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Ultrastructural and immunohistochemical studies on the ontogenic development of bronchus-associated lymphoid tissue (BALT) in the rat: special reference to follicular dendritic cells

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ABSTRACT: The ontogenic development of lymphoid and non-lymphoid cells in bronchus-associated lymphoid tissue (BALT) of the rat was studied ultrastructurally and immunohistochemically. In the late foetal period, only the alveolar macrophages showed a weak positivity for leucocyte common antigen, but no immune region associated (Ia) antigen was detected by monoclonal antibody, MAS 043. Mast cells were present. At 6 days of age, Ia-positive cells were observed in the alveolar wall and peribronchial interstitial tissue, and ultrastructurally there was an aggregation of the fibroblastoid mesenchymal cells. By 10 days of age, the aggregation of lymphoid cells together with S-100-positive reticulum cells had formed a BALT-like periarterial lymphoid sheath. In the adult animals, an obvious B-cell area was present in the central part and subepithelium of BALT, whilst in this area, S-100-positive, strongly Ia-positive cells with a dendritic form were observed. These dendritic cells appeared to be identical to the follicular dendritic cells (FDC) seen in the secondary follicles of lymphoid organs. Those cells may be derived from the fibroblastic reticulum cells, and may function to present antigen to lymphocytes.

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The defence mechanisms of the respiratory tract include mucociliary clearance, humoral and cell-mediated immunity, and the alveolar macrophages [1]. Collections of lymphoid cells are seen in the bronchus-associated lymphoid tissue (BALT) and in aggregates of lymphoreticular cells, as well as in the hilar and paratracheal lymph nodes [2]. In several mammalian species, BALT appears as condensed lymphoid aggregates to the bronchial wall as described by BIENENSTOCK *et al.* [3]. In humans, secondary lymphoid infiltration of the bronchi has been observed in such pathological conditions as silicosis or chronic bronchitis, but not in normal bronchi [4, 5]. Accordingly, we investigated the ontogenic development of BALT in the rat.

Follicular dendritic cells (FDC) are present in the germinal centres of lymphoid organs. Whilst their origin and precise role have still to be determined, they are thought to be *in situ* cells that function in presenting antigen to the lymphocytes [6]. It is not clear whether such cells are present in non-lymphoid organs, such as the lung. Our objective was to investigate the ontogenic development of lymphoid and non-lymphoid cells in BALT, with special reference to the dendritic cells.

Material and methods

Animals

A total of 55 Sprague-Dawley (SD) rats were kept in an ordinary environment with food and water provided *ad libitum*. The age and number of animals evaluated was as follows: late foetal period (12); Day 0 (3); postpartum Days: 1–3 (6), 6 (6), 7–8 (5), 9–13 (5), 14–28 (4), 42–70 (5), 112–140 (4), and ≥140 (5). Data were subsequently evaluated according to foetal period and postpartum weeks 1–4, and adult period over 6 weeks of age. Furthermore, a total of 12 Specific Pathogen Free (SPF) SD rats were obtained from Clea Japan, Inc. (Tokyo, Japan). The age and number was as follows: postpartum Days 21–28 (6) and 35–45 (6). Animals were sacrificed by cervical dislocation or by the intraperitoneal injection of a lethal dose of barbital.

Lung tissue preparation

The lungs were quickly removed, dissected in a longitudinal plane and fixed in 8% paraformaldehyde for preparation of wax sections.

Ultrastructural observation

Specimens were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer for 2–4 h, postfixed in 2% osmium tetroxide for 2 h, dehydrated in graded ethanol, and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate, and observed with a JEM 100-C electron microscope.

Immunostaining and quantitation of cell populations

The indirect immunoperoxidase or immunoalkaline-phosphatase methods were performed as described previously [7]. The first layer monoclonal antibodies used in these experiments were as follows: MAS 026 (Sera-lab, Sussex, UK) to anti-rat leucocyte common antigen [8]; MAS 027 (Sera-lab) to anti-rat Thy.1 [9]; MAS 043 (Sera-lab) to anti-rat immune region associated antigen (Ia) [10]; MCA 340 (Serotec, Oxford, UK) anti-rat B-cells [11]; and polyclonal anti-bovine S-100 protein (Dacopatts, Glostrup, Denmark) [12, 13]. Density of Ia-positive cells·mm^{-2} proximal bronchial epithelium was determined by counting stained cell in three different fields of view, with the aid of a calibrated microscope. Results were expressed as mean and standard deviation.

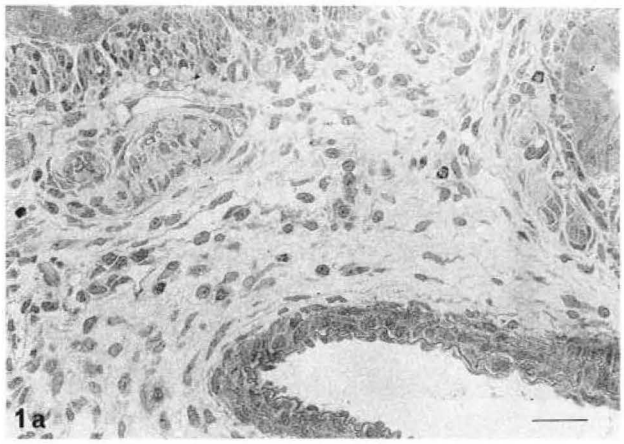
Results

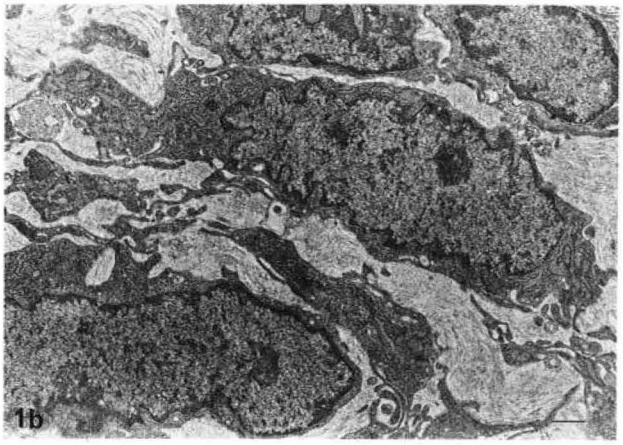
Late foetal period

Rats in the late foetal period (21 days gestation) showed a poor differentiation of the bronchial epithelium. Spindle-shaped cells could be observed between the main bronchus and the associated vessels. Macrophages were present in the alveolar spaces. Immunohistologically, leucocyte common antigen (LCA) was expressed only weakly in the alveolar macrophages. Mast cells were detected in the interstitial tissue by metachromasia with toluidine blue staining. Ultrastructural identification of the lung tissue was difficult except for the immature epithelial cells with poorly-developed cilia, a few lamellar inclusions, and wide areas containing glycogen.

One week of age

Differentiated bronchial epithelia surrounded by interstitial cells were observed on Day 6. In semi-thin sections, the aggregation of reticulum cells, and an increased number of mast cells were observed (fig. 1a). Ultrastructural examination showed fibroblastoid mesenchymal cells with intermediate filaments in the cytoplasm (fig. 1b). LCA-positive cells were present in the alveolar wall and, more frequently, around the main bronchus. The Ia-positive cells showed a similar distribution. Large cells expressing Ia were seen in the interstitial tissue (fig. 1c).





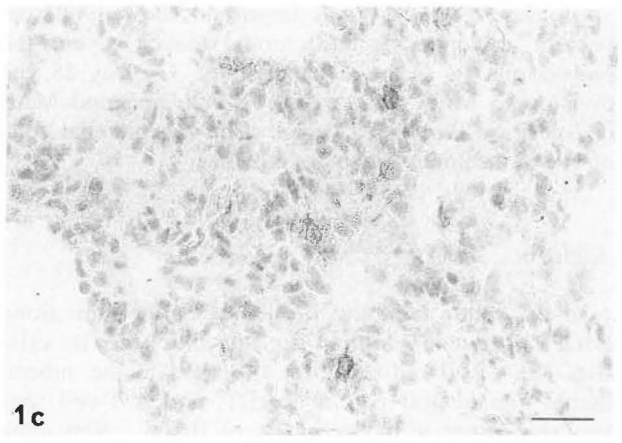


Fig 1. – a) Semi-thin section of the lung of a 6 day old rat stained with toluidine blue. Note the aggregation of reticulum cells. Mast cells showing metachromasia of the granules are frequently observed. (Scale bar=30 μm); b) Ultrastructure of the lung of a 6 day old rat. Subepithelial fibroblastoid mesenchymal cells with intermediate filaments are seen in the cytoplasm. (Scale bar=1 μm); c) Immunoperoxidase staining for Ia antigen in the lung of a 6 day old rat. Ia-positive large cells with dendritic process are seen in the interstitial tissue. (Scale bar=20 μm). Ia: immune region associated antigen.

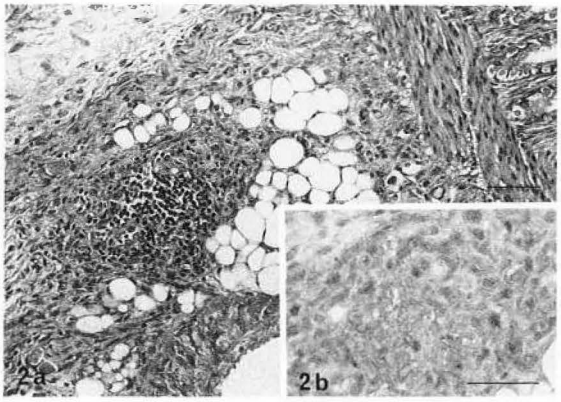


Fig. 2. – a) Haematoxylin and eosin staining of the lung of a 10 day old rat. Lymphoid aggregation together with reticulum cells is observed between the main bronchus and the accompanying vessels. A periarterial lymphoid sheath has formed. (Scale bar=50 μm); b) Immunoalkaline-phosphate staining of S-100 protein in the same specimen as figure 2a. Cells weakly positive for S-100 are observed in the centre and the periphery of BALT. (Scale bar=50 μm). BALT: bronchus-associated lymphoid tissue.

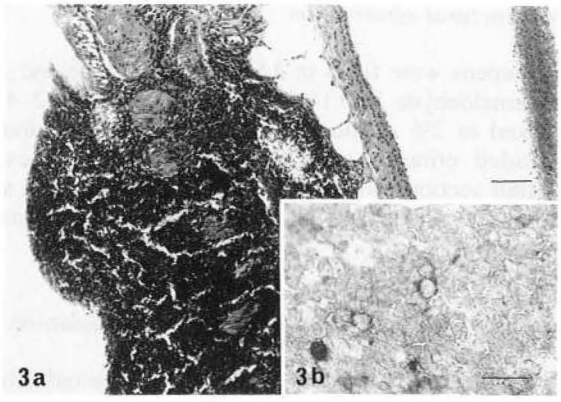


Fig. 3. – a) Haematoxylin and eosin staining of BALT in the lung of a 16 week old rat. Infiltration of lymphocytes is observed in the subepithelium and the lymphepithelium lacking cilia. (Scale bar=50 μm); b) Immunoalkaline-phosphatase staining for S-100 protein in the same specimen as figure 3a. S-100 is expressed more intensely as compared with figure 2b (10 day old rat) on the cells in the centre of BALT. (Scale bar=20 μm). BALT: bronchus-associated lymphoid tissue.

Two weeks of age

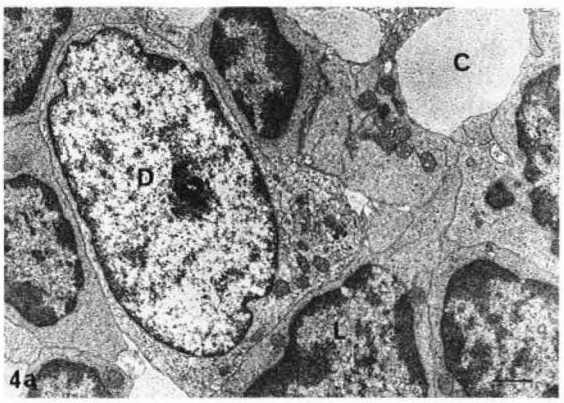
From Day 9, there was obvious lymphoid aggregation around a branch of the pulmonary artery, resembling the periarterial lymphoid sheath of the splenic white pulp (fig. 2a). Reticulum cells in BALT were weakly positive for S-100 protein, observed in both the marginal area of BALT and in the centre of lymphoid aggregation (fig. 2b).

Three to four weeks of age

On Day 20, BALT was large and showed internal vessels. Lymphocyte infiltration extended beyond the propria mucosa to the subepithelium. On Day 28, the division to MCA 340-positive B-cell area and MAS 027-positive T-cell area began. The subepithelial zone of every specimen was a B-cell area.

Adult rats

In the adult rats, the bronchial epithelium along BALT showed lymphepithelium, lacking in cilia (fig. 3a). A B-cell area was observed in the subepithelial and central part of BALT, and a T-cell area was recognized in the periphery of BALT. Reticulum cells that were strongly Ia-positive were seen in the centre of BALT. The intensity of staining for S-100 was increased in the reticulum cells (fig. 3b). Ultrastructurally, these reticulum cells were seen to contact the lymphocytes by means of dendritic cytoplasmic processes (fig. 4a and b). The BALT was richly innervated and "high endothelial venules" and lymphocytes were observed to pass through it.



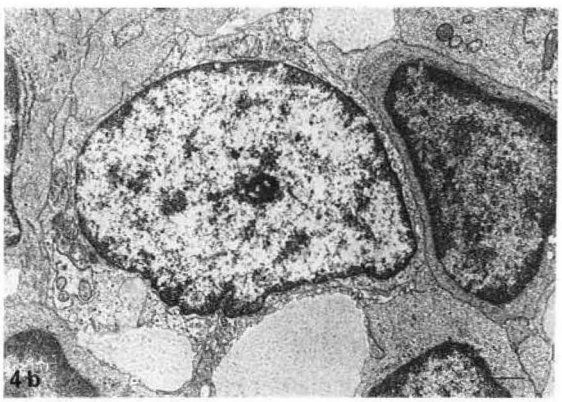


Fig. 4. – a) Ultrastructure of the central part of BALT (16 week old rat). Follicular dendritic cells (FDC) are observed with dendritic cytoplasmic processes which contact the lymphocytes. The nuclei of FDC have an euchromatic structure. The cytoplasm consists of a thin layer which contains some mitochondria, small strands of rough-surfaced endoplasmic reticulum, Golgi apparatus and vesicles. Collagen fibres are present between the cells. D: follicular dendritic cell; L: lymphocyte; C: collagen fibre; BALT: bronchus-associated lymphoid tissue. (Scale bar=1 μm); b) Ultrastructural appearance of lymphocytes and FDC. FDC with slender processes, forming a web-like structure, are observed. (Scale bar=1 μm).

SPF rats

SPF rats on Day 21 showed less and smaller BALT compared with the animals kept under conventional conditions. A specimen contained no more than 1 BALT, in which the number of cells was <400. Furthermore, 255±19 cells·mm^2 were Ia-positive outside BALT. In age-matched conventionally kept rats, 5–7 BALT per specimen contained a total of 1,500–1,800 cells, and 617±113 cells·mm^2 were Ia-positive outside BALT (SPF *vs* conventional: $p<0.05$ by Student's t-test). On Day 35, subepithelial infiltration of lymphocytes began. Number (2–6) and size (1,300–4,200 cells) of BALT were identical to conventionally kept rats, but 344±14 cells·mm^2 were Ia-positive outside BALT, still less than in animals conventionally kept (669±66 cells·mm^2) ($p<0.05$).

Discussion

BALT develops postnatally in response to an antigen stimulus. In the lung of 6 day old rats, there is aggregation of the reticulum cells between the main bronchus and the accompanying vessels, with lymphoid aggregation occurring by day 9. An important role of antigen stimulus in young rats was manifested by making a comparison between SPF and conventionally kept animals. Although no obvious secondary follicles are present, BALT shows a distinct B-cell area which includes FDC, as was confirmed both immunohistochemically and ultrastructurally. Dendritic cells [14, 15] are classified as leucocytic [16–20] and fibroblastic [21]. FDC are thought to be derived from the fibroblastic reticulum cells [22]. From our observations, the aggregation of fibroblastic reticulum cells precedes the formation of BALT, and develops into a dendritic morphology.

Pulmonary lymphoid tissue has recently been studied immunohistochemically. Although lymphocytes have been demonstrated at birth [23, 24], we found that very little LCA antigen was expressed by alveolar macrophages in the lungs of rat foetuses. A previous study of T-cells and B-cells associated with the development of BALT showed that, at about the 4th week, the B-cell area constantly forms in the subepithelial zone [24]. From our observations, a large part of BALT was B-cell area. Most of the cells present in BALT are Ia-positive. In the rat, the macrophages are Ia-negative [25], although in the mouse, 10–30% of macrophages are Ia-positive [26]. Ia-positive dendritic cells have been observed in the tracheal epithelium and in the alveolar septal walls in rat lung, and have an intimate relationship to macrophages [27, 28]. From one week of age, Ia-positive cells were recognized around the main bronchus and in the alveolar wall. An immunohistochemical study on interdigitating dendritic cells reported their existence in the T-cell area of BALT [29]. Similar studies have been performed on gut- or nasal-associated lymphoid tissues [30–32]. It has been shown that Ia-positive non-lymphoid cells are increased in nasal lymphoid tissue during postnatal development; some of those cells may be antigen-presenting (dendritic) cells [32]. These cells have to be confirmed ultrastructurally.

Few ultrastructural studies have been performed on BALT [33, 34]. The existence of reticulum cells in BALT has been reported [34]. We suggest that these reticulum cells are identical to the dendritic cells, the FDC observed in the B-cell area. They appear to function as antigen-presenting cells and, together with the lymphocytes, may play an important role in local defences.

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