Microscopic, submicroscopic characterization of pro- and anti-inflammatory cell phenotypes of the lungs in conditions of experimental allergic inflammation

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The aim is to study the microscopic and submicroscopic characteristics of pro- and anti-inflammatory cell phenotypes of the lungs under conditions of experimental allergic inflammation.

Material and methods. We used histological and electron microscopic methods to study the lungs of 48 male guinea pigs in experimental ovalbumin-induced allergic inflammation, simulated by subcutaneous sensitization and subsequent intranasal inhalation with ovalbumin. Submicroscopic changes of respiratory endocrine cells, goblet cells, exocrine bronchiolar cells, mast cells, macrophages, eosinophils, endothelial cells of guinea pigs lungs were determined.

Results. The most significant reactive submicroscopic changes were established on the 23rd and 30th days of observation in the form of an increase in the functional activity of exocrine bronchiolar and goblet cells, as evidenced by the presence of a light nucleus with a predominance of euchromatin, nucleoplasm of low electron-optical density, nucleoli, developed granular endoplasmic reticulum and an increase in the number of goblet cells secretory mucous granules by electron microscopic examination. The revealed ultramicroscopic features of respiratory endocrine cells (an increase in a number of "empty" core dense vesicles), eosinophilic granulocytes (piecemeal degranulation), an increase in the number of mast cells granules, numerous pseudopodia in macrophages are the confirmation of the active participation of these cell phenotypes in the initiation of inflammation during the early period of the allergic inflammatory process in lungs.

Conclusions. A significant reaction of the innate nonspecific and adaptive immunity occurs in airways during the experimental ovalbumin-induced allergic inflammation, consisting primarily of the functional activation of eosinophilic granulocytes, mast cells, and macrophages, as well as an increase in the secretory activity of exocrine bronchiolar cells and goblet cells, which is confirmed by the changes investigated by electron microscopic examination and are accompanied by reactive changes in the vessels of microcirculatory bed.

Key words: electron microscopy, exocrine bronchiolar cell, mast cell, respiratory endocrine cell, goblet cells, guinea pigs.

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Мікроскопічна, субмікроскопічна характеристика про- та антизапальних клітинних фенотипів легень в умовах експериментального алергічного запалення

С. С. Попко

Мета роботи – з'ясувати мікроскопічну, субмікроскопічну характеристику про- та антизапальних клітинних фенотипів легень в умовах експериментального алергічного запалення.

Матеріали та методи. Застосувавши експериментальний, гістологічний та електронномікроскопічний методи, вивчили легені 48 самців морської свинки в умовах гострого овальбумін-індукованого алергічного запалення, яке моделювали шляхом підшкірної сенсибілізації та наступної інтраназальної інгаляції овальбуміном. Визначали субмікроскопічні зміни дихальних ендокриноцитів, келихоподібних і бронхіолярних екзокриноцитів, макрофагів, мастоцитів, еозинофілів та ендотеліоцитів судин гемомікроциркуляторного русла легень морських свинок.

Результати. Найістотніші реактивні субмікроскопічні зміни виявили на 23 і 30 доби спостереження, а саме підвищення функціональної активності бронхіолярних і келихоподібних екзокриноцитів у складі епітелію дихальних шляхів. Про це свідчили наявність світлого ядра з переважанням еухроматину, нуклеоплазми низької електроннооптичної щільності, ядерця, розвинута гранулярна ендоплазматична сітка та збільшення кількості секреторних слизових гранул келихоподібних екзокриноцитів під час електронномікроскопічного дослідження. Визначили ультрамікроскопічні особливості дихальних ендокриноцитів: збільшення кількості «порожніх» везикул зі щільною серцевиною, еозинофільних гранулоцитів – ознаки часткової дегрануляції; збільшення кількості гранул високої електроннооптичної щільності в цитоплазмі мастоцитів; численні псевдоподії макрофагів є підтвердженням активної участі цих клітинних фенотипів в ініціації запалення протягом раннього періоду розвитку алергічного запального процесу в легенях.

Висновки. В експериментальній моделі овальбумін-індукованого алергічного запалення дихальних шляхів виникає суттєва реакція з боку клітинної ланки вродженого неспецифічного й адаптивного імунітету, що полягає передусім у функціональній активації еози-

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Key words: electron microscopy, exocrine bronchiolar cell, mast cell, respiratory endocrine cell, goblet cells, guinea pigs.

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нофільних гранулоцитів, мастоцитів і макрофагоцитів, а також збільшенні секреторної активності бронхіолярних і келихоподібних екзокриноцитів; це підтверджується змінами, що спостерігали під час електронномікроскопічного дослідження та супроводжувалися реактивними змінами судин мікроциркуляторного русла.

Ключові слова: електронна мікроскопія, бронхіолярний екзокриноцит, мастоцит, дихальний ендокриноцит, келихоподібний екзокриноцит, морська свинка.

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Nowadays three main processes responsible for the clinical manifestations of bronchial asthma are well known: airway allergic inflammation, bronchoconstriction, and mucus hyperproduction. However, the underlying histopathophysiological mechanisms responsible for these processes are complex and multifaceted, including diverse cellular phenotypes and cytokines [1–3]. In addition, the activity and influence of each cellular and molecular component varies considerably between individuals and may change with time, response to drug therapy, and environmental exposure, so it is extremely important to study the histophysiology of the allergic inflammatory process in a chronobiological aspect.

Among various cell phenotypes, some key cells that are usually involved in the initiation of the main histophysiological processes in the lungs can be identified, namely respiratory endocrine cells, eosinophils, mast cells as pro-inflammatory cell phenotypes [4–6]. Mast cells are involved in both innate and adaptive immune responses to allergens. Thanks to the presence of heparin, secreted by perivascular mast cells into the intercellular substance of the connective tissue, the permeability of microvessels increases, which in case of allergic inflammation causes the release of lymphocytes and plasma cells into the perivascular intercellular substance [6]. In addition, according to scientists, mast cells contribute to the maintenance of the chronic allergic inflammatory process of the respiratory tract and play a central role in the initiation of the immune response to the allergen, during which they transmit signals that stimulate the synthesis of IgE by plasma cells and the differentiation of Th2 lymphocytes [7].

Exocrine bronchiolar cells (EBCs) are antagonists of pro-inflammatory cellular phenotypes with an immunomodulatory effect in the form of suppression of the allergic inflammatory process. EBCs perform specialized functions necessary to protect the body in a normal state, but retain the ability to proliferate in response to damage. Some EBCs provide renewal of the epithelial line after damage to ciliated cells and other cell phenotypes [8]. EBCs also exert an anti-inflammatory immunomodulatory effect: their secretory protein CC16 regulates the immune response to various infectious agents and allergens in the lungs [8].

Detailing the ultrastructure of these cellular phenotypes of airways and lungs during the experimental allergic inflammatory process in the chronobiological aspect is necessary for a better understanding of the histophysiology of allergic inflammation, but to date it is not described enough.

Aim

The aim of this research is to study the microscopic and submicroscopic characteristics of pro- and anti-inflammatory cell phenotypes of the lungs under conditions of experimental allergic inflammation.

Materials and methods

The experiment was performed on 48 male guinea pigs, which were involved under standard macroenvironmental conditions in the animal facility of Zaporizhzhia State Medical University. All experimental procedures and animal care were carried out according to ethical guidelines (Strasbourg, 1986; Kyiv, 2001).

Experimental model of allergic airway disease. Induction of airway allergic inflammatory process was performed by subcutaneous sensitization and airway challenge through nasal inhalation with OVA (0.5 mg/mL per animal) mixed with aluminum hydroxide (10 mg/mL in saline per animal) on days 0, 7 and 14. From 21 to 28 days animals were exposed for 15 min to an aerosol of OVA (10 mg/mL in saline) using a nebulizer (Little Doctor International, Singapore, LD-211C) attached to a plastic chamber [9].

Experimental design. Animals were assigned equally into six experimental groups of 8 guinea pigs each. Group I–IV were guinea pigs sensitized and challenged with ovalbumin (OVA) (Sigma Aldrich, USA) with alum as an adjuvant (AlumVax Hydroxide vaccine adjuvant, OZ Biosciences, France), dropped out of the experiment respectively on the 23rd, 30th, 36th and 44th days after its start. Group V – guinea pigs sensitized and exposed to saline, served as control. Group VI – intact animals (norm).

Lungs removed and fixed in 10 % neutral buffered formalin. Formalin-fixed, paraffin wax embedded lung specimens were selected for histological preparation, prepared as 4 μm thick sections and stained with hematoxylin, and eosin for routine examination. Histological study was carried out on Carl Zeiss Primo Star microscope equipped with the Axiocam digital microphoto attachment using the ZEISS ZEN 2011 software.

Electron microscopy was performed on glutaraldehyde-fixed 1 × 1 mm specimens of lung tissue followed by processing in a 1 % solution of osmium tetroxide. Subsequently, the pieces were dehydrated in a series of graded ethanol up to 100 % according to histological standards, acetone with additional contrasting for 2 hours in 2.5 % uranyl acetate at 700 C. Pouring into the block was carried out by gradual impregnation of the material with acetone oxide with Eponym (2:1, 1:1, 1:2) and poured into pure Epon. The resin polymerization was carried out in two stages at 36 °C (12 hours) and 56 °C (24 hours). Ultra-thin (55–65 nm) sections were obtained on a "Power-Tome RMC Boeckeler" ultratom and contrasted with Reynolds lead citrate for 25 minutes at room temperature [10]. Ultrathin sections were viewed on a PEM-100-01 electron microscope.

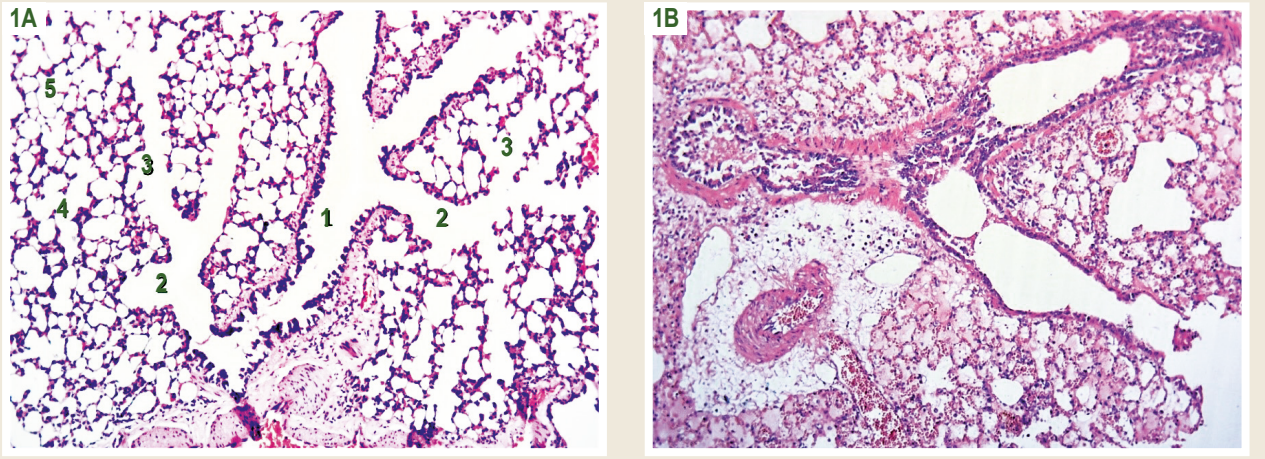

Fig. 1. Internal architecture of a guinea pig lung in the norm (A) and in the early stage of the allergic inflammatory process (B). **A.** Intact group. **1:** terminal bronchiole; **2:** respiratory bronchiole; **3:** alveolar duct; **4:** alveolar sac; **5:** alveoli. **B.** Experimental group I (23rd day after the start of the experiment). Stain: hematoxylin and eosin, ×100.

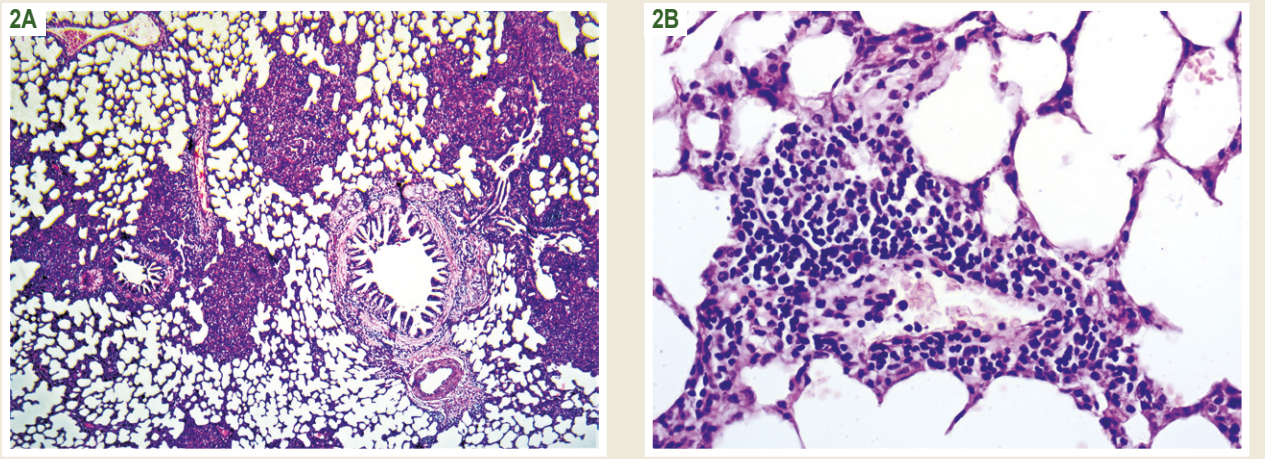

Fig. 2. Histological changes in the guinea pigs lungs in the late stage of the allergic inflammatory process. Experimental group IV (44th day after the start of the experiment). Stain: hematoxylin and eosin. **A:** ×100; **B:** ×400.

The ultrastructure of dense core vesicles (DCV) of respiratory endocrine cells was identified. We studied the ultrastructural features of goblet cells and EBCs, macrophages, mast cells, eosinophils, and endothelial cells of blood vessels of the pulmonary hemomicrocirculatory bed during the early and late stages of the experimental allergic inflammatory process.

Results

The structural basis of the internal structure of the guinea pigs' lungs consists of pyramidal or cone-shaped lung lobes. Dichotomous branching of small bronchi and bronchioles continues into the pulmonary lobe. The lobes are separated from each other by thin layers of connective tissue, poorly developed in guinea pigs. Inside the lung lobes, the inner diameter of the small bronchi decreases and as a result of their branching, they become terminal bronchioles – the final part of airways (*Fig. 1a*).

Terminal bronchioles branch, in turn, into respiratory bronchioles, the last generations of which already pass into cellular ducts, which receive 2–3 alveolar sacs, which blindly end into alveoli. Each lung lobe consists of 12–13 pulmonary acini. According to the internal architecture, guinea pig lungs can be attributed to the "light" or alveolar type of lungs – with a developed and large alveolar part, which provides a larger respiratory surface, and a poorly developed connective tissue stroma. However, the lungs of this type have a developed elastic framework. In OVA-sensitized guinea pigs during histological examination in the early period of the development of experimental allergic inflammation, we found thickening of the wall of small bronchi and terminal bronchioles, changes in the structure of pulmonary acini, and vessels of the hemomicrocirculatory bed, an increase in the number of immunocompetent cells, compared to the control group (*Fig. 1b*).

The detected changes are exudative-inflammatory in nature and coincide with the most pronounced clinical manifestations of allergic inflammation in guinea pigs (asphyxia, tachypnea, orthopnea). The degree of manifestation of inflammatory changes increases as the caliber of the bronchi decreases, reaching its maximum in the terminal bronchioles. During the late period of the development of

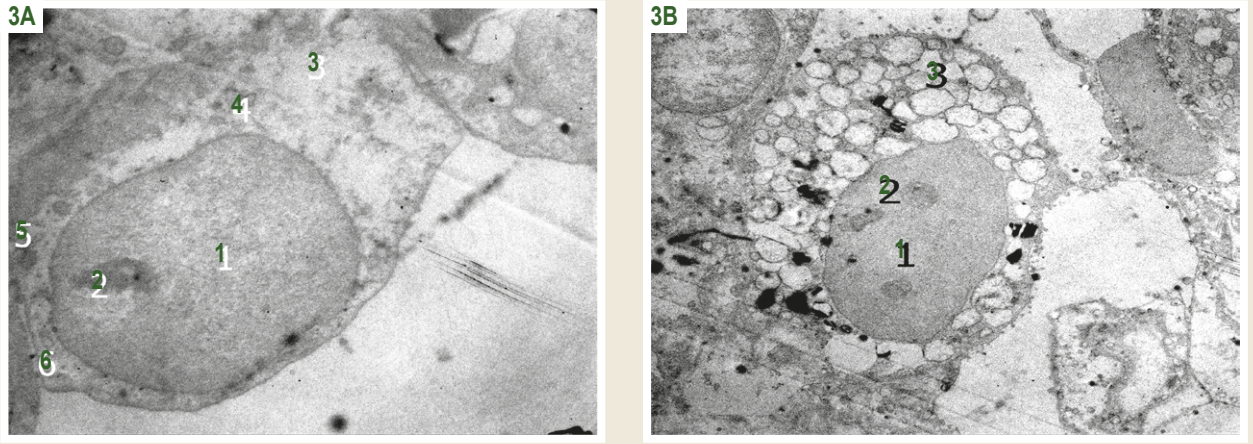

Fig. 3. Submicroscopic changes of epitheliocytes in airways under conditions of experimental allergic inflammation. **A.** Exocrine bronchiolar cell (experimental group I, 23rd day of the experiment). **1:** nucleus; **2:** nucleolus; **3:** cytoplasm; **4:** mitochondria; **5:** lysosome; **6:** granular endoplasmic reticulum. **B.** Goblet cell (experimental group II, 30th day of the experiment). **1:** nucleus; **2:** nucleolus; **3:** an increase in the number of secretory mucous granules in the cytoplasm. Transmission electron microphotos, ×6000.

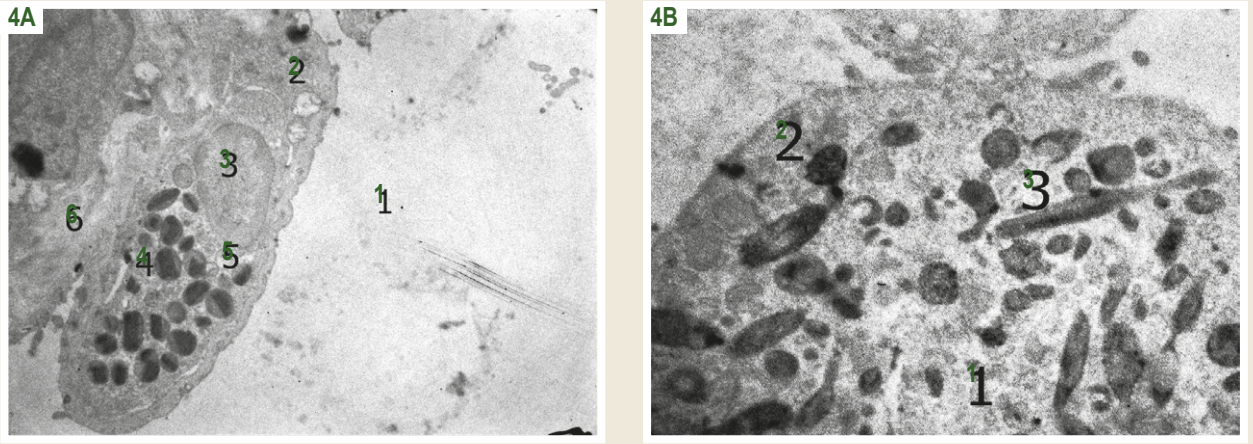

Fig. 4. Ultrastructural features of eosinophilic granulocytes in guinea pigs lungs in the early stage of the allergic inflammatory process. **A.** Control group. **1:** lumen of a blood vessel; **2:** eosinophilic granulocyte; **3:** nucleus; **4:** large eosinophilic granule; **5:** small eosinophilic granule; **6:** endothelium of a blood vessel. **B.** Experimental group I (23rd day after the start of the experiment). **1:** cytoplasm; **2:** lysosome; **3:** "eosinophilic sombrero-vesicle". Transmission electron microphotos. **A:** ×6000; **B:** ×8000.

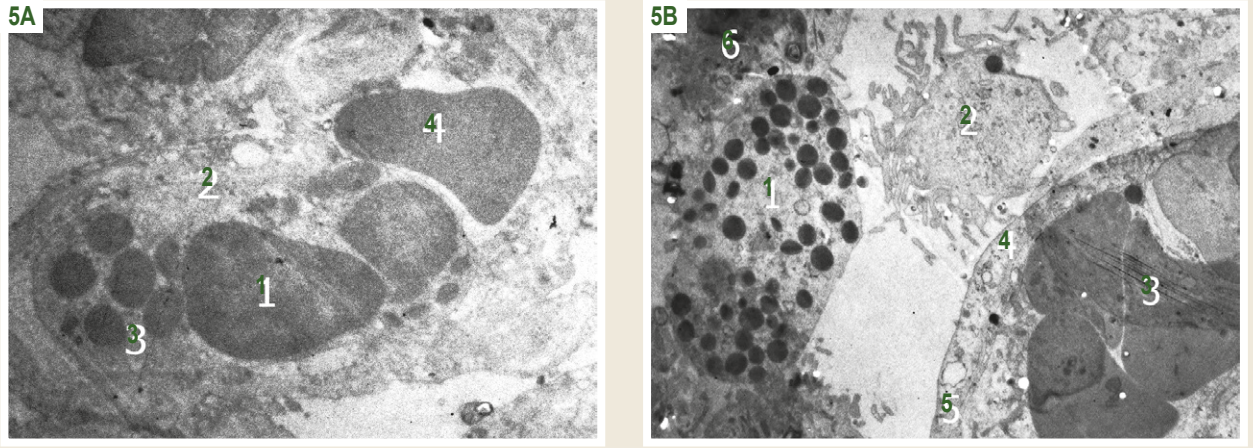

Fig. 5. Perivascular mast cell (**A**) and respiratory endocrine cell of the "open" type (**B**) in the lungs of a guinea pig. **A.** Experimental group III (36th day of the experiment). **1:** nucleus; **2:** cytoplasm; **3:** basophilic granules; **4:** erythrocyte in the lumen of a blood capillary. **B.** Experimental group I (23rd day of the experiment). **1:** respiratory endocrine cell; **2:** activated macrophage; **3:** erythrocytes in the lumen of the postcapillary venule; **4:** endothelial cell of the postcapillary venule; **5:** a pinocytotic vesicle in the cytoplasm of the endothelial cell; **6:** bronchiolar epithelium. Transmission electron microphotos. **A:** ×2200; **B:** ×3000.

the allergic inflammatory process in the guinea pig lungs, proliferative changes appear in the form of an increase in the development of broncho-associated lymphoid tissue against the background of the expansion of alveoli and the thinning of interalveolar septa that occur with emphysematous changes (*Fig. 2b*). Areas of emphysema alternate with areas of atelectasis (*Fig. 2a*).

EBCs are localized mainly in terminal and respiratory bronchioles and are the main cellular phenotype of the distal airways of the guinea pig. The cells are pyramidal in shape with the apical part protruding into the lumen of the terminal bronchiole. The light oval-shaped nucleus is large, has a medium electron-optical density, with a predominance of euchromatin, contains a nucleolus. Mitochondria, granular endoplasmic reticulum, and lysosomes with signs of degeneration are present in the cytoplasm. There are dense intercellular contacts with neighboring cells. In the conditions of the allergic inflammatory process, we observed signs of increased functional activity of EBCs: the presence of a light nucleus with a predominance of euchromatin, nucleoplasm of low electron-optical density, nucleoli, and a developed granular endoplasmic reticulum (*Fig. 3a*). We observed the most significant submicroscopic changes in goblet cells in airways against the background of sensitization and aeroallergization with ovalbumin in the early stage of allergic inflammation. At the submicroscopic level, we found an increased number of granules with mucous secretion in the cytoplasm of goblet cells (*Fig. 3b*).

At the ultrastructural level, in the cytoplasm of eosinophilic granulocytes, there are specific (secondary) large eosinophilic granules of an oval shape of different electron-optical density. Specific granules contain a centrally located crystalloid body of high electron-optical density and a peripheral matrix of low electron-optical density surrounded by a membrane. Nonspecific (primary) small eosinophilic granules are smaller in size and contain Charcot-Leyden crystalloid protein. It is interesting that the circulating eosinophilic granulocyte, before penetrating the lung tissue, "sticks" to the endothelium of a blood vessel and "rolls" on its surface (*Fig. 4a*). During the early stage of the allergic inflammatory process, we observed submicroscopic signs of piecemeal degranulation (PMD) in eosinophilic granulocytes. Vesicles (both round and tubular) are released from specific granules and move to the cell membrane for exocytosis of their contents. Tubular vesicles twist into an elongated ring-like structure described by the term "eosinophilic sombrero-vesicles" (*Fig. 4b*).

During the ultramicroscopic examination, we found the presence of perivascular mast cells and mast cells of the mucous membrane of granules in the cytoplasm, which are larger in size than eosinophilic granules, but less numerous. After sensitization with ovalbumin, the number of secretory granules in the cytoplasm of mast cells increased (*Fig. 5a*). On electron microscopic examination, the identification of small cytoplasmic, usually spherical secretory granules, otherwise known as dense core vesicles (DCV), is the main ultrastructural characteristic of respiratory endocrine cells. According to the results of our research, the size and appearance of the granules vary from 70 nm to 200 nm. Granules contain a core of variable electron-optical density in the center, usually separated from the surrounding thin, electron-transparent zone by a three-layer membrane. Granules are observed throughout the cytoplasm, but, in our opinion, are more often concentrated in the perinuclear and basal parts of the cytoplasm of the respiratory endocrinocyte (*Fig. 5b*). Depending on the ultramicroscopic features, vesicles with a dense core of respiratory endocrine cells of guinea pigs can be divided into two types. DCV type 1 has a wedge-shaped or oval shape with a dense amorphous core and a diameter of approximately 130 nm. There is usually no halo between the dense core and the membrane. In contrast, type 2 DCV are approximately 100 nm in diameter, more circular in shape, and have a smaller core that is surrounded by a distinct 15 to 20 nm halo. "Empty" vesicles of respiratory endocrine cells were more often observed in experimental groups I and II in the early period of the development of allergic inflammation in direct contact with the extracellular space. Differences in the morphology of DCV in individual cells are interpreted depending on the stage of their formation or secretion. Other characteristics of respiratory endocrine cells that we found at the ultrastructural level include a different number of free ribosomes and mitochondria, the latter usually smaller in size than in neighboring cells. The Golgi apparatus is well visualized and is located in the supranuclear zone. Granular and agranular endoplasmic reticulum and inclusion of glycogen in a small amount.

During the development of the experimental allergic inflammatory process in the lungs of the guinea pig, we also established submicroscopic changes, which were manifested by the decompensation of the processes of barrier function and selective permeability of the wall of blood capillaries and extracapillary venules. We determined the swelling of endotheliocytes, which led to the formation of protrusions, folds, and as a result of which the shape of the vascular lumen changed, the blood formed blood elements in the lumen of the capillary venules. Therefore, of these morphological changes at the submicroscopic level, we found that in the regions of protrusions and folds of the cytoplasm of endotheliocytes, the fusion of pinocytotic vesicles and the formation of vacuoles with their subsequent separation into the lumen of vessels took place. Macrophages had submicroscopic signs of increased functional activity: the presence of numerous pseudopodia, lysosomes, and autophagosomes in the cytoplasm (*Fig. 5b*).

Discussion

The most significant morphological changes at the light-optical level of the intrapulmonary bronchi and lungs of experimental animals were observed in the distal parts of the intrapulmonary airways and in the alveoli in the early stage of the inflammatory process (23rd and 30th days after the start of the experiment). The late (36th and 44th days after the start of the experiment) period of the allergic inflammatory process in the lungs is accompanied by a gradual decrease

in the activity of eosinophilic inflammation. Attention was drawn to the decrease in the diameter of the lumen of small bronchi and terminal bronchioles, probably due to hyperplasia and hyperreactivity of the smooth muscle component. Similar changes were observed by other scientists [11].

Our work demonstrates submicroscopic signs of an increase in the functional activity of mast cells of the mucous membrane of the respiratory tract during the early period of the development of experimental allergic inflammation, as evidenced by the presence of large granules of high electron-optical density in their cytoplasm. A similar trend is present in the works of other scientists [6,7]. In conditions of OVA-sensitization, the submicroscopic changes of the perivascular mast cells are more significant, which, in our opinion, determines the histochemical changes of the surrounding microvessels of the connective tissue and the morphometric changes of the vessels of the pulmonary hemomicrocirculatory bed in the animals of the experimental groups that we discovered earlier [12,13]. After all, together with respiratory endocrine cells, mast cells contribute to the maintenance of local homeostasis of the lungs in normal conditions and after the action of environmental factors, which is consistent with the opinion of other scientists [7,14,15].

The most significant reactive submicroscopic changes were established on the 23rd and 30th days of observation in the form of an increase in the functional activity of exocrine bronchiolar and goblet cells in airway epithelial lining. In our opinion, this is related to the activation of nonspecific resistance of airways epithelial lining in response to OVA-sensitization. Hypertrophy and increase in a number of secretory granules in goblet cells is a morphological confirmation of the development of bronchial hyperreactivity as a result of the action of an allergen, which is primarily associated with the action of CGRP (calcitonin gene-related peptide) of respiratory endocrine cells – pro-inflammatory cells, as well as innate lymphoid cells of type 2, confirmed by the results studies by other authors [16].

Under the influence of their cytokines, eosinophils are activated, first of all, their number increases in the bronchiolar epithelium and connective tissue of the lungs. The latter, in turn, increases mucus secretion by goblet cells and stimulates hypertrophy and contraction of the smooth muscle component of the bronchi. IL-13 of type 2 innate lymphoid cells directly affects the goblet cells of the mucous membrane of the respiratory tract, stimulating their hyperplasia and increased secretion of mucus. Cytokine IL-5 activates eosinophils, increases their number and their secretion of leukotriene C4 cytokines and platelet-activating factors. The latter increases mucin secretion by goblet cells and stimulates contraction of the smooth muscle component of bronchi and blood vessels [17]. IL-13 stimulates goblet cell hyperplasia and mucus secretion. The results of our morphometric and electron microscopic research on hyperplasia and increased secretory activity of goblet cells with an increase in the number and secretory activity of respiratory endocrine cells are confirmed by the data of other scientific studies [4,14,18].

Conclusions

1. A significant reaction of the innate nonspecific and adaptive immunity occurs in airways during the experimental ovalbumin-induced allergic inflammation, consisting primarily of the functional activation of eosinophilic granulocytes, mast cells, and macrophages, as well as an increase in the secretory activity of exocrine bronchiolar cells and goblet cells, which is confirmed by the changes investigated by electron microscopic examination.

2. Ultrastructural changes of mast cells are accompanied by reactive changes in the vessels of microcirculatory bed, manifested by decompensation of barrier function processes and selective permeability of the wall of blood capillaries and postcapillary venules.

Prospects for further research. We are planning an electron microscopic study of the components of the lymphoid tissue associated with the bronchi of guinea pigs in the conditions of the early and late periods of the experimental allergic inflammatory process.

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