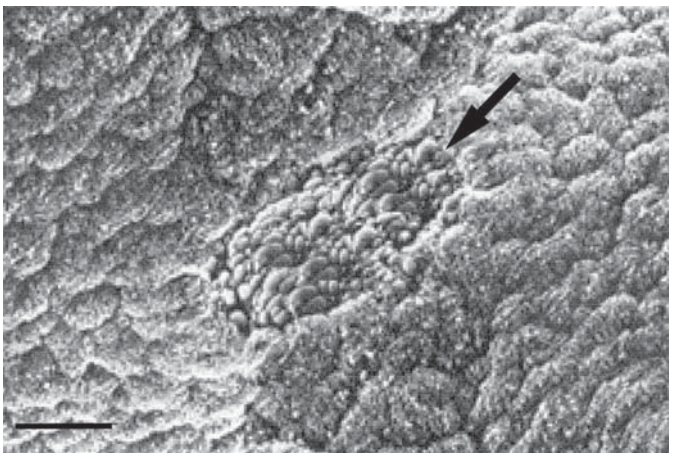

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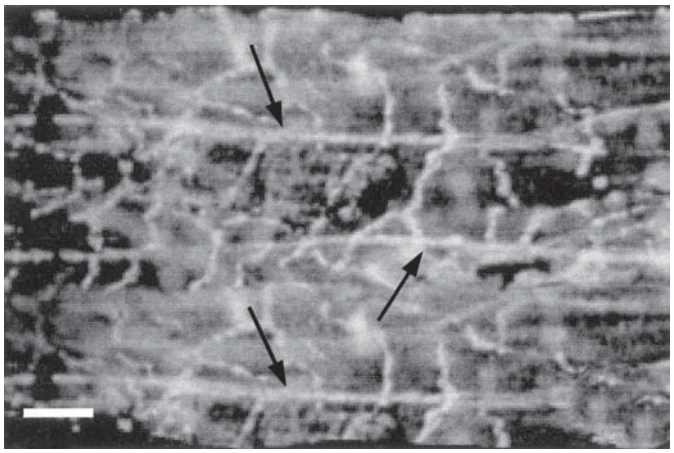
Fig. 1. Light microscopy: A small autopsy specimen of lung was stained with hematoxylin-eosin. BALT aggregates (arrow) are contained between two bronchiolar forks (B) and are surrounded by lung parenchyma. Magnification ×460.

Fig. 2. Scanning electron microscope picture of a small fragment of the human lung. A small islet of BALT (arrow; formed of about 200–300 cells) is surrounded by bronchial epithelial cells. Magnification ×1,380.



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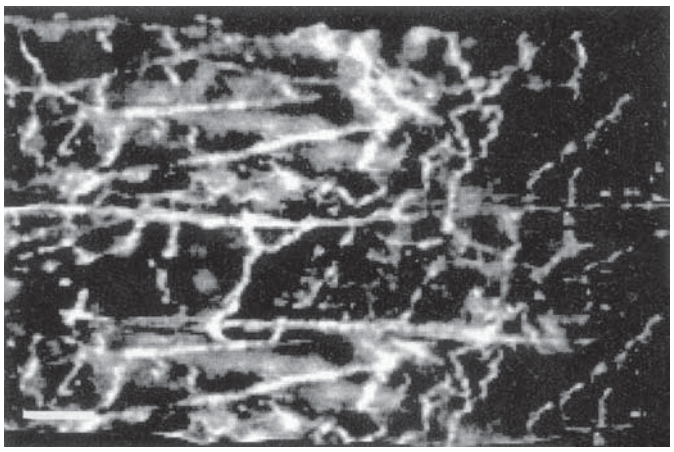

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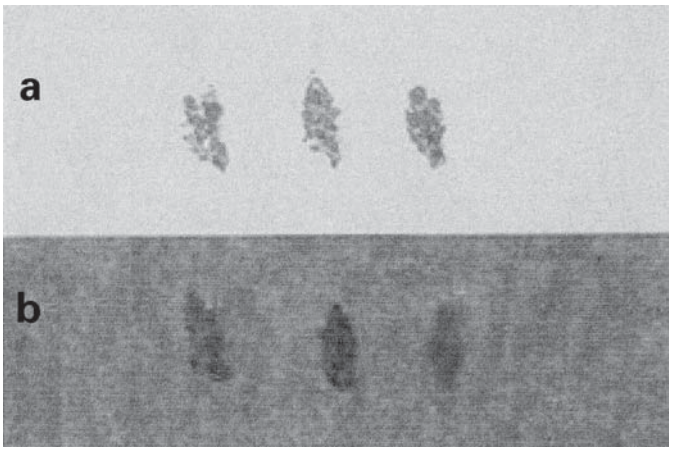

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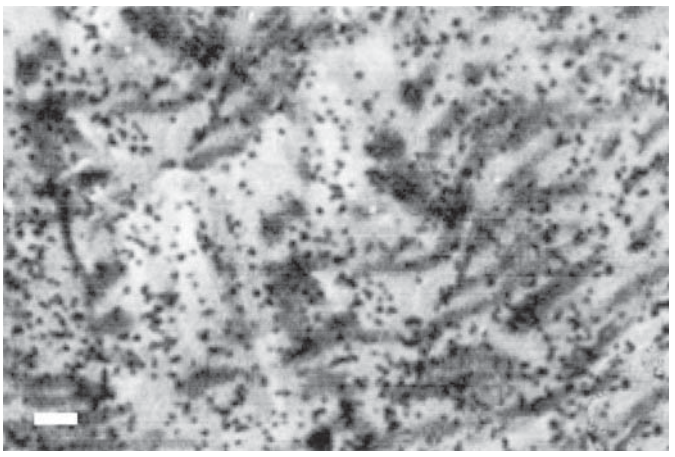

6

Fig. 3. Falck staining: Many fluorescent nerve fibers (arrows), running in the human BALT, were stained with formaldehyde vapors. These fibers showed crossings and varicosities along their course. Magnification ×920.

Fig. 4. Fluorescence histochemistry: Exposure of experimental sections to GIF caused the development of yellow-green fluorescent structures in BALT. The majority of fluorescent structures were nerve fibers, showing varicosities and crossings along their course. Magnification ×920.

Fig. 5. A section of human BALT treated with ^{125}I-PIN. **a** Slice with sample treated with labeled PIN and stained with hematoxylin-eosin. **b** The radiographic film impressed, indicating the presence of radiolabeled sites and/or the presence of β-adrenoreceptors. The overlapping autoradiography is presented at magnification 1:1.

Fig. 6. At higher magnification (1,380×) the results of light microscopy autoradiography were shown. The dark points indicate the receptor sites for the radiolabeled drug and, therefore, they represent the β-adrenergic receptors which bind ^{125}I-PIN.

Table 1. Human BALT: values of QAI (CU) performed on light-microscopic images

Total BALT area[a]	258.4 ± 16.6
Total fluorescent area	61.6 ± 3.4
Number of varicosities	19.4 ± 1.2
Number of crossings	15.3 ± 1.6

Values are means ± SEM. $p \leq 0.001$, calculated as reported in Methods.

[a] Twenty BALT areas were harvested and examined.

Table 2. Human BALT: values of QAI performed on light-microscopic autoradiographies

Total BALT area[a], CU	258.4 ± 16.6
-Adrenergic receptors, CU	34.6 ± 1.8
$_1$-Adrenergic receptors, %	26.4 ± 1.3[b]
$_2$-Adrenergic receptors, %	73.6 ± 2.4[b]

The values are means ± SEM.

[a] Twenty BALT areas were harvested and examined.

[b] These values are expressed as % of the value reported in line 2 (34.6 ± 1.8 CU).

Table 2 deals with the amount of β-adrenergic receptor in human BALT. Each value was obtained by means of QAI performed on autoradiographic images. Total β receptors in twenty samples of BALT are 34.6 ± 1.8 CU; 26.4% of these are β_1 and 73.6% are β_2 receptors.

Discussion

Adrenergic nerve fibers and β-adrenergic receptors have been identified in the human BALT. Morphological observations and histochemical staining for catecholamines demonstrated that BALT possesses a rich supply of sympathetic nerve fibers, while overlapping and light microscopy autoradiography demonstrated that β-adrenergic receptors are mainly present in the most richly innervated part of the BALT.

Respiratory surfaces that come into contact with the outside world possess a specialized immune system, a large amount of which consists of BALT. This immune system is made up of nonencapsulated submucosal lymphoid nodules and diffuse lymphocytic infiltrates in the submucosa of the respiratory tracts [20]. Each structure becomes populated with lymphoid cells only after coming in contact with environmental microbes and antigens and there has even been a suggestion that they are essential stimulants for differentiation of immunoglobulin genes [21].

BALT can be divided into several lymphoid aggregates that are completely separated from each other and differ in color from other surrounding cells even though a connective capsule does not exist. The presence of BALT in specific sites along the respiratory tract suggests that a special microenvironment is required for the induction of its immune role [22–24].

Airways are supplied with double parasympathetic and sympathetic autonomic nerve fibers. Parasympathetic nerve fibers of airways and lungs arise from the vagus nerve. Sympathetic nerve fibers of the airways and lungs arise from the T3 and T4 segments of spinal cord. Ganglionic sympathetic neurons are located in the inferior cervical ganglion in the first 4–5 sympathetic ganglia of the thoracic chain [1]. Sympathetic nervous control of airways plays a key role in the respiratory apparatus: in particular it relaxes airway smooth muscle and constricts pulmonary and bronchial vessels. Moreover, impaired noradrenergic transmission is considered to be a factor in the pathogenesis of several respiratory disturbances [25, 26].

In conclusion, our results provide direct evidence of the presence of β-adrenergic receptors in the BALT. β-Adrenergic receptors are principally present in the most richly innervated part of the BALT, and are therefore in close relationship with related adrenergic nerve fibers.

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