

Leukocyte Infiltration of the Periarterial Space of the Lung after Allergen Provocation in a Rat Asthma Model

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Key Words

Asthma model · Eosinophils · Inflammation · Lung · Periarterial space · Ultrastructure

Abstract

The periarterial space has recently been described and its physiological and pathophysiological role during inflammatory and allergic reactions has been reviewed. The present studies used a light-/electron-microscopic approach to characterize the periarterial space in an asthma model in Brown Norway rats. After repeated sensitization with ovalbumin and heat-killed *Bordetella pertussis* bacilli, airway challenge was carried out after 1 further week. Four or 24 h after challenge, rats were fixed by perfusion or instillation and processed for microscopy. Several periarterial capillaries and connective tissue characterized the tissue between small pulmonary arteries, bronchioles and alveolar septa. Additionally, a partly pronounced interstitial edema was seen independent of the kind of fixation. Not only small arteries but also arterioles and venules were partly surrounded by edematous fluid already visible by light microscopy. Within the connective tissue and within the periarterial fluid,

numerous leukocytes, predominantly eosinophils, were found. However, leukocytes were detected only rarely in the vascular lumen. Only sporadically were eosinophils seen in the wall of small arteries or venules. Eosinophils transmigrating the endothelium of capillaries or arterioles were not visible 4 or 24 h after challenge. Thus, granulocytes transmigrate in the periarterial space very rapidly or even earlier than 4 h after challenge. The location of transmigration in the periarterial space needs further investigation.

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Introduction

Allergen provocation of the lung leads to inflammation-dependent alterations not only in the bronchial wall and the small airways but also in the alveolar areas [1, 2]. In the asthma model of the Brown Norway (BN) rat it was shown that lymphocyte infiltration into lung parenchyma was in part responsible for the increase in lymphocyte numbers in the bronchoalveolar lavage fluid [3]. A subgroup of lymphocytes, the T-helper-2 cells, play a key role in orchestrating asthmatic airway inflammation [4]. Cytokines secreted from these cells, e.g. interleukin (IL)-4, IL-5 and IL-13, promote airway eosinophilia and secretion of IgE [5]. Furthermore, during allergic immune re-

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actions, leukocytes migrate into different compartments of the lung [6, 7]. Lymphocytes may reach preferentially lung compartments supplied by bronchial vessels, whereas leukocytes may emigrate preferentially into postcapillary venules of pulmonary vessels and in particular into capillaries of the pulmonary system [7]. Recently, a third pathway for entry of leukocytes into the lung, the periarterial space, was proposed [7] and reviewed for many species and inflammatory situations [8]. The leukocyte infiltration of the periarterial space was strain dependent in mice [9]. Scanning electron microscopy has revealed a thin network of capillaries around pulmonary arteries lying in the vicinity of bronchi [10, 11]. In different models of lung inflammation, this periarterial space exhibits edema and leukocyte infiltration [12–14]. So far, no data exist on the ultrastructural appearance of the periarterial space after inflammation. To characterize the possible role of the periarterial space during inflammation, the well-established model of allergen-induced airway hyperresponsiveness in BN rats was used. This asthma model reflects many features of human allergic asthma [3], including both early- and late-phase reactions, increase in antigen-specific IgE following active immunization and a neutrophilic and eosinophilic inflammation [15]. Electron microscopy was employed to characterize (1) the ultrastructure of the periarterial space, (2) possible alterations in structure, (3) the type and location of inflammatory cells within the periarterial space and (4) the point of transmigration.

The composition of this space was investigated 4 and 24 h after challenge to study possible differences after the onset and during advanced late allergic responses [16].

Materials and Methods

Male BN rats ($n = 20$, mean weight 260 g) were kept under specific pathogen-free conditions and had ad libitum access to food and water. The animals were divided into two groups. Nine BN rats served as controls without any sensitization, and 11 BN rats were used for asthma induction. The experiments were approved by the local governmental authority and met the NIH Guidelines for the Care and Use of Laboratory Animals (NIH Publication No. 85, reprint 2002).

Asthma Model

BN rats were sensitized and challenged according to the asthma model of BN rats as described before [3]: (1) first sensitization with 1 mg ovalbumin + 200 mg $\text{Al}(\text{OH})_3$ in 1 ml 0.9% NaCl solution, applied subcutaneously, and 5×10^9 heat-killed *Bordetella pertussis* bacilli (kindly donated by Chiron Behring, Marburg, Germany) in 0.4 ml 0.9% NaCl solution, given intraperitoneally as an adjuvant; (2) second sensitization with the same allergen and adjuvant

1 week later, and (3) airway challenge (provocation) after another week, consisting of inhalation of an aerosol of 5% ovalbumin/saline solution (0.25 l/min) [the rats were placed in a Plexiglas tube for 15 min; a flexible latex mask was placed over the nose and connected to a nebulizer (Pariboy; Ritzau, Starnberg, Germany)] [17, 18], or instillation of 250 μl of a 0.5% OVA/saline solution.

No significant differences in the infiltration of the periarterial regions were seen after provocation by inhalation compared with instillation. Other studies using the BN rat asthma model have shown that 4 h after challenge neutrophil numbers and 24 h after challenge eosinophil numbers were significantly increased. Therefore, rats were processed for light microscopy 4 or 24 h after challenge.

Fixation Protocol

Fixation by Airway Instillation. Control ($n = 4$) and challenged rats ($n = 6$) were anesthetized with ether. After opening the abdominal wall, the animals were killed by aortic exsanguinations, and the lungs were dissected from the chest and fixed by instillation in a special instillation device, followed by immersion fixation for at least 24 h.

Fixation by Vascular Perfusion. Control ($n = 5$) and challenged rats ($n = 5$) were anesthetized with ether and intraperitoneal injection of 100 mg ketamine hydrochloride (Ketanest[®]; WDT, Garbsen, Germany) and 4 mg xylocaine hydrochloride/kg of body weight (Rompun[®]; Bayer, Leverkusen, Germany). After tracheotomy and intubation, lungs were ventilated with a tidal volume of 5 ml. After thoracotomy, the pericardial cavity was opened and a cannula was pushed forward into the pulmonary artery via the right ventricle. The lung was flushed with a preperfusion solution containing 0.9% NaCl, 2.5% polyvinylpyrrolidone, 0.5% procaine-HCl, 25,000 IU Liquemin[®] (pH 7.35) to remove blood. Immediately after preperfusion, the lung was fixed in distension followed by perfusion with fixation solution. The left atrium was opened for volume relief throughout this procedure. Perfusion with both solutions was carried out with a hydrostatic pressure of 15 cm H_2O . At the end of fixation, the trachea was tightened and the heart-lung block excised. The latter was immersed in cold fixative for 24 h before further processing. The fixation solution consisted of 4% paraformaldehyde and 0.1% glutaraldehyde in 0.2 M HEPES buffer.

Sampling

Further processing and sampling of lungs was carried out as described elsewhere [2, 19]. In brief, after separation into the right and left single lung, each lung was embedded in 2% aqueous agar. The organ was then cut from apical to caudal into 3-mm-thick parallel transverse slices by means of a tissue slicer. The slices were taken for light and electron microscopy in an alternating manner: from each single lung four to five slices were obtained. For electron microscopy, tissue blocks with recognizable parts of the air conducting system were sampled.

Tissue Processing

(1) Specimens were dehydrated with a graded series of ethanol and embedded in methacrylate resin (Technovit 7100; Kulzer, Heraeus, Germany). Sections of 1.5- μm thickness were stained with hematoxylin and eosin. (2) Specimen processing was carried out as previously described [3]. Briefly, after repeatedly rinsing in 0.1 mmol/l cacodylate buffer, tissue blocks were osmicated for 2 h in 1% osmium tetroxide (OsO_4) in 0.1 mmol/l cacodylate buffer

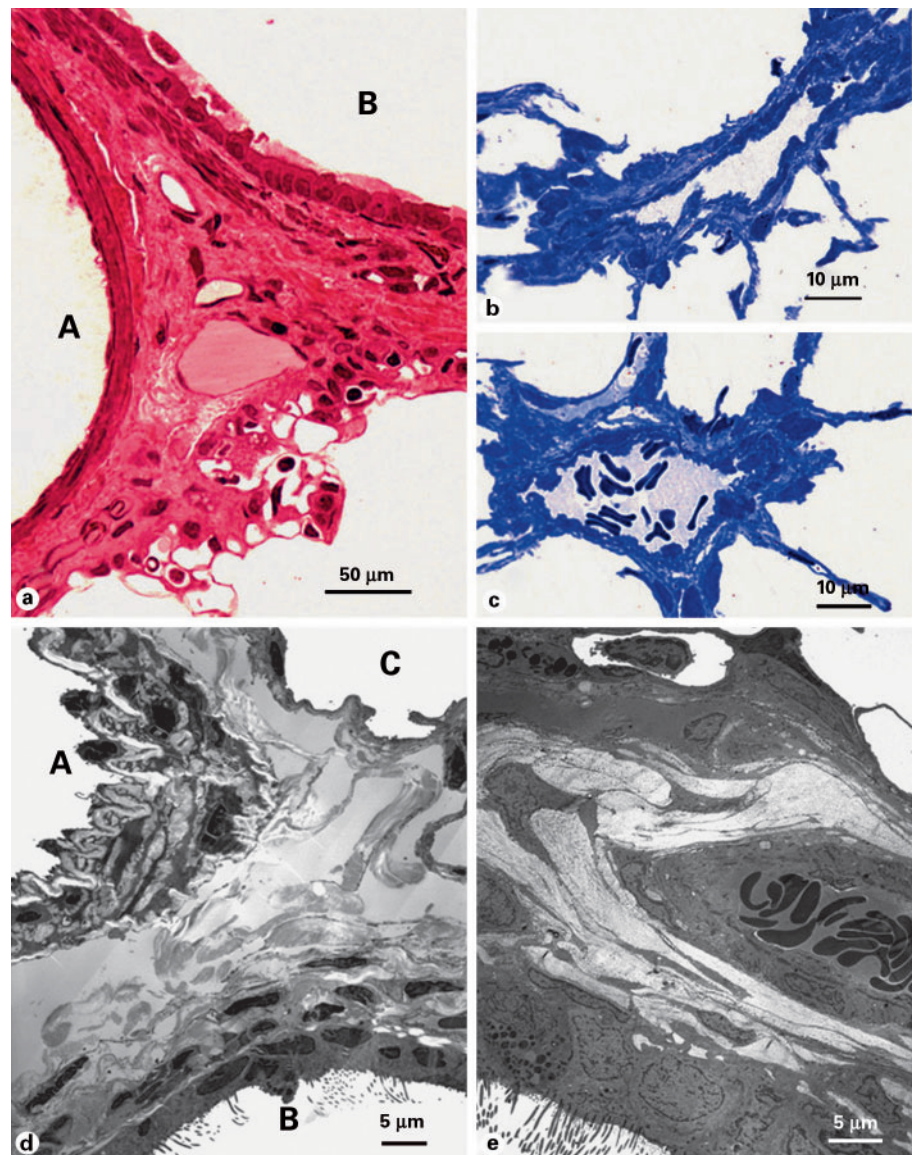


Fig. 1. Control rats: light microscopy after perfusion fixation. **a** Region between an artery and a bronchus. The periarterial space shows capillaries without cell infiltrations. A = Artery; B = bronchi. **b** Parenchymal vessel without perivascular edema and infiltrations. **c** Small artery within lung parenchyma. No edema and no cell infiltrations are visible. Electron microscopy after perfusion fixation. **d** Periarterial space without cellular infiltrations. A = Small artery; B = bronchiolus; C = alveolar space. **e** Interstitial space between bronchiolus and alveolar septum. No inflammatory cells are seen at the small arteriole.

and repeatedly rinsed in 0.1 mmol/l cacodylate buffer (six changes over 60 min). After twice rinsing in distilled water, specimens were stained en bloc overnight (12–18 h) with a mixture of equal portions of uranyl acetate and water [half-saturated aqueous uranyl acetate solution (1:1)] at 4–8°C. Specimens were dehydrated in an ascending series of acetone (70, 90 and 100%) and embedded in Epon. Semithin sections (1 µm) and ultrathin sections (70 nm) were cut by an ultramicrotome (Reichert Ultracut S, München Germany). Semithin sections were stained with methylene blue and ultrathin sections with lead citrate and uranyl acetate.

Evaluation

Qualitative investigations of the periarterial regions were carried out using a light microscope (Orthoplan; Leitz, Wetzlar, Germany) and an electron microscope (EM 10; Zeiss, Oberkochen,

Germany). Representative areas were documented. A semiquantitative overview was obtained by determining the percentage of test fields containing perivascular edema with and without infiltrations.

Results

Light Microscopy

Controls. Normally only few cells were seen within the tissue between arteries and bronchi (fig. 1a). Smaller periarterial vessels and capillaries normally contained no inflammatory cells in their walls or within their lumina

Fig. 2. Asthmatic rats: light microscopy after perfusion fixation. **a** Region between a small artery (A) and bronchi (B). The periarterial space shows capillaries and cell infiltration (arrows). In the lumina of capillaries, some leukocytes are visible (arrow-heads). **b** A partly pronounced periarterial edema (arrows) between artery (A), bronchiolus (B) and alveoli is recognizable as a pink-stained layer (HE). Light microscopy after instillation fixation. Periarterial edema (arrow) and cell infiltration (arrow-head) around a venule within the lung parenchyma (**c**) as well as around a small artery near bronchioles and lung parenchyma (methylene blue; **d**). Edematous regions show a light blue staining.

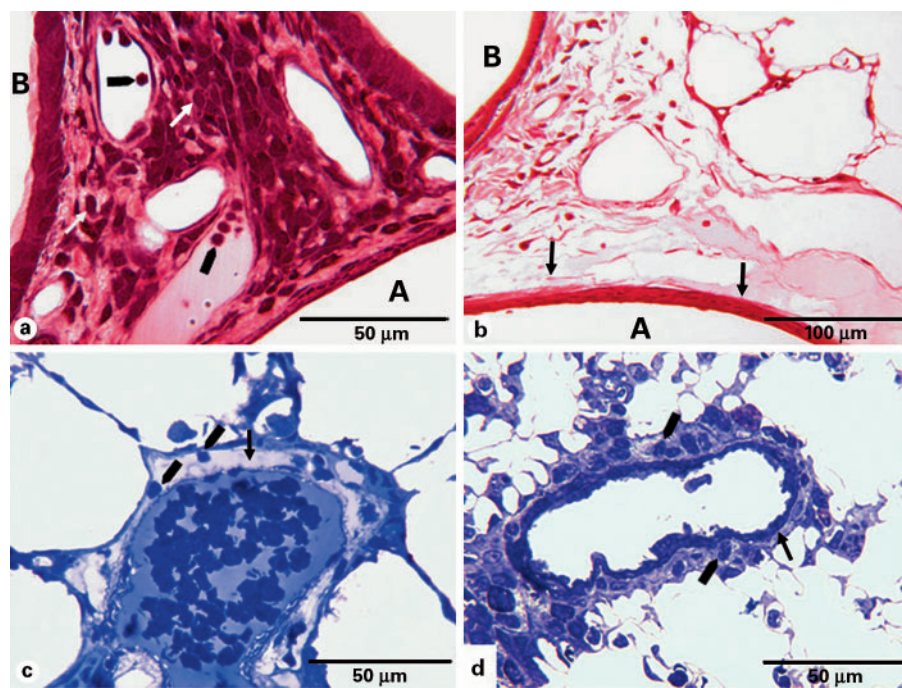
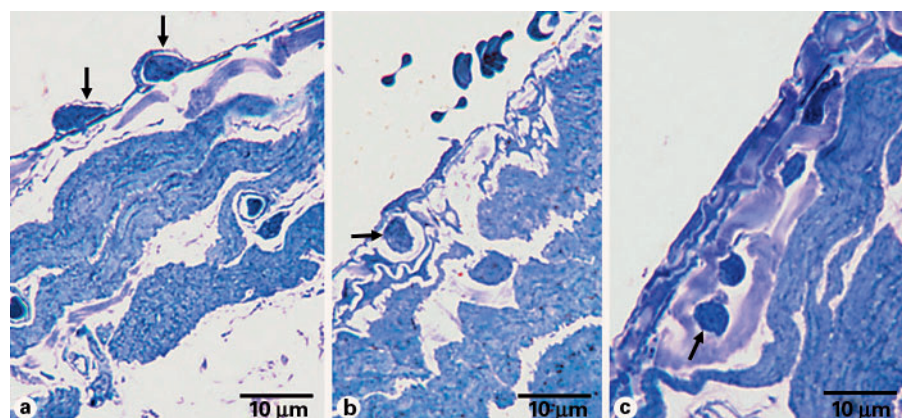


Fig. 3. Asthmatic rats: light microscopy after instillation fixation. Leukocytes within the wall of an artery (arrows) below the endothelium (**a**), between intima and media (**b**) and within the media (**c**; methylene blue).



(fig. 1a–c). A semiquantitative overview (controls: $n = 6$) has shown that after perfusion fixation in $13 \pm 7\%$ (SD) of test fields with vessels near bronchi or within parenchyma a more or less pronounced perivascular fluid accumulated without noteworthy cell infiltration, probably depending on perfusion pressure during preperfusion and or fixation. Remarkable cell infiltration of the perivascular regions was visible in only 1% of test fields.

Asthma Induction. Compared to controls, numerous cells were visible after challenge within the tissue between arteries and bronchi (fig. 2a) as well as between small arteries and bronchioles. The cells surrounded the periarterial smaller vessels and capillaries and interspersed the

connective tissue independent of the fixation technique (fig. 2a, b). The arteries and venules near bronchi and bronchioles as well as within the lung parenchyma exhibited more or less pronounced periarterial edema (fig. 2b–d). In hematoxylin-eosin-stained methacrylate sections, these edematous periarterial regions were recognizable as pink-stained areas (fig. 2b), and in methylene-blue-stained semithin sections as a light blue coat (fig. 2c, -d). Numerous cells partly infiltrated the edematous fluid (fig. 2c, d). In the lumina of some of these vessels, other cell types were visible apart from erythrocytes (fig. 2a, c). In some vessels, a few leukocytes lay immediately below the endothelium and within the middle layer of the arterial wall

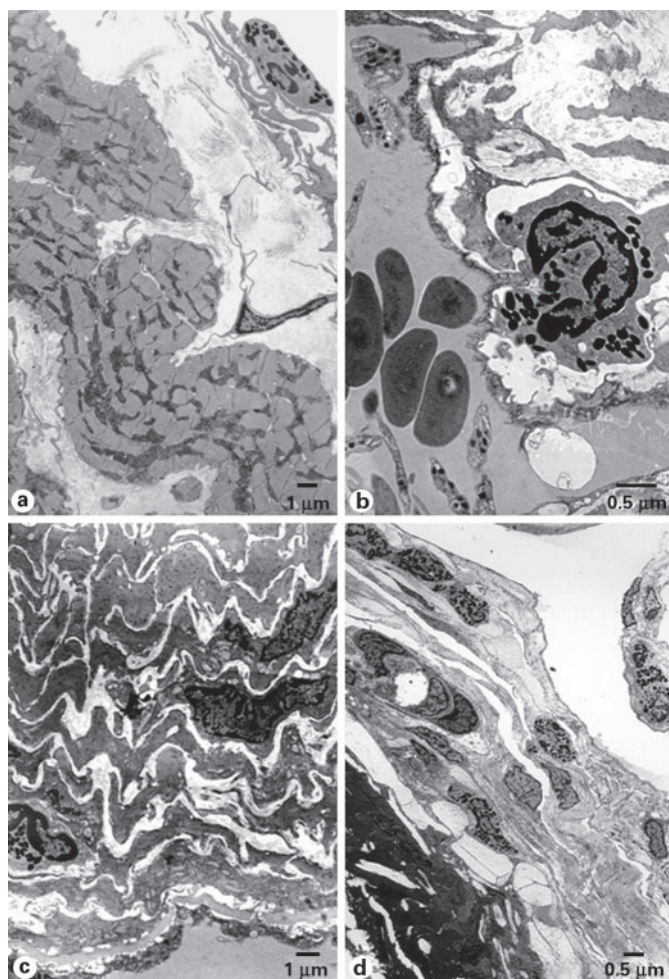


Fig. 4. Asthmatic rats: electron microscopy. **a** Eosinophil at the endothelium of the same artery shown in figure 3. The medial wall of this vessel is characterized by cross-striated muscle cells. Eosinophils below the endothelium of a vein (**b**), within the wall of an elastic artery (**c**) and in the periarterial space of an artery (**d**).

(fig. 3a–c). A semiquantitative overview ($n = 5$) has revealed more or less pronounced perivascular edema with remarkable cellular infiltrations in nearly $46 \pm 24\%$ (SD) of vessels containing test fields; 4% of test fields showed perivascular fluid accumulation without infiltrations.

Electron Microscopy

Controls. The perivascular space between bronchi or rather bronchioli and arteries normally has shown no inflammatory cells (fig. 1c, d). Eosinophils occurred only sporadically in the perivascular space or in alveolar septa.

Asthma Induction. The electron-microscopic studies showed that the rare leukocytes seen in the vessel walls by light microscopy were eosinophils (fig. 4a–c). Among the transmigrated cells, eosinophils were prevalent in the periarterial regions of many sections (fig. 4d). These were detected abundantly predominantly in the edematous space near arteries and around arterioles (fig. 5a) and capillaries (fig. 5b). Sometimes, eosinophils were visible between pericytes and endothelial cells within the basement membrane (fig. 5b). Furthermore, numerous eosinophils were seen surrounding periarterial capillaries and small venules (fig. 5c), between the periarterial space and alveolar septa (fig. 4d) as well as within capillaries of alveolar septa (fig. 5e). The connective tissue between small arteries and bronchi also contained other leukocytes and lymphocytes (fig. 5c). Local accumulations of lymphocytes were found predominantly below the bronchiolar wall. In this region, apoptotic cells were occasionally noted (fig. 5f). Neutrophils were only sporadically identified. However, in samples from lungs fixed 4 h after challenge, neutrophils occurred more frequently than in lungs fixed 24 h after challenge. In the lumen of small arteries or arterioles and venules, leukocytes were only rarely identified even 4 h after challenge. In capillaries, very few lymphocytes were seen (fig. 5c). Apart from occasional eosinophils in the wall of small arteries and venules (fig. 4a–c), no frequent infiltration of the arterial wall was detectable. Furthermore, the bronchiolar wall was not infiltrated by leukocytes. Neither 4 nor 24 h after challenge were leukocytes transmuting through the endothelial cells of blood vessels or capillaries detectable. However, eosinophils were predominantly identified near arteries and periarterial capillaries. The ultrastructure of vascular endothelium and bronchiolar epithelium did not show any visible alteration after challenge.

Discussion

As early as 4 h as well as 24 h after provocation of ovalbumin-sensitized rats, the periarterial space around small branches of pulmonary arteries partially exhibits a pronounced fluid accumulation and leukocyte infiltration. This has been mentioned in passing in various animal models of asthma. Electron microscopy revealed that most of the leukocytes invading this space at these time points are eosinophils. Eosinophils were seen in the lumina and walls of small arteries, venules and capillaries, but not transmuting vascular endothelium. Eosinophils are often also visible in lung parenchyma near the

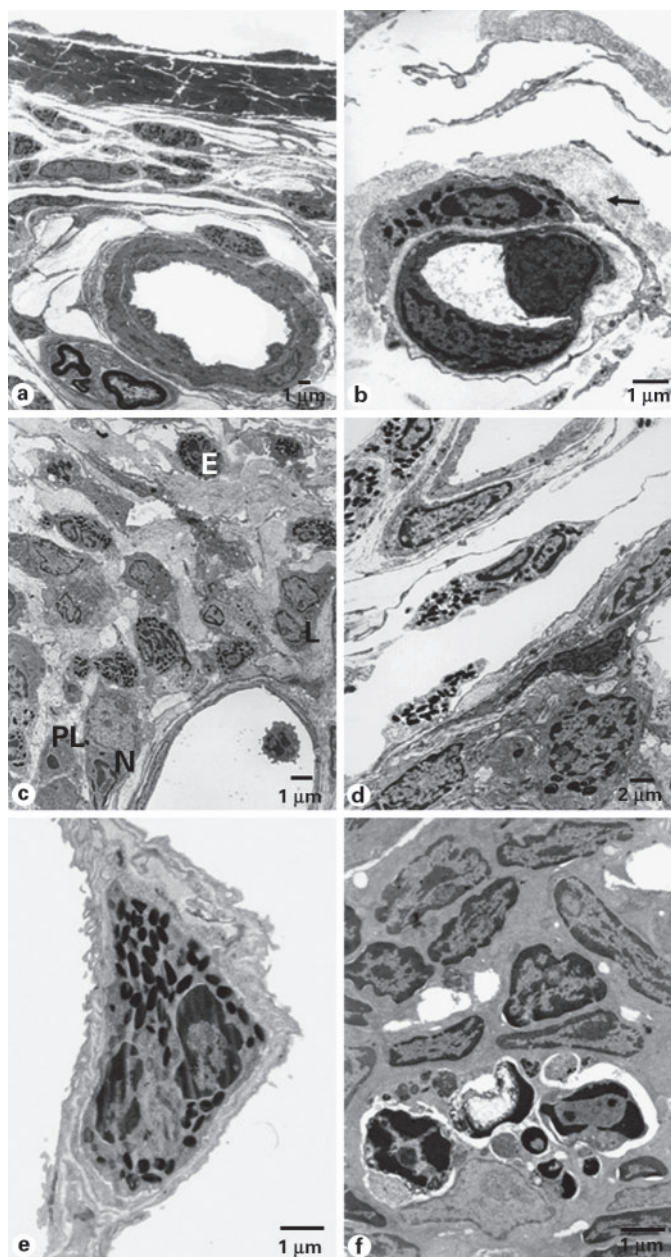


Fig. 5. Asthmatic rats: electron microscopy. Capillaries in the periarterial space. Endothelial cells and basement membrane show no significant alterations. **a** Predominantly eosinophils contribute to periarterial cell infiltration. They also surrounded an arteriole. The connective tissue between small arteries and bronchioles contained different leukocytes and lymphocytes. **b** Eosinophil within the basement membrane (arrow) of a capillary. **c** Many eosinophils, some lymphocytes and a few neutrophils are located in the periarterial space near arteries and bronchioles. E = Eosinophil; N = neutrophil; L = lymphocyte; PL = plasma cell. Eosinophils are located between the periarterial space and alveolar septa (**d**) and in capillaries of alveolar septa (**e**). **f** Local accumulations of lymphocytes are visible below the bronchiolar wall. In this region, apoptotic cells also occur occasionally.

periarterial space but not in the bronchiolar wall, which is typical for asthma in humans. Lymphocyte accumulation was occasionally detected in the connective tissue near the bronchiolar wall. The portion of neutrophils 4 h after challenge was higher than 24 h after challenge. This observation is in accordance with others determining a higher number of neutrophils recovered from lung tissue than after 24 h [20].

Leukocyte accumulations were also mentioned not only in various allergy models but also after inflammation by bacteria, e. g. Lewis and Sprague-Dawley rats sensitized to house dust mite antigen showed periarterial infiltration 2 days after challenge [21]. Continuous exposure of mice to house dust mites, unlike ovalbumin, elicited severe and persistent periarterial but also peribronchial eosinophilic infiltration [17]. Periarterial mononuclear infiltration around parenchymal vessels was occasionally found in neonatal/juvenile mice exposed to ovalbumin with residual oil fly ash [18]. Intratracheal injection of ovalbumin-pulsed dendritic cells followed by exposure to ovalbumin aerosol also resulted in an accumulation of mononuclear cells in the periarterial space [22]. Exposure to different bacteria led to periarterial infiltrations, too [23, 24]. However, all these authors did not use electron microscopy and partly mentioned this phenomenon only incidentally.

Our electron-microscopic findings are in accordance with the results of other authors [25, 26]. In a chronic ovalbumin model, Nishida et al. [25] showed that eosinophils were frequent around the arteries and that lymphocytes left capillaries in the peribronchiolar interstitial space. Studies by Ichikawa et al. [26] discovered periarterial accumulation of leukocytes in the lung after intratracheal administration of sheep erythrocyte antigen to primed mice. They described intravascular leukocytes migrating to the periarterial space in the lung, in particular from arteries, by migrating between endothelial cells to reach the internal elastic lamina of arteries and by further penetrating the smooth muscle layer to the interstitium.

The present detailed electron-microscopic analysis revealed eosinophils predominantly in the periarterial space and not in the vascular lumina or walls and suggests that extravasations of eosinophils into the periarterial space occurred less than 4 h after ovalbumin challenge.

However, our findings cannot exclude the proposed entry sites of leukocytes, i.e. (1) leaving the vascular bed through the wall of small pulmonary arteries, venules or periarterial capillaries and accumulating in the periar-

rial space, or (2) transmigration through septal capillaries or lymphatics. From the periarterial space, an invasion into septal lung interstitium is also possible.

The following mechanisms leading to transmigration into the periarterial space are possible. (1) Eosinophils may have an increased inflammation-dependent metabolic activity and elevated capacity to migrate across vascular walls due to the presence of elevated circulating factors, i.e. the cytokines IL-3, IL-5 and granulocyte-macrophage colony stimulating factor [27]. (2) The emigration process may be supported by the expression of vascular adhesion protein-1, which has already been shown to be involved in adhesion and transmigration of lymphocytes across endothelial cells in the liver and other organs [28]. (3) The entrapment and transmigration process may be supported by the expression of endothelial intercellular adhesion molecule-1 and alterations in capillary diameter [29].

The more or less pronounced periarterial edema around branches of pulmonary arteries, found in our asthma model and reviewed for other models [7, 8], was not mentioned in any other inflammation or allergy model. The mechanism of periarterial fluid accumulation following asthma induction is unsolved. Our findings show no obvious structural alterations in the vessel walls. It remains to be studied how the allergen situated in the airway lumen sends signals to the endothelium of the vessels in the periarterial space enabling lymphocytes to adhere and emigrate. Inflammation-dependent alterations

in the expression of some aquaporins [30], increasing the transport of water into the periarterial space, are one possibility.

Conclusions

Asthma induction in BN rats leads to inflammation-dependent leakage from vascular walls and periarterial edema formation followed by leukocytes infiltrating the periarterial space. The few leukocytes, which were visible in the wall and lumina of small branches of the pulmonary artery and capillaries within the periarterial space, support emigration in this region. However, neither after 4 h (associated with an increased number of neutrophils [20]) nor after 24 h (associated with an increased number of eosinophils recovered from lung tissue [3, 20]) did neutrophils accumulate in the walls of arteries or capillaries. Thus, transmigration into the periarterial space happened very rapidly in this model, or the predominant transmigration process occurred in less than 4 h.

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