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Histological and Ultrastructural Changes in the Lungs of Guinea Pigs Exposed to the Extract of *Erwinia herbicola*

Zmiany histologiczne i ultrastrukturalne w płucach świnek morskich po inhalacji extraktem *Erwinia herbicola*

Гистологические и ультраструктуральные изменения в легких морских свинок ингалированных экстрактом *Erwinia herbicola*

Many authors regard microbial antigens in organic dusts as chiefly responsible for extrinsic allergic alveolitis — hypersensitivity pneumonitis (17, 18). Among the factors causing the lesions of farmer's lung, thermophilic actinomycetes *Micropolyspora faeni* and *Thermoactinomyces vulgaris* developing in wet and mouldy vegetable products have been proved to have particular etiological importance. Clinical observations showed, however, that the contact with threshing dusts of dry fresh grain also caused clinical and radiological symptoms similar to those reported in farmer's lung (11).

The nature of pathogenic allergens in grain dust is still not clear and our recent findings indicate the possible role of some bacteria. Dutkiewicz (4) demonstrated that in the air highly polluted with grain dust the concentration of microbial propagules is large (10^5—$10^6/m^3$); the prevalent organisms are Gram negative rods belonging to the species *Erwinia herbicola* (synonym: *Enterobacter agglomerans*) which constitute 22—58% of the isolated strains. In our further research, *E. herbicola* was found to produce biologically active endotoxins (3) and to show strong antigenic properties (5). Among the exposed populations of farmers and grain workers, the percentage of skin and serological reactions to the antigen of these bacteria was high (5, 11). Moreover, farmers who developed allergic alveolitis as a result of exposure to grain dust had positive reactions to the antigen

of *E. herbicola* in a bronchial provocation test and in several immunological tests — agar-gel precipitation, complement fixation, immunofluorescent test, skin test (11). This points to the etiological role of these bacteria in the pathogenesis of the disease.

Erwinia herbicola is the species widely distributed in nature and prevails in epiphytic microflora of grain, especially immediately after harvest. These bacteria are also found on seeds, fruit and leaves of many plants, such as clover, grass, flax, beans and apples. They are common in cotton dust and may constitute as much as 50% of hay microflora. The features distinguishing this species from other Gram negative bacteria are: yellow pigmentation, peritrichal flagellation and the ability to fermentate carbohydrates.

In allergic alveolitis patients, the most frequent histopathological pulmonary changes are: interstitial inflammation, inflammatory exudate into the lumen of alveoli, granulomas and allergic inflammation of blood vessels (7, 13, 16). Similar changes were reported by the authors studying experimentally the action of different antigens in animals (17, 18, 19). Such studies proved to be important for the elucidation of the pathogenesis of the disease and for the identification of new etiological factors (12).

The purpose of the present study was to find out whether the inhalation challenge of guinea pigs with the antigen of *E. herbicola* may bring about pulmonary lesions similar to those observed in humans in the course of allergic alveolitis.

MATERIALS AND METHODS

110 guinea pigs of random sex weighing 300—400 g, all of the same bred, were used in experiments.

For the inhalation challenge, saline extract of the cell mass of *E. herbicola* in dilution of 1 : 1000 (1 mg/ml) was used. The extract was prepared from the *E. herbicola* strain M-10-3 which was isolated from the air of a rye mill and which had been used as a standard strain in our earlier immunological studies (5). Bacteria were grown on a nutrient agar for 48 hrs at 34°C. The growth was harvested by washing with distilled water. Subsequent stages of producing the preparation included: extraction of the bacterial mass with 0.85% NaCl, thermal disintegration, supernatant centrifugation, concentration of supernatant and lyophilization. The lyophilized extract was dissolved in 0.85% NaCl at the concentration of 1 mg/ml and Seitz-filtered to obtain a solution ready for inhalation. Before the experiment, the extract was cultured on microbiological media and injected intraperitoneally to mice. The tests proved sterility and lack of lethality of the extract was aerosolized with an ultrasonic nebulizer (type TUR-USI 3, product of East Germany) and administered into the exposure chamber, which was a plastic container with a tight-fitting glass lid. Thus, animals could easily be observed during the experiment. As much as 5 ml of the extract was aerosolized for each inhalation challenge.

Two experiments were performed, each on 40 guinea pigs. In the first experiment aerosol was administered repeatedly for 60 min.; in the second one — for 6 min. The scheme of both experiments is presented in Table 1. The animals

which received aerosol for 60 min. a day were killed after 1, 2, 10 and 20 days of the experiment; those challenged for 6 min. were killed after 10, 20, 60 and 80 days of the experiment having received, respectively, 10, 20, 40 and 50 short aerosol exposures.

A control group (Table 1) was composed of 30 guinea pigs, five of which were not challenged at all and another five were chellenged with aerosolized saline (0.85% NaCl) once for 60 min. Ten guinea pigs were given an aerosol of saline 60 min. a day for 20 days and another ten pigs were challenged 6 min. a day for 20 days. Saline aerosol was administered with another TUR-USI 3 apparatus not used for the aerosolization of bacterial extract. All the experimental and control animals were killed 24 hrs after the last aerosol challenge. It was done by an intraperitioneal injection of sodium pentothal (Morbital, Polfa) in the amount of 0.05 mg/g animal's weight.

Table 1. Scheme of the experiment — experimental and control groups

Experiment I				Experiment II				Control group		
Subgroup	Exposure time* min.	Number of exposures	Experiment duration days	Subgroup	Exposure time* min.	Number of exposures	Experiment duration days	Subgroup	Exposure time** min.	Number of exposures
I A	60	1	1	II A	6	10	10	C-A	-	-
I B	60	2	2	II B	6	20	20	C-B	60	1
I C	60	10	10	II C	6	40***	60	C-C	60	20
I D	60	20	20	II D	6	50***	80	C-D	6	20

* Exposed to the aerosol of the saline extract of the cell mass of *Erwinia herbicola* in dilution 1 : 1000.

** Exposed to the aerosol of saline (0.85% NaCl).

*** Animals exposed every day for the first 20 days; then every second day.

There were 10 guinea pigs in each subgroup except for control subgroups C—A and C—B which had 5 guinea pigs each.

After thoracotomy blood was collected from the heart. Next, lungs and trachea were removed *en block*, infused intratracheally with buffered 4% formalin and placed into a jar with formalin. A few hours later, middle parts of cross sections of right lobes were separated for histopathological examination. The fixation of the sections was completed in 4% buffered formaline; they were subsequently embedded in parafin. Tissue sections were stained with hematoxylin and eosin (by Van Gieson and Heidenhain's method), and silver-impregnated according to Gomori.

Tissue material for electron microscopy was taken from middle parts of the cross sections of right lobes immediately after animal's death. Random sections were taken from two guinea pigs in each experimental and control group. The sections were fixed in 4% glutaraldehyde buffered with 0.1 M cacodylate buffer to *pH* 7.2—7.4. For further fixation 1% osmium tetroxide in Michaelis buffer of *pH* 7.2—7.4 was used. The sections were subsequently dehydrated in ethylic alcohol of increasing concentration and embedded in Epon 812. Sections were cut on Tesla

BS-490A ultramicrotome. In the semithin sections 1 μm thick, stained with toluidine blue, places were selected for ultrastructural examination. The ultrathin sections were additionally stained with uranyl acetate and lead citrate, and viewed in a Tesla BS-500 electron microscope at 90 kV.

RESULTS

HISTOLOGY

Histopathologic studies showed occasional small foci of interstitial inflammation in the lungs of animals of all control groups. The presence of such lesions in healthy guinea pigs was observed by other authors (18).

In experiment I (60 min. aerosol challenge), pathologic changes of small intensity were observed in the lungs of animals killed after one exposure; they covered one-third of the studied cross section. The bronchial tree outline was conpicuous. Histopathologically, interstitial inflammatory lesions were found; they were only little more intense than those in the control group. Numerous erythrocytes were observed in the lumen of alveoli. Around bronchi and bronchioli there was inflammatory infiltrate composed of mononucleated cells. In the group exposed twice, larger interstitial inflammatory lesions were observed, as well as inflammatory exudate into the lumen of alveoli composed of erythrocytes, eosinophils and neutrophils. Numerous macrophages were present in the lumen of alveoli and in interalveolar septa. Macroscopic examination of lungs after 10 exposures showed multifocal, confluented inflammatory lesions, usually covering more than 2/3 of the studied cross section. In other parts of the lungs interstitial inflammatory lesions prevailed. Microscopically, inflammatory exudate was viewed in the lumen of alveoli which contained large amounts of fibrin, numerous macrophages and less numerous neutrophils and eosinophils. Focal necrosis of interalveolar septa was seen with numerous neutrophils and macrophages around it. Macrophages grouped in some places forming granulomas (Fig. 1). Lymphocytes and macrophages prevailed in the inflammatory infiltrates situated in interalveolar septa. In silver-impregnated preparations, a slight increase of reticulin fibres was found. There were focal necrotic lesions in small blood vessels. In bigger vessels, hyperthrophy of media and intima was seen, subendothelial edema, and numerous perivascular inflammatory infiltrates of monoculeated cells. Micro- and macroscopic lesions in lungs after 20 days of the experiment were similar to those observed after 10 days. Fibrosis within interalveolar septa was more intense, though.

In experiment II (6 min. aerosol challenge), macroscopic obser-

vations of lungs derived from animals of subgroup II A (10 exposures) revealed small and medium pathologic lesions covering 1/3 of the cross section examined. Focally, big emphysematous vesicles were observed, resulting from the rupture of several alveoli. Microscopically, mainly interstitial infiltrate was found, sometimes accompanied by hemorrhage into the lumen of alveoli. The macroscopic picture of lungs in subgroup II B (20 exposures) was similar to that in subgroup II A. Interstitial infiltrate was found in all cases, whereas inflammatory exudate into the lumen of alveoli consisting of erythrocytes, neutrophils and eosinophils was observed only occasionally. Interalveolar septa of silver-impregnated preparations had an increased amount of reticulin fibres. The inflammatory infiltrate localized in interalveolar septa contained very numerous lymphocytes, less macrophages, and single fibroblasts, plasma cells and neutrophils. Proliferation of media and intima was observed in arteries and arterioles. In subgroup II C (40 exposures), signs of fibrosis and small inflammatory infiltrates were found in interalveolar septa. In bronchi, papillomatous hyperplasia of the mucous membrane with features of mucus overproduction was sometimes observed. Thickening of media and intima in the arteries and perivascular inflammatory infiltrates could be seen. An increase in the number of reticulin fibres as well as traits of focal lymphoplasia were observed in interalveolar septa. The microscopic lung picture in animals of subgroup II D (50 exposures) was similar to that in subgroup II C. However, the signs of the fibrosis of interalveolar septa were more conspicuous.

ULTRASTRUCTURAL EXAMINATION

The ultrastructural examination of alveoli and bronchi was performed in both control and experimental animals. In guinea pigs exposed to the extract of *E. herbicola* once for 60 min. (subgroup I A), coagulative necrosis of endothelial cells was a characteristic ultrastructural lesion (Fig. 2, 3). Amorphous electron-dense fibrin deposits were frequently found within the lumen of lung capillaries (Fig. 2). Type II epithelial cells (type II pneumocytes) showed advanced degeneration: dilated rough endoplasmic reticulum and swelling of mitochondria (Fig. 4). The release of lamellar granules from type II epithelial cells into the lumen of alveoli was also observed (Fig. 4). Scare type II epithelial cells were also found in the lumen of alveoli. A few eosinophils and neutrophils were seen in alveolar walls and sometimes in the lumen of the alveoli. Changes observed in the electron microscope in the lungs of subgroup I B animals (2 exposures) were similar to those in subgroup I A, yet more advanced. Some parts of the lungs were atelectatic, whereas the alveoli in other

parts were dilatated. Eosinophils and neutrophils were more frequently found in interalveolar septa and the lumen of alveoli. Type II epithelial cells were swollen and showed a decrease in the amount of lamellar granules. Numerous alveolar macrophages and type II pneumocytes were present in the lumen of alveoli. Foci of atelectasis and compensatory emphysema, as well as features of interstitial inflammation were found in the lungs of the animals of subgroup I C (10 exposures) and I D (20 exposures). Thickened interalveolar septa contained numerous eosinophils, lymphocytes, neutrophils and fairly numerous plasma cels (Fig. 5). In plasma cells, the canals of rough endoplasmic reticulum were dilated and flocculent material was present in their lumen (Fig. 6). The increased number of macrophages in the interalveolar septa was observed. The shape of some macrophages suggested their passage through the alveolar wall (Fig. 7). Sporadically, both types of pneumocytes were noticed to be replaced by macrophages (Fig. 8). Type II pneumocytes containing a considerable amount of lamellar granules grew in number. The increase of collagenic fibres was observed in the amorphous granular zone between the basement membrane of the endothelial cell and the pneumocytes.

In the second (II) experimental group (6 min. exposure to the extract of *E. herbicola*), the ultrastructural changes in the lungs of the guinea pigs were similar to those in group I, but less intense. Inflammatory infiltrates in interalveolar septa were composed mainly of eosinophils; there were fewer neutrophils and only single plasma cells. In subgroup II C (40 exposures) and II D (50 exposures), the interalveolar septa were lined with numerous type II epithelial cells containing large amounts of lamellar granules (Fig. 9). Type II epithelial cells were also found in the lumen of the alveoli. The number of alveolar macrophages increased considerably, and some of them showed signs of migration through the alveolar wall. Amorphous electron-dense masses were only occasionally seen in the lumen of the alveolar capillaries, or within macrophages. The number of lymphocytes, plasma cells and eosinophils in the walls of interalveolar septa was greater than in subgroups II A and II B. In alveolar walls, thick bunches of collagenic fibres were found, some of which ringed granular pneumocytes or inflammatory cells.

DISCUSSION

It has been well documented that thermophilic actinomycetes and fungi occurring in organic dusts evoke histological and ultrastructural changes in the lungs of experimental animals which resemble the changes

in the lungs of experimental animals which resemble the changes observed in the course of allergic alveolitis in humans (17, 18, 19). The presented results prove that such changes may as well be caused by antigenic substances produced by Gram-negative bacteria naturally contaminating organic dusts. Our results correspond well with those of Rylander et al. (14, 15), who proved by means of lung lavage technique that Gram-negative bacteria occurring in cotton and their endotoxins may induce cell influx into lungs and expressed the opinion that these agents represent a potential cause of byssinosis in cotton mill workers.

According to the number and duration of aerosol challenges, various pictures of acute and chronic inflammatory lesions in lungs were observed in the light and electron microscope, mainly in the form of interstitial inflammation. The ultrastructural examination showed that the acute changes observed in animals of group I (subjected to longer challenges) were characterized by the presence of fibrin deposits in the lumen of alveolar capillaries and by the necrosis of numerous endothelial cells. Type II epithelial cells showed features of swelling and the loss of the continuity of the cytoplasmic membrane with the release of lamellar granules into the lumen of alveoli. These changes were observed at 24 hrs after a single exposure to the antigen; they were intensified in the group of animals exposed twice to the antigen. In both experimental subgroups, the increase of the number of alveolar macrophages in the interalveolar septa was found, as well as the ultrastructural manifestation of the migration of macrophages through the interalveolar septa. At 24 hrs after a single exposure to the antigen there were present — in bronchial walls, in interalveolar septa and in the lumen of alveoli — numerous inflammatory cells, mainly neutrophils and eosinophils. The number of the inflammatory cells increased considerably after two exposures to the antigen.

Damage to alveolar capillaries is exceptionally rare in the course of acute non-specific pathogenic processes affecting lung parenchyma (20). In such cases the necrosis of pneumocytes is usually observed, mainly of the type I pneumocytes, and at more advanced stages also of type II pneumocytes. Our observatoins in the electron microscope suggest that the thrombohemorrhagic phenomena visible after a single and double exposure to the antigen were evoked by the endotoxin present in the extract (10). It is known that endotoxins may damage endothelial cells of blood capillaries, cause the formation of intravascular fibrin microthrombi (1) and increase the penetration of blood cells into the lumen of alveoli (15). The absence of such changes in the lungs of control animals excludes lethal shock as a casual factor. It is possible, however, that

these changes may be also due, at least partly, to the anaphylactic reaction. This assumption is confirmed by the presence of numerous neutrophils and eosinophils, as well as by the changes of the hemorrhagic pneumonitis type observed in the light microscope already after a single exposure to the antigen. Hemorrhagic pneumonits with hemorrhagic exudate into the lumen of alveoli was observed by other authors in animals exposed to other antigens occurring in organic dusts (10, 18, 19, 20). They were also found in the lung biopsy material collected from patients with acute farmer's lung (7, 16).

In the animals of group II subjected for a long period to short challenges with the antigen of *E. herbicola,* the increased amount of reticulin fibres and collagen in the lungs was found in the light microscope. Early lesions typical for progressive pulmonary fibrosis were noticed, as well as the formation of granulomas. A variety of vascular and perivascular changes were found, such as edema of blood vessel walls, proliferation of endothelial cells, thrombosis leading sometimes to the closing of the lumen of a vessel. Numerous lymphocytes were present in perivascular infiltrates. In the ultrastructural examination of the lungs of animals exposed many times to the antigen, thickening of interalveolar septa was observed, the septa containing numerous neutrophils, lymphocytes, eosinophils and plasma cells. The number of macrophages increased both in interalveolar septa and in the lumen of the alveoli. It was observed that macrophages penetrated through the interalveolar septa into the lumen of the alveoli, and they could replace the epithelial cells of the alveoli (substitution). The amount of type II epithelial cells containing lamellar granules considerably increased. So did the amount of collagenic fibres in interalveolar septa. After 80 days of the experiment (50 short exposures), thick bunches of collagenic fibres surrounded type II epithelial cells or inflammatory cells.

The presented data show that in both acute and chronic phases of the observed process alveolar macrophages not only grow in number, but they also migrate through the interalveolar septa and substitute their epithelium. This behaviour of macrophages is often observed as a reaction to factors evoking acute or chronic lesions (8). Alveolar macrophages are known to be the primary defense cells of the lungs against inhaled noxious agents. Activated macrophages initiate cellular reactions that may lead to the development of pathologic changes (15). The presented results reveal that the diluted extract of *Erwinia herbicola* exhibits a strong ability of macrophage activation. This phenomenon, according to Harris et al. (9), plays an important role in the pathogeny of allergic alveolitis, and — according to Rylander (15) — in pulmonary diseases evoked by inhaling endotoxins. The presented histopathological

results correspond well with those in our earlier paper in which we reported a significant increase of the number of macrophages in the pulmonary lavage fluid guinea pigs exposed for one hour to the aerosolized extract of *E. herbicola* (6).

We did not find typical complexes antigen-antibody, similarly to Burke et al. (2). The absence of subendothelial and subepithelial immunological deposits and the presence of numerous lymphocytes and macrophages may account for the predominant role of cellular immunological mechanisms in the devlopment of morphologic changes. The presence of flocculent material in dilated rough endoplasmic reticulum points to the intensified production of immunologublins (2). It is possible that in the course of the disease non-precipitating immunological complexes are formed, or that the formed complexes are quickly eliminated (10).

The presented results indicate that the antigen of *E. herbicola* causes pulmonary changes characteristic of allergic alveolitis, what supports the view that this bacterial species represents one of the etiological agents of this disease in grain workers (11). Similar changes were observed in the biopsy material collected from patients with acute and chronic form of farmer's lung (7, 13, 16). Our results are close to those obtained by other authors studying the mode of action of different pathogenic factors present in organic dusts (17, 18, 19). It should be pointed out, however, that these authors exposed animals to large amounts of viable organisms, whereas in ous experiments a highly diluted, sterile extract was used. It seems that the considerable intensity of the changes observed in our experiment results from the ability of *E. herbicola* to produce active endotoxins (3).

On the basis of our observations it can be assumed that the process bringing about lesions in the course of allergic alveolitis has a complex nature being mainly the result of altered cellular reactivity. The acute changes occurring after 1 or 2 longer exposures seem to be evoked mainly by the action of the endotoxic component of the used bacterial extract. The chronic changes occurring after many shorter exposures seem to be the result of hypersensitivity phenomena.

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EXPLANATION TO FIGURES

Fig. 1. Grouping macrophages from early granuloma (H+E). Magn. 100×.

Fig. 2. Ultrastructural lung picture after one exposure to *E. herbicola* extract. Necrosis of endothelial cells (EnN) in many capillaries. Fibrin deposits (Fib) in the lumen of vessels. Type II pneumocytes (PII) with few lamellar granules. Additional symbols: PI — type I pneumocyte, Er — erythrocyte, BM — capillary basement membrane. Magn. 8000×.

Fig. 3. Necrotizing endothelial cells (EnN) in the lumen of capillary. Outlines of nuclei (Nuc) and mitochondria (Mi) are seen. Type II pneumocyte (PII) present in the lumen of alveolus. Additional symbols: LG — lamellar granules, PI — type I pneumocyte, BM — capillary basement membrane. Magn. 22,000×.

Fig. 4. Release of lamellar granules (LG) into the lumen of alveolus after breaking of cell membrane continuity in type II pneumocyte. Dilatation of smooth and rough endoplasmic reticulum in type II pneumocyte. Mitochondrial matrix of some mitochondria in this pneumocyte are focally cleared. Additional symbols: PI — type I pneumocyte, CF — collagenic fibres, BM — capillary basement membrane. Magn. 20,000×.

Fig. 5. Abundant inflammatory cells in interalveolar septa and in the lumen of alveolus: neutrophils (Gr), eosinophils (E), lymphocytes (L) and plasma cells (PL). Additional symbols: Al — lumen of alveolus, Cl — lumen of capillary, Er — erythrocyte, PI — type I pneumocyte, PII — type II pneumocyte. Magn. 10,000×.

Fig. 6. Plasma cells (Pl) in interalveolar septum. Dilated canals of rough endoplasmic reticulum (RER) in the plasmocyte are filled with flocculent material. Additional symbols: Nuc — cellular nucleus, PI — type I pneumocyte, Al — lumen of alveolus. Magn. 36,000×.

Fig. 7. Alveolar macrophage (PAM) with clearly deformed nucleus migrating through interalveolar septum. Additional symbols: CF — collagenic fibres, BM — capillary basement membrane, Cl — lumen of capillary, Al — lumen of alveolus, Er — erythrocyte, PI — type I pneumocyte, En — endothelial cell. Magn. 20,000×.

Fig. 8. Alveolar macrophages (PAM) present in the lumen of alevolus. A macrophage replacing an epithelial cell of alveolus (PAM-1) is seen. Type II pneumocyte (PII) contains abundant lamellar granules. Additional symbols: En — epithelial cell. Magn. 16,000×.

Fig. 9. Two type II pneumocytes (PII) with numerous lamellar granules (LG) are seen. Between pneumocytes a streak of collagenic fibres (CF) is seen. Magn. 16,000×.

STRESZCZENIE

Przebadano metodami histologicznymi i elektronowomikroskopowymi płuca świnek morskich, które poddano uprzednio inhalacji silnie rozcieńczonym (1 : 1000) wodnym ekstraktem masy komórkowej bakterii *Erwinia herbicola*, występujących obficie w pyle zbożowym. W zależności od czasu trwania i liczby inhalacji obserwowano ostre i przewlekłe zmiany zapalne o różnym nasileniu, przeważnie wykazujące obraz śródmiąższowego zapalenia płuc, spotykany w alergicznym zapaleniu pęcherzyków płucnych. Charakter zmian sugeruje, że są one głównie wynikiem kompleksowej zmiany reaktywności immunologicznej, spowodowanej przez endo-

toksyczne i alergizujące działanie *E. herbicola*. Wskazuje to na fakt, że bakterie te winny być traktowane jako potencjalna przyczyna alergicznego zapalenia pęcherzyków płucnych u ludzi narażonych na działanie pyłu zbożowego.

РЕЗЮМЕ

Была проведена оценка гистологическим и электрономикроскопическим методом легких морских свинок, которые предварительно ингалировано заметно разбавленным (1:1000) водным экстрактом бактерии *Erwinia herbicola* обильно присутствующих в зерновой пыли. В зависимости от течения времени и числа ингаляции обнаружено острые и хронические воспалительные изменения о различном напряжении. Преимущественно мы обнаружили интерститиальное воспаление легких, которое можно встречать в аллергическом воспалении пузырьков легких (*alveolitis allergica*). Характер изменений внушает, что эти изменения возникают в последствии комплексного изменения иммунологической реактивности вызванной эндотоксической и аллергизирующей деятельностью *E. herbicola*. Это свидетельствует о том, что эти бактерии вызывают аллергическое воспаление пузырьков легких (*alveolitis allergica*) у людей подвергнутых действию зерновой пыли.

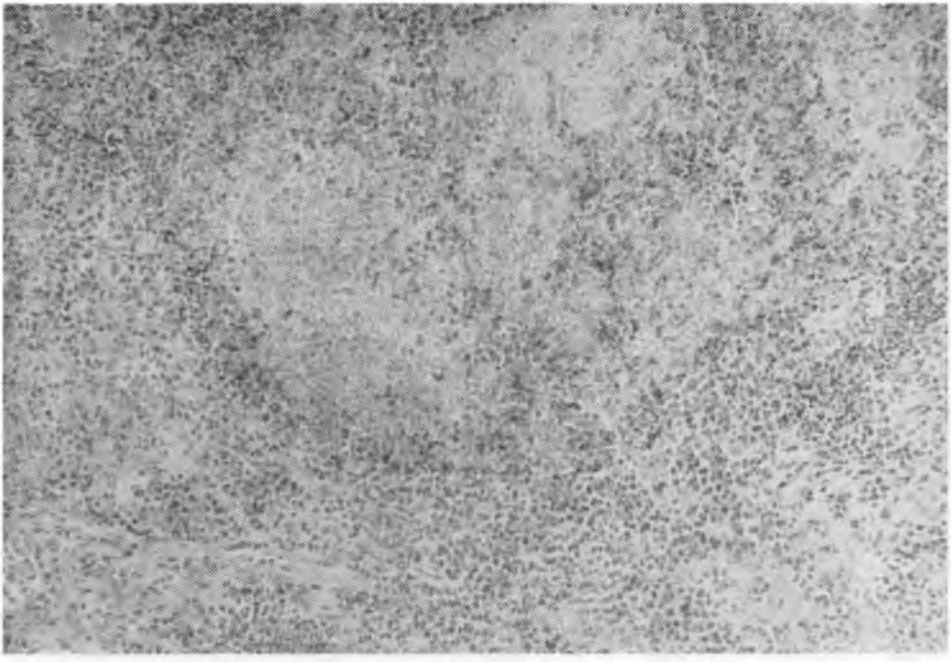

Fig. 1

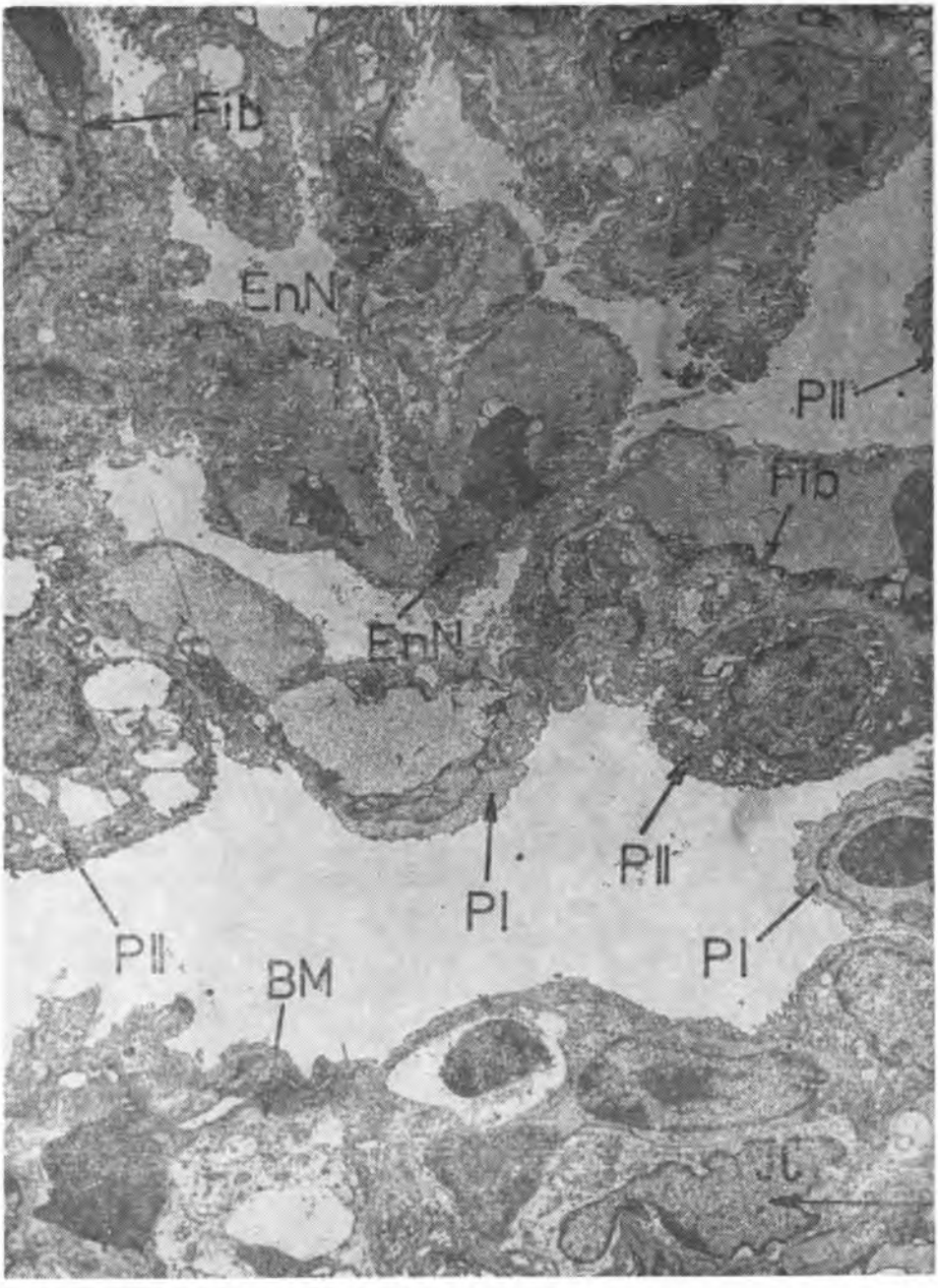

Fig. 2

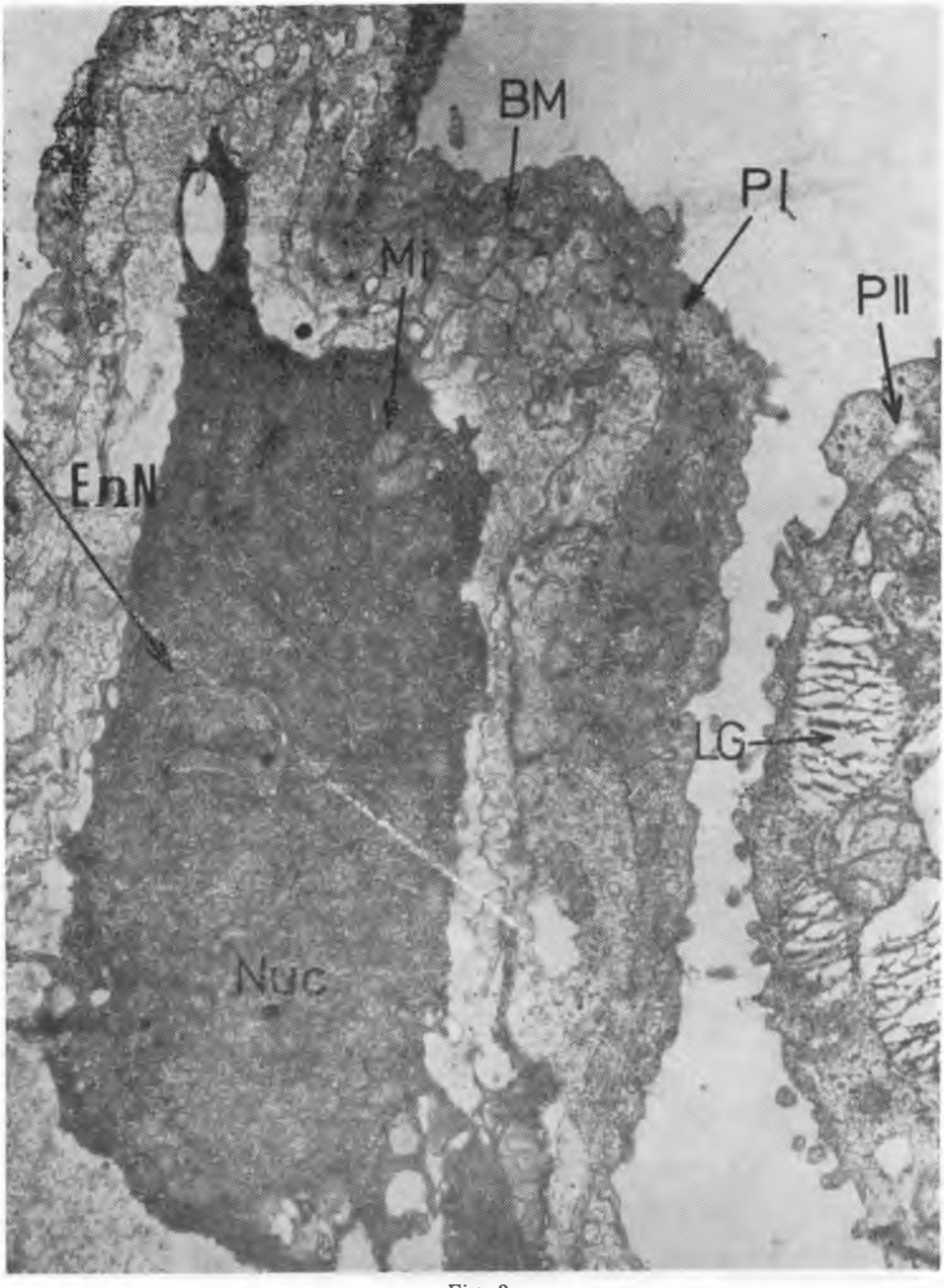

Fig. 3

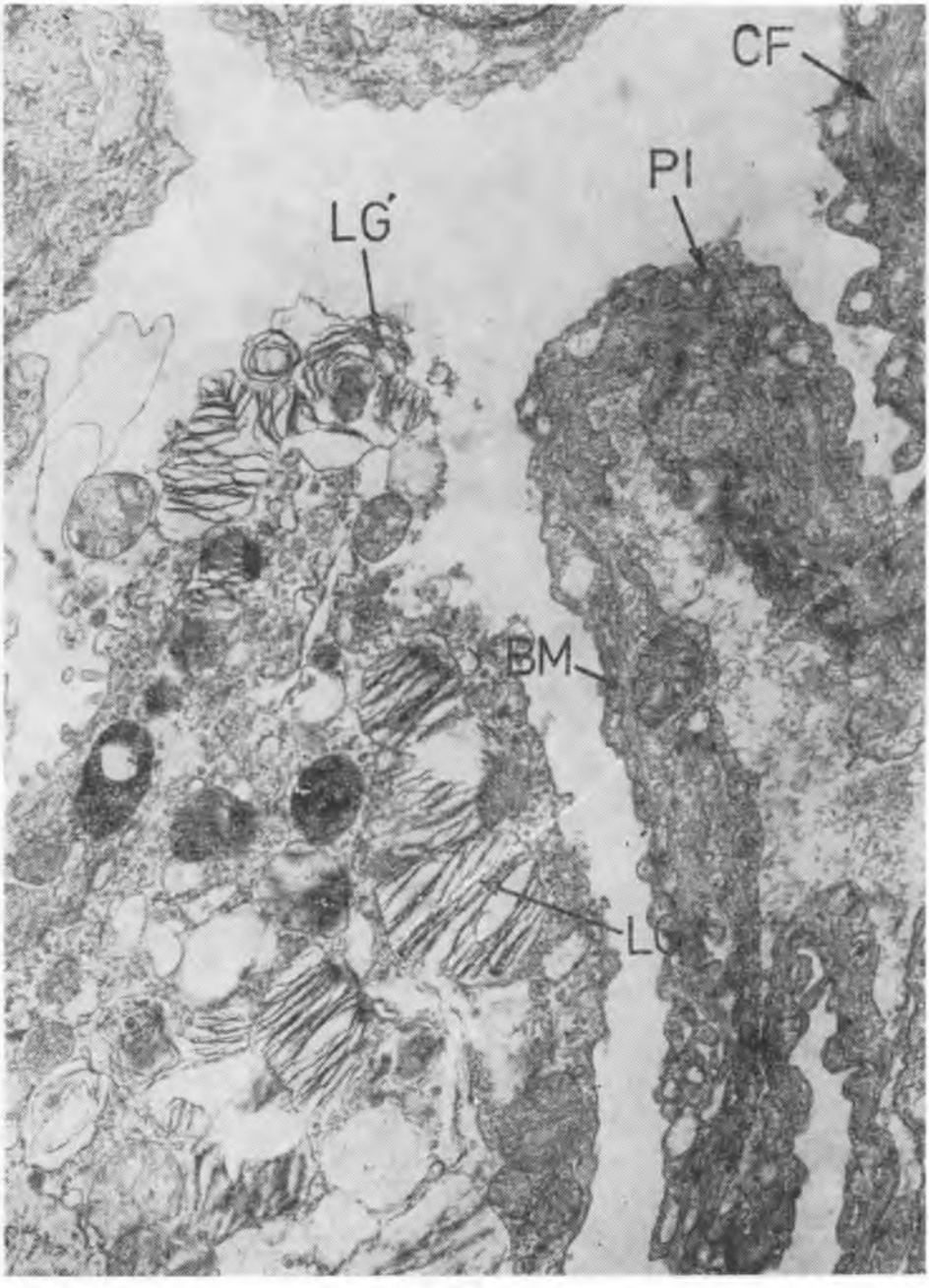

Fig. 4

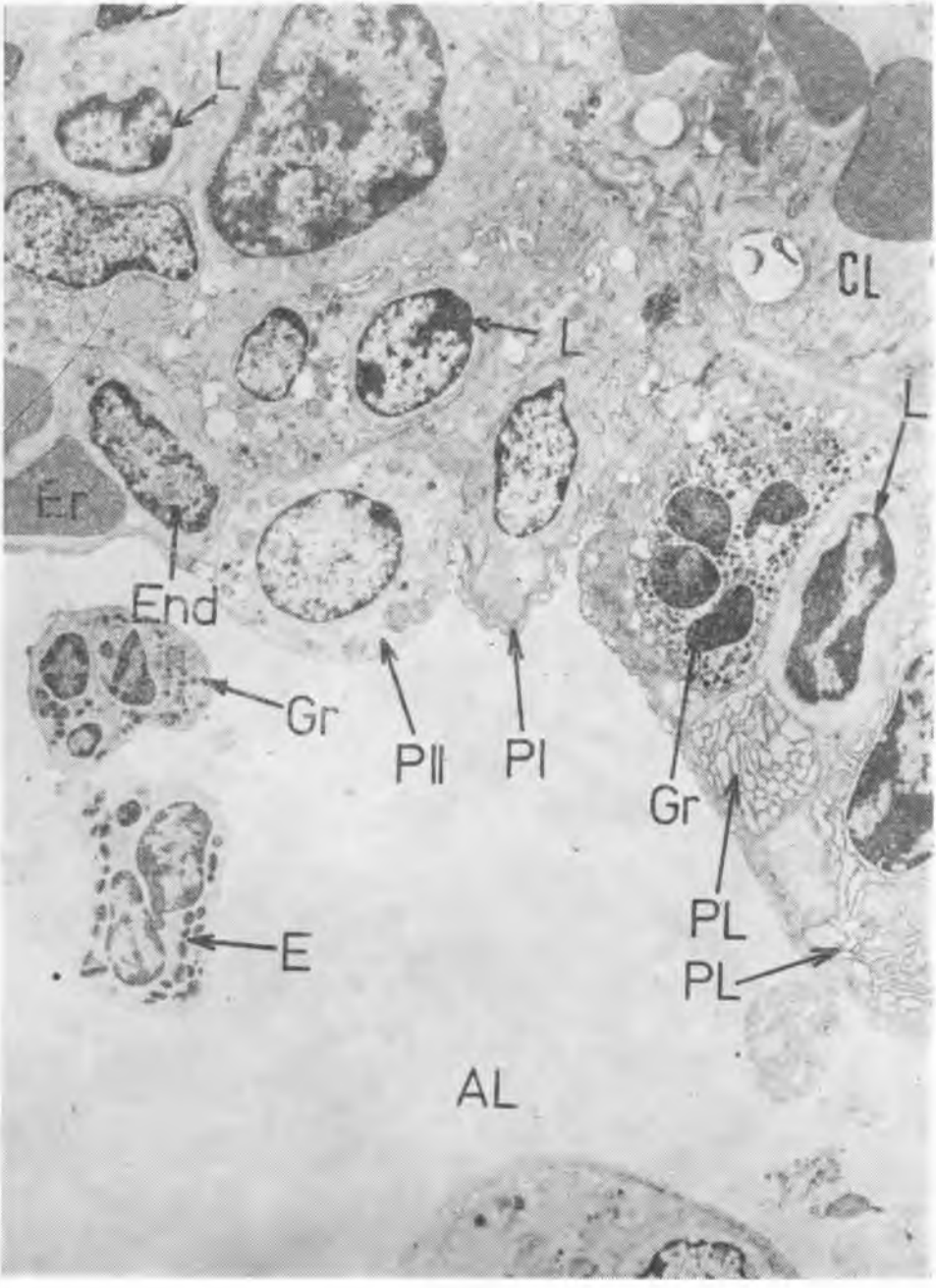

Fig. 5

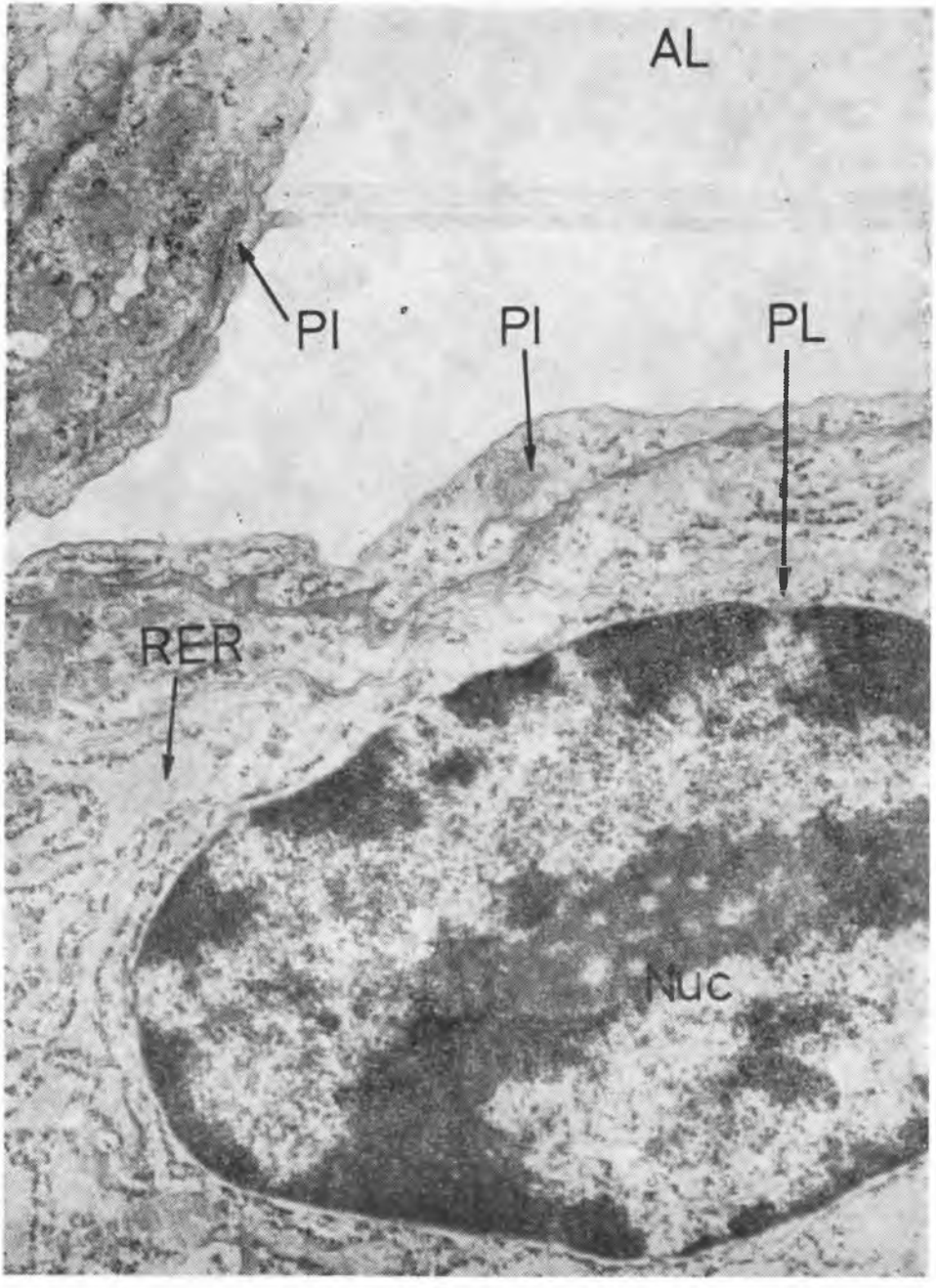

Fig. 6

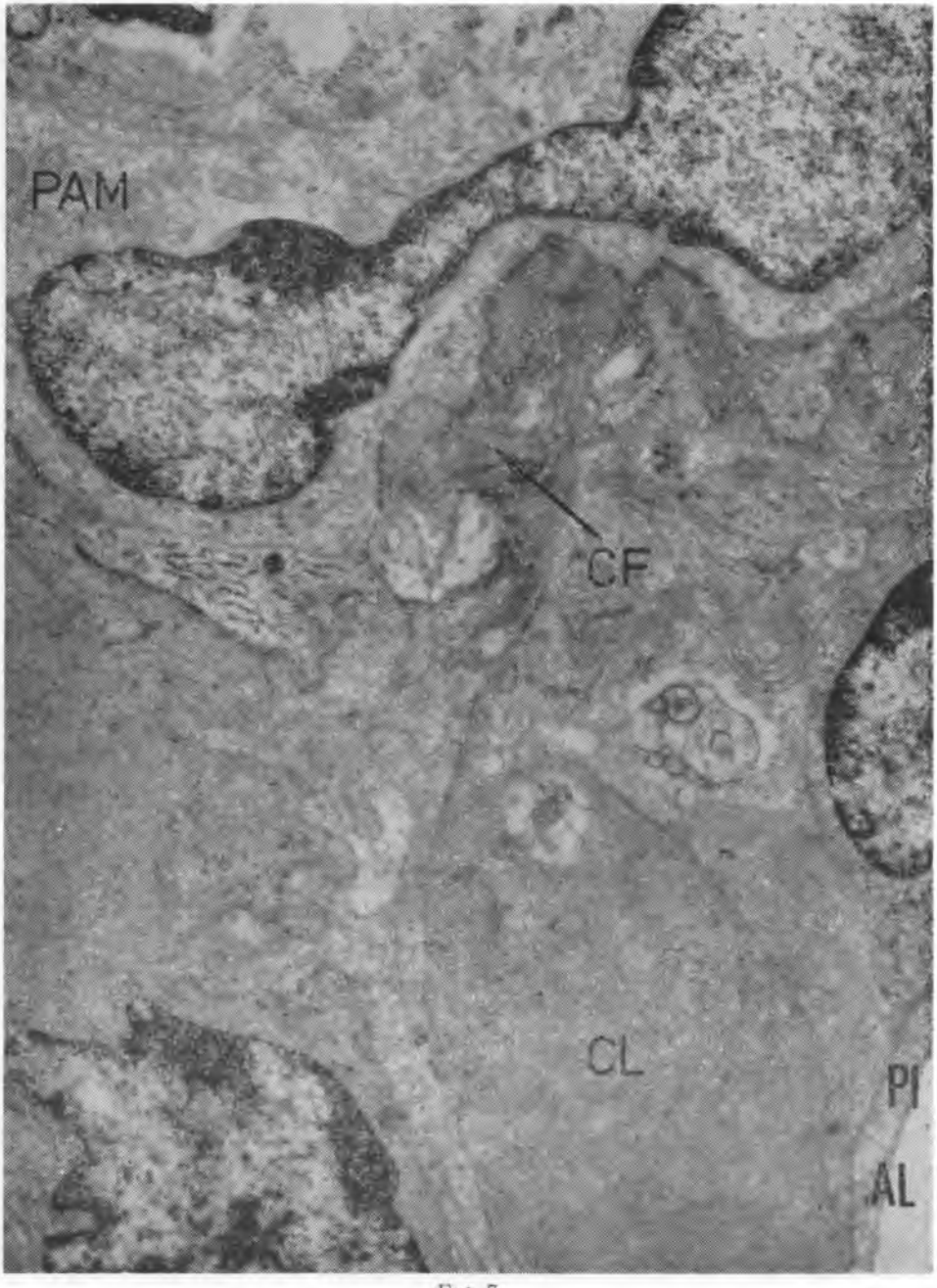

Fig. 7

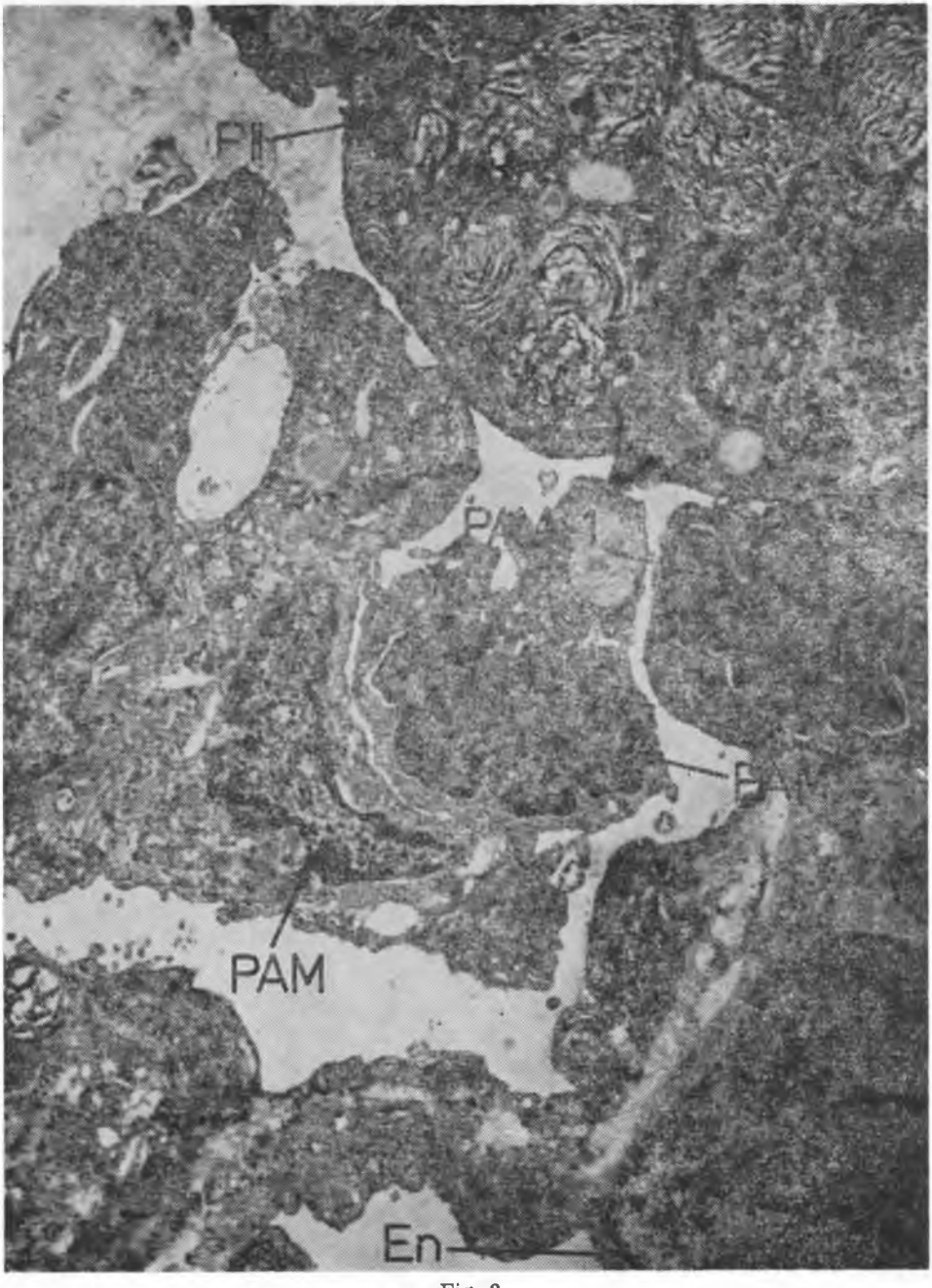

Fig. 8

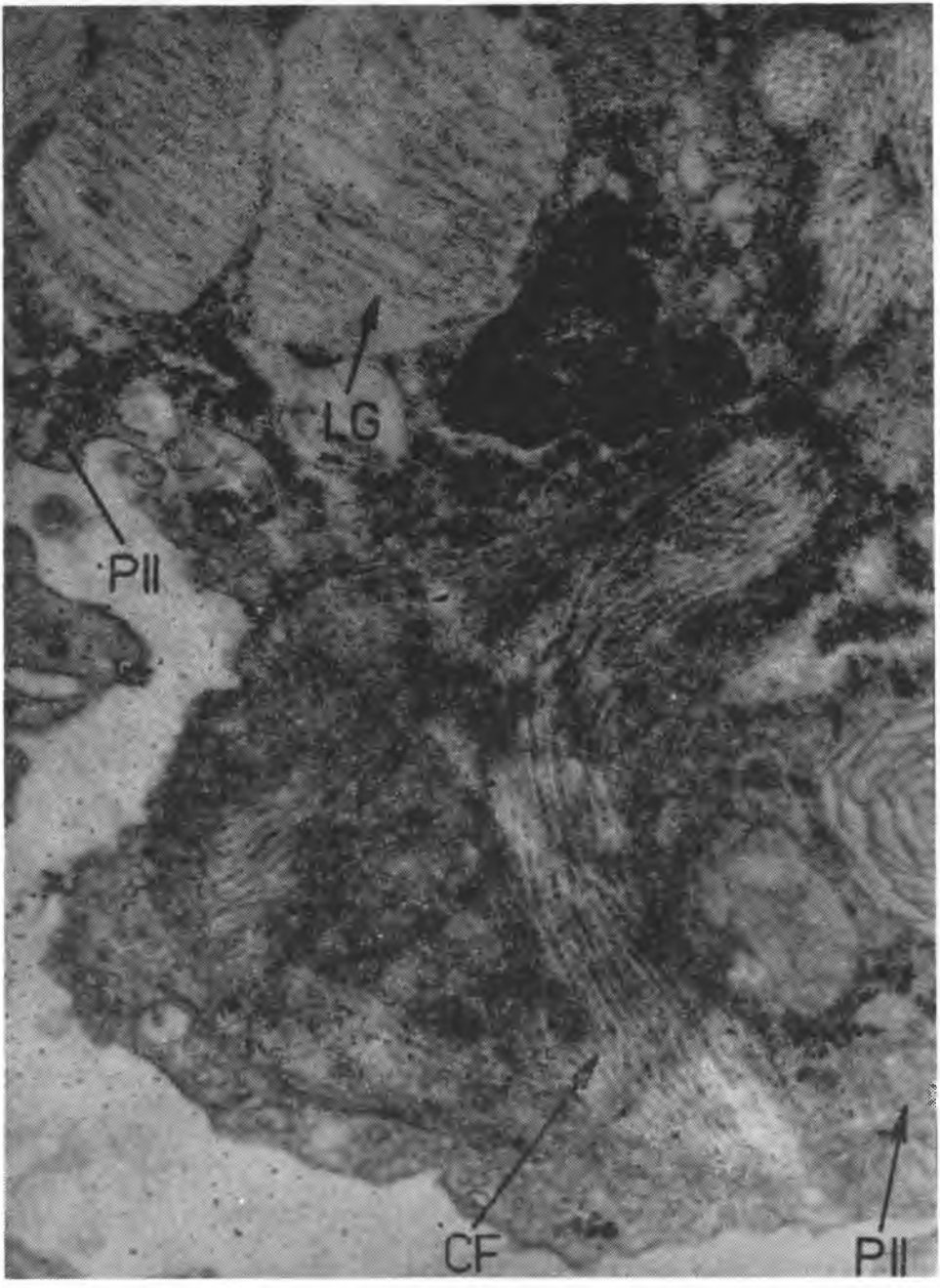

Fig. 9