

Responses of Pulmonary Intravascular Macrophages to 915-MHz Microwave Radiation: Ultrastructural and Cytochemical Study

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ABSTRACT *Background:* Microwave (MW) radiation is being increasingly used as a source of heat supplementation during early postnatal development of pigs. Although MW radiation does not cause deleterious physiological effects, no specific information exists regarding its impact on immune cells such as macrophages. Pulmonary intravascular macrophages (PIMs) are emerging as important inflammatory cells due to their endocytic and secretory potential. An *in vivo* study was conducted to evaluate the effects of infrared, and low and high power MW radiation on the PIMs of pigs.

Methods: Pigs were exposed to infrared (IR), low MW (LMW; 6.1 mW cm^{-2}), and high MW (HMW; 11.4 mW cm^{-2}) radiation at 915 MHz ($n = 2$ for each treatment) for 24 hr. The controls ($n = 2$) were exposed to natural light for the same period of time. Lung tissues were processed for ultrastructural examination and acid phosphatase (AcPase) cytochemistry. In addition, rough endoplasmic reticulum (RER) as a fraction of cytosol of the PIMs was counted.

Results: Ultrastructural and numerical data suggested enhanced secretory activity in the PIMs of LMW-treated pigs as indicated by the increased RER:cytoplasm ratio, prominent Golgi complex profiles, and accumulation of secretory vesicles in conjunction with microtubules as compared with the control, IR, and HMW-exposed pigs. High MW treatment induced some damage to pulmonary interstitium as deduced from the presence of extracellular AcPase precipitates and disrupted collagen matrix. Intracellular globules were noticed in the PIMs of IR and LMW-treated pigs but not in the control and HMW-radiated animals.

Conclusions: Elaboration of structural signs of secretory activity in the PIMs by LMW radiation in the absence of pulmonary pathological changes indicates its potential for cell activation in addition to the already established role of LMW in heat supplementation. This activation could be due to either increased core body temperature or initiation of intracellular signaling by the LMW radiation. This study also shows that the HMW radiation is capable of inducing pathology in the form of changes in the pulmonary interstitial matrix and may not be a good source of supplementary heat.

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Key words: Golgi complex, Lung, Microwave radiation, Pulmonary intravascular macrophages, Pig, Rough endoplasmic reticulum

The lungs of domestic animal species including pigs contain resident population of immune cells such as pulmonary intravascular macrophages (PIMs; Warner and Brain, 1990; Atwal et al., 1992; Staub, 1994). The PIMs form adhesion plaques with the capillary endothelium, and in pigs they cover approximately 25% of the pulmonary capillary surface (Winkler, 1988; Warner and Brain, 1990). These cells influence pulmonary responses including hemodynamics and may initiate lung inflammation due to their endocytic and secretory potential (Albertine and Staub, 1986; Warner

et al., 1988; Winkler, 1988; Sierra et al., 1990; Atwal et al., 1994). Porcine PIMs have been implicated in *Haemophilus pleuropneumoniae* and African swine fever virus infections and in the generation of inflammatory

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substances (Bertram, 1986; Bertram et al., 1989; Sierra et al., 1990; Chitko-McKowan et al., 1991).

Recently, microwaves have been used for heat supplementation in the early stages of development of food-producing animals in cold climates (Braithwaite et al., 1994). Microwave (MW) radiation causes oscillations of bipolar molecules, increasing their kinetic activity with consequent generation of heat (Michaelson and Lin, 1987). Given their high water content, biological organs are the ideal targets for the effects of MW radiation. One of the most commonly used frequencies is 915 at wavelength of 32.8 cm in the air. The penetrating ability of radiation is determined by the wave frequency and the composition of material, but the heating capacity depends on its intensity or incident power density, which is commonly expressed as mW cm^{-2} (Schwan, 1971). The penetrating capability of 915-MHz mW is 17.7 cm into low-water-containing tissue (e.g., fat and bone) and 3.04 cm into high-water-containing tissue (muscle; Johnson and Guy, 1972).

Several studies address the consequences of hyperthermia and other athermic effects when euthermic animals are subjected to substantial MW power (Justesen, 1975; Tell and Harlen, 1979; Gordon, 1987). Exposure of dogs to a power density of 165 mW cm^{-2} of 2.86-GHz MW caused classic thermal overload when housed under thermoneutral conditions but not when they were maintained at 11°C (Michaelson, 1973). Comparable results of rectal temperature elevation during exposure to microwaves have been reported for rats, rabbits, squirrels, and rhesus monkeys (Ely et al., 1964; Phillips et al., 1975; deLorge, 1978). Furthermore, abnormal electrocardiographic, hematologic, enzymatic, and teratogenic changes have been added to the inventory of microwave effects associated with hyperthermia (Phillips et al., 1975; Roux et al., 1986; Lu et al., 1987). Pigs exposed to 2,450 or 915 MHz tended to reduce locomotive activity and to sleep more without any effect on their growth performance (Braithwaite et al., 1992; Foote et al., 1995).

Although substantial work has been done on the physiological impact of MW radiation on animals, no information about its cellular structural effects exists. In this study, responses of pig pulmonary vascular cells, especially PIMs to MW and infrared (IR) radiation, were investigated. The IR is commonly used as a control in the MW radiation studies, and we wanted to evaluate its possible effects on the pulmonary vascular cells. The PIMs were used because of their potential to initiate pulmonary inflammatory responses to biological and chemical challenges. Moreover, MW radiation may activate macrophages (Norton et al., 1990; Liu and Olszewski, 1993). Ultrastructural, numerical, and cytochemical data demonstrate that the low microwave (LMW) radiation induced enhanced expression of biosynthetic machinery in most of the vascular cells including PIMs without associated lung pathology. High microwave (HMW) radiation induced possible lung damage as indicated by acid phosphatase (AcPase) precipitates in the capillary lumen and interstitium, and disorganization of extracellular matrix.

MATERIALS AND METHODS

Ten Yorkshire X Landrace 3–4-week-old crossbred pigs were obtained from a local minimum-disease com-

mercial farm. The experiment protocol used in this study was approved by the Animal Care Committee of University of Prince Edward Island, Charlottetown, Canada. All animals were housed at 25°C in individual pens of $122 \times 50 \text{ cm}$ within a stainless steel weaner deck with a solid lid cover, sides of wire mesh with holes of $0.64 \times 0.64 \text{ cm}$, and rubberized expanded metal flooring with perforations of $2.5 \times 1 \text{ cm}$. Each deck contained three pens separated by a 6-mm microwave transparent plexiglass separation. Animals had water and feed ad libitum and received an 18% protein standard starter ration.

The weaner decks were designed to function as echoic chambers for 915-MHz MW radiation. The microwaves were generated by a 4-kW water-cooled magnetron acquired from D'Ossone Canada Ltd. (Charlottetown, PEI). The generator could be regulated to dispense the required microwave intensity. Microwaves were transferred from the magnetron to the echoic chambers by a 300Ω coaxial cable connected to an antenna that delivered the microwaves into a 28-cm-long, $26 \times 12.6\text{-cm}$ waveguide. The cages were fitted with two safety switches to turn the magnetron off if the lid was not properly sealed. The influx of microwaves to the cages was measured with two on-line Bird RF directional thru-line wattmeters (Model 43, Cleveland, OH). Infrared-exposed animals were housed in identical pens that had a 25.4-cm diameter perforation in the lid for a 250-W infrared lamp instead of the waveguide for MW dispensation.

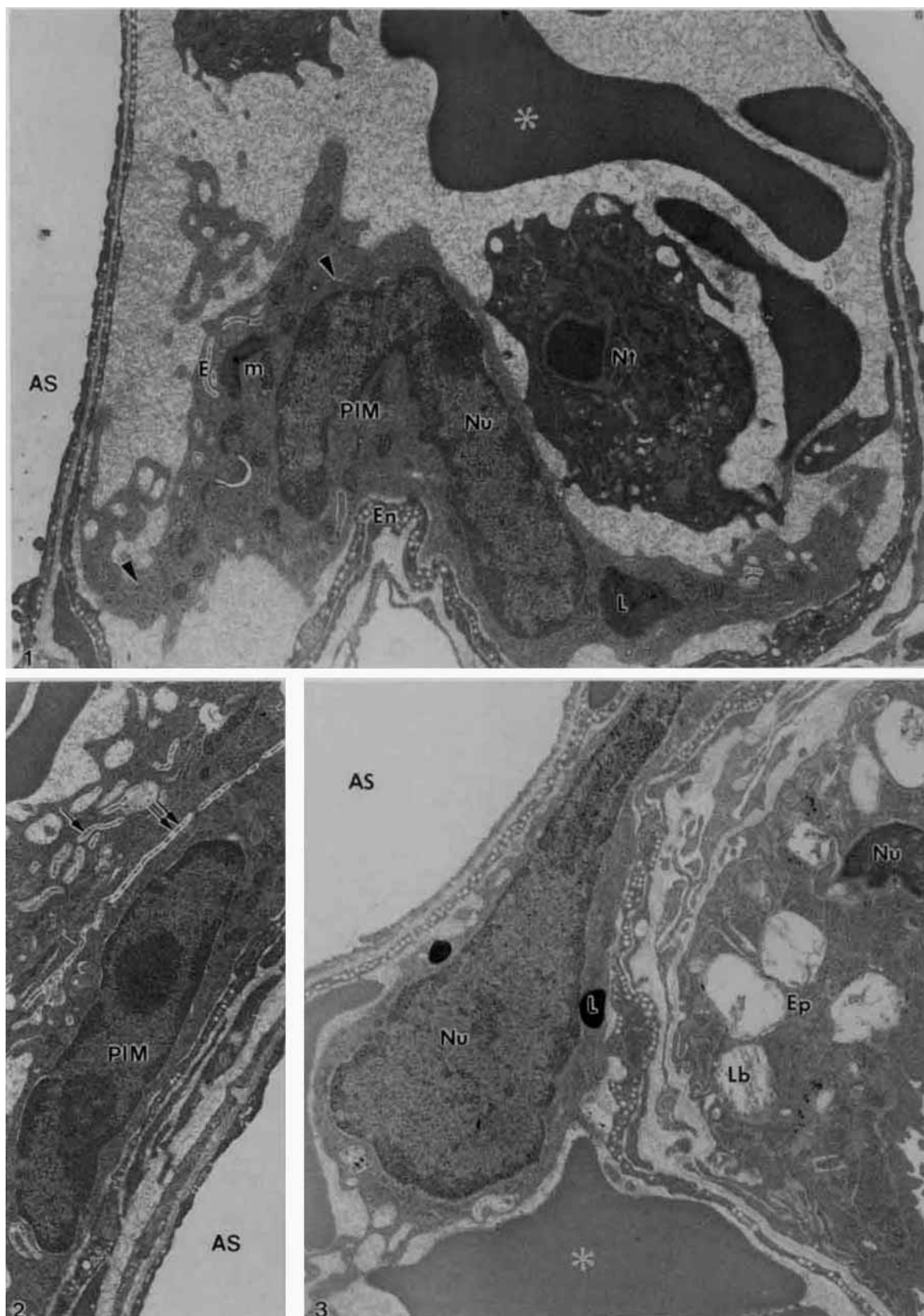
After 4 days of acclimatization to the environment, an indwelling catheter was placed in the jugular veins of eight pigs under halothane anesthesia. A second period of 4 days was allowed for recovery prior to exposure to experiment protocols. These cannulated piglets were assigned randomly to four groups ($n = 2$): control, IR, LMW, and HMW. Two uncannulated piglets were also studied to evaluate effects of cannulation on the PIMs.

Animals in the IR group were housed at $24\text{--}25^\circ\text{C}$ for 8 days and then at $16\text{--}18^\circ\text{C}$ for 24 hr while supplemented with a 250-W infrared lamp placed 50 cm above the floor. Weaners in the LMW were treated similarly to those in the IR but were exposed to 6.1 mW cm^{-2} of continuous 915-MHz MW radiation for 24 hr. Animals in the HMW were treated with 11.4 mW cm^{-2} instead of only 6.1 mW cm^{-2} . Blood samples were collected from the cannulated animals to evaluate endocrine pa-

Fig. 1. Control. A pulmonary intravascular macrophage (PIM) is attached to the capillary endothelium (En) and carries fuzzy electron-dense material in endosomes (E). Small patches of rough endoplasmic reticulum (arrowheads) and mitochondria (m) are scattered throughout the cytoplasm. Neutrophil (Nt) and red blood cells (asterisk) are seen in the vicinity of this PIM. L, lysosome; Nu, nucleus; AS, alveolar space. $\times 12,500$.

Fig. 2. Control. A PIM carries fuzzy electron-dense material in its endosomes (single arrow) and plasma membrane invagination (double arrows). AS, alveolar space. $\times 12,500$.

Fig. 3. Control. A PIM shows AcPase-reactive spherical lysosomes (L) in the cytoplasm and faint cytochemical reactivity in its nucleus (Nu). Asterisk, red blood cell; AS, alveolar space; Ep, type II alveolar epithelial cell; Lb, lamellar body. $\times 12,500$.



Figs. 1-3.

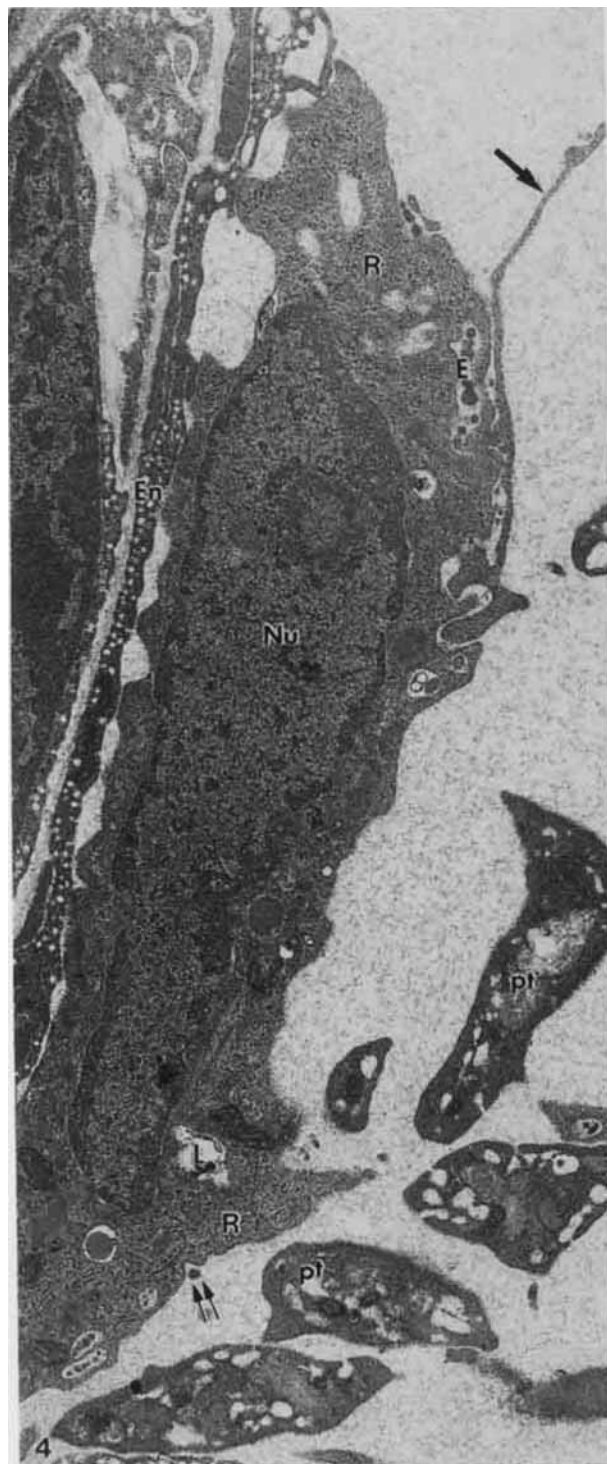


Fig. 4. Infrared treatment. This micrograph depicts a PIM along the capillary endothelium (En). Plasma membrane is devoid of coat globules except at a coated pit (double arrows), but endosomes (E) contain multiple globules. Rough endoplasmic reticulum (R) is spread throughout the cytoplasm. Platelets (pt) are aggregated in the vicinity of this PIM. Nu, nucleus; single arrow, a pseudopod. $\times 15,000$.

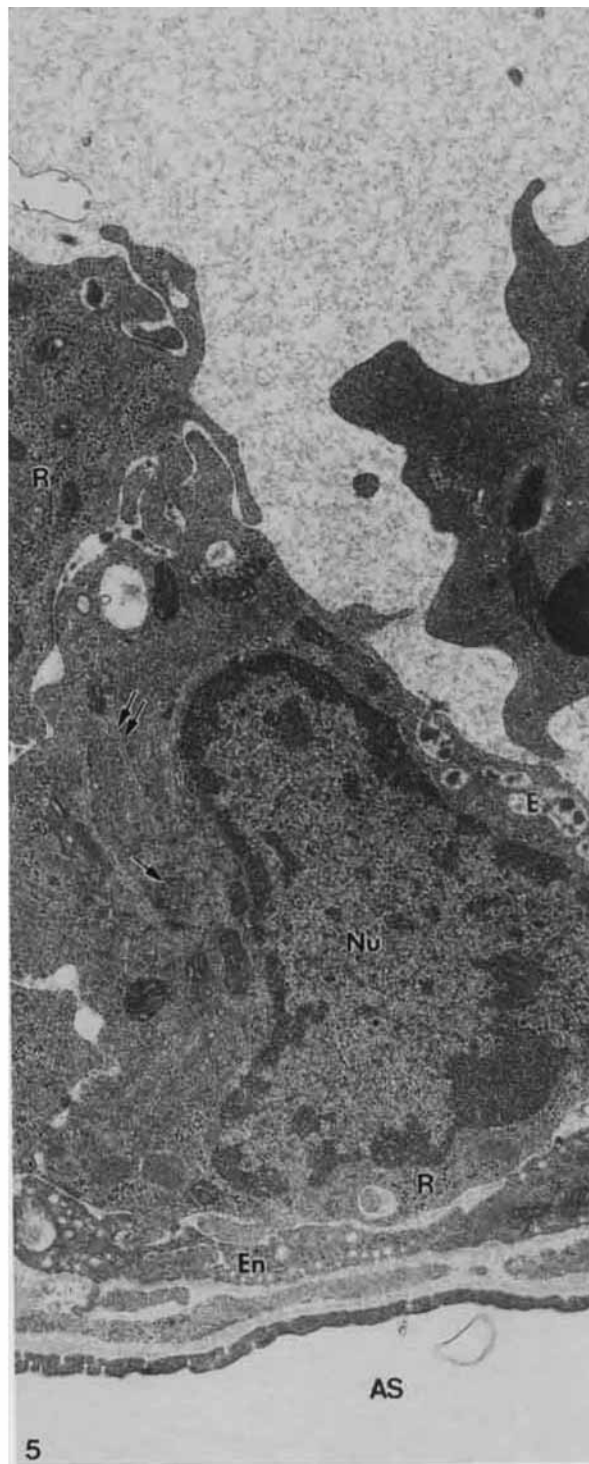


Fig. 5. Infrared treatment. A PIM attached to the endothelium (En) shows a centriole (single arrow) and associated microtubules (double arrows), multiple globule-containing endosome (E), and rough endoplasmic reticulum (R). Nu, nucleus; AS, alveolar space. $\times 20,000$.

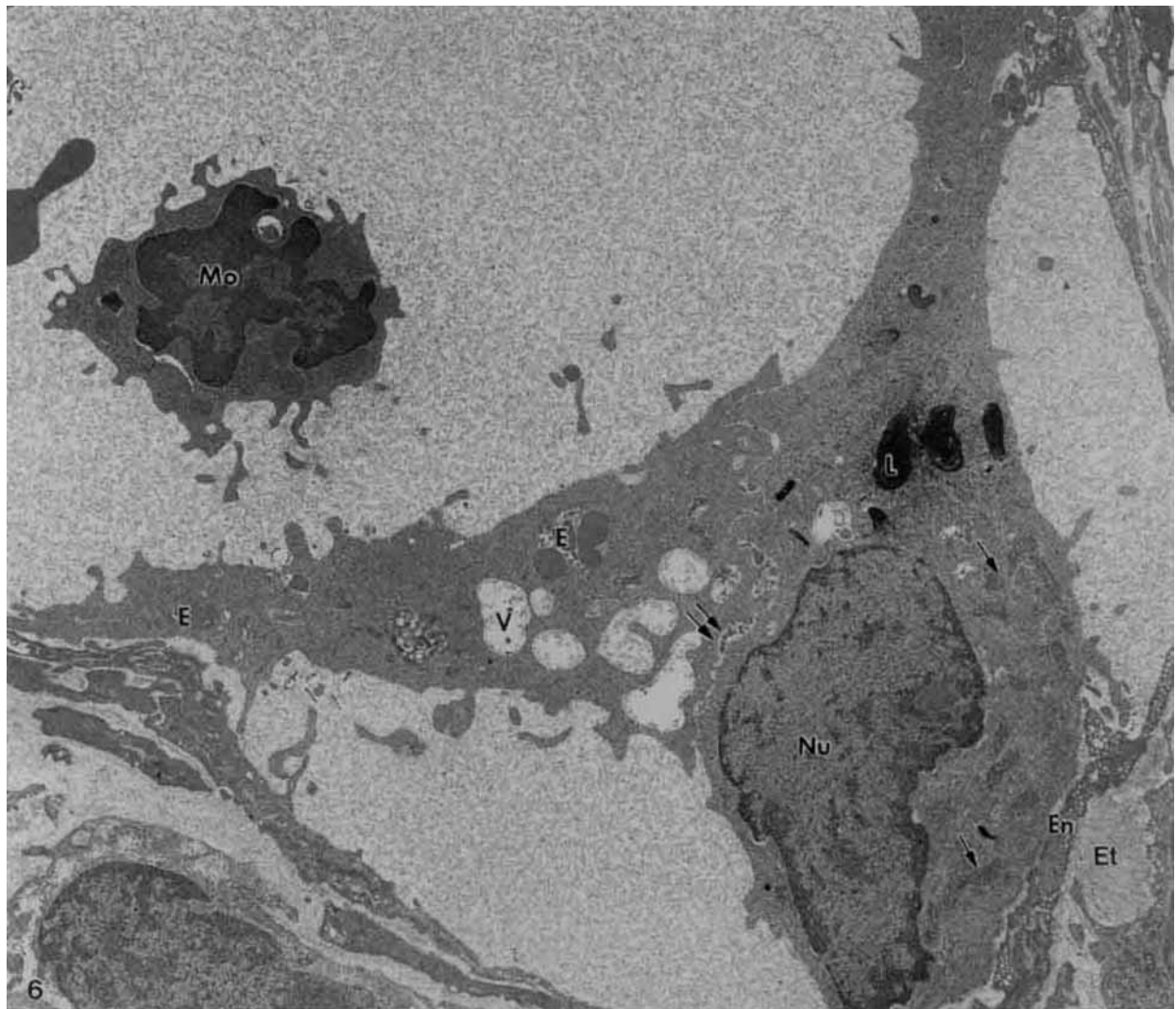
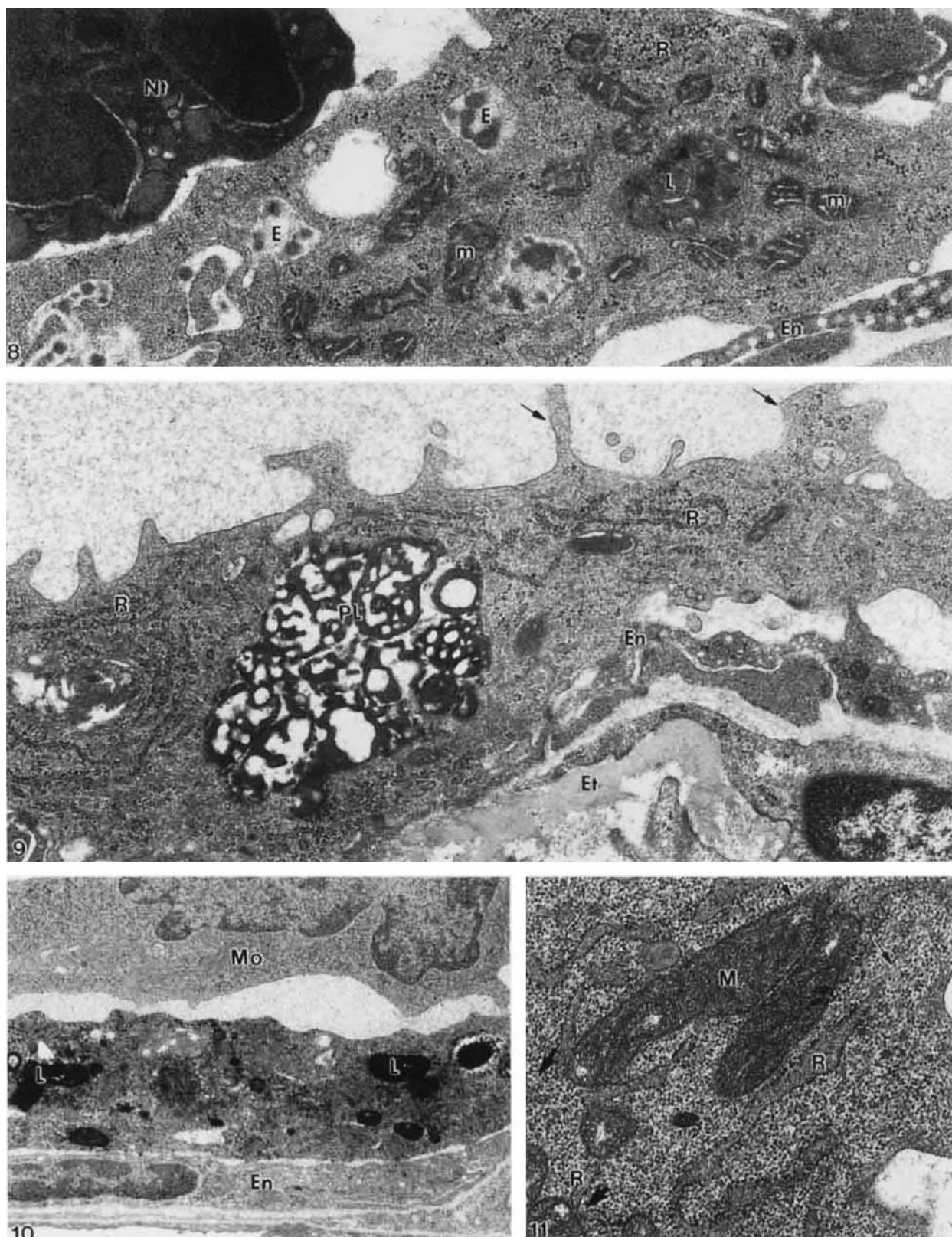


Fig. 6. Infrared treatment. A PIM showing AcPase reactivity in the lysosomes (L) but not in the globule-carrying endosomes (E) or an endocytic channel (double arrow). Nucleus (Nu) of this PIM and a monocyte (Mo) is faintly reactive for AcPase staining. Mitochondria (single arrows) are aligned along insinuating microtubules. V, vacuoles; En, endothelium; Et, elastin. $\times 12,000$.

Fig. 7. Infrared treatment. A view of fine organization of interstitial matrix with collagen (Co), elastin (Et), and a fibrocyte (FC) with its AcPase-reactive nucleus (Nu). $\times 15,000$.



Figs. 8-11.

rameters to be reported elsewhere. Final evaluation of the data demonstrated that cannulation did not influence the PIMs, thus the data will be reported as those of controls, IR, LMW, and HMW.

Lung Fixation

Pigs were killed with an overdose of pentobarbital sodium (Euthanyl[®]). Tracheotomy was performed, and lungs were fixed *in situ* via airways with 2% glutaraldehyde and 2.5% paraformaldehyde in 0.1 M Na-cacodylate buffer for 60 min. Pigs were kept in sternal recumbency during fixation. Tissues were collected from cranial, caudal, and middle lobes of the lungs and were immersion fixed for 3 hr. Some of the tissues were incubated with 0.5% tannic acid to increase the density of lipid-containing structures.

Acid Phosphatase Cytochemistry

The lung tissues were processed for AcPase cytochemistry as described elsewhere (Borgers et al., 1991; Singh et al., 1995). Briefly, tissues were incubated for 3 hr with sodium β -glycerophosphate (Sigma Chemical Co., St. Louis, MO) and lead nitrate in 0.5 M sodium acetate buffer (pH 5.0). The controls were treated for the same duration in a solution without sodium β -glycerophosphate.

Transmission Electron Microscopy

Lung tissues were dehydrated in graded concentrations of ethyl alcohol and propylene oxide and embedded in Epoxy resins (J.B. Em Inc). Thick sections of 90 nm were cut on a Reichert-Jung Ultracut-E microtome and evaluated under a light microscope. The ultrathin sections of 20 nm were obtained, stained with uranyl acetate and lead nitrate, and examined in a Hitachi-7000 transmission electron microscope at 80 kV.

Numerical Analysis of the Rough Endoplasmic Reticulum Volume

Numerical counts were performed using established techniques (Weibel, 1979). Six representative micrographs from each treatment (three from each animal) were selected and printed to a final magnification of $\times 15,000$. A square grid was placed randomly on each micrograph. Number of grid intersections on rough en-

doplasmic reticulum (RER) profiles and total number of intersections in the cytoplasm were counted and RER:cytoplasm volume ratios were determined. The average value of the RER:cytoplasm volume ratios for both animals in each group were obtained and compared between groups.

RESULTS

The PIMs in the control pigs were attached to the capillary endothelium. The PIMs resembled fully differentiated macrophages and showed indented nuclei, lysosomes, and electron-dense material in their endosomes and plasma membrane invaginations (Figs. 1, 2). The AcPase cytochemistry revealed acid hydrolases in the lysosomes and faint reactivity in the nuclei of PIMs from animals in the control groups (Fig. 3). Monocytes also showed AcPase precipitate in their nuclei.

The PIMs of pigs exposed to IR showed plasma membrane ruffles, intracellular globules, RER profiles, and platelet aggregation in their vicinity (Fig. 4). A few PIMs exhibited centrioles and associated tracts of microtubules (Fig. 5). Acid phosphatase cytochemistry produced punctate reaction in the nuclear heterochromatin of PIMs and other lung cells and a dense reaction in acid hydrolase-containing lysosomes (Figs. 6, 7). Pulmonary interstitium showed typical arrangement of collagen, elastin, and other cellular components following IR exposure (Fig. 7).

Low-MW-treated PIMs exhibited plasma membrane modifications, cytoplasmic vesicles with luminal globules, and conspicuous RER profiles, suggesting increased volume (Figs. 8, 9). Some of the large lysosomes had partially digested lipidlike material (Fig. 9). Acid phosphatase reaction demonstrated multiple lysosomes in the PIMs (Fig. 10). The RER contained electron-dense proteinaceous material in its dilated lumen; RER and mitochondria were associated with microtubules in these cells (Fig. 11). The average RER:cytoplasm ratio in the PIMs of LMW-treated pigs (0.36) was higher than that in the control (0.21), IR (0.17) or HMW-exposed animals (0.17). Golgi complexes of the majority of the PIMs were associated with centriole, microtubules, and secretory vesicles, and their nuclei contained interchromatin granules (Figs. 12, 13). Granular AcPase reaction was localized to the nuclear heterochromatin of LMW-exposed PIMs (Fig. 14). Pulmonary parenchyma of pigs treated with LMW radiation appeared unaltered (Fig. 15).

Lungs of HMW-exposed pigs contained PIMs that had circular plasma membrane ruffles, partially digested lipidlike material in the lysosomes, and no intracellular globules (Figs. 16, 17). Extracellular acid hydrolases were seen in the capillaries and pulmonary interstitium of the lung tissues processed for AcPase cytochemistry (Figs. 18, 19). The lung interstitium displayed patches of disorganized collagen and elastin elements (Fig. 19).

DISCUSSION

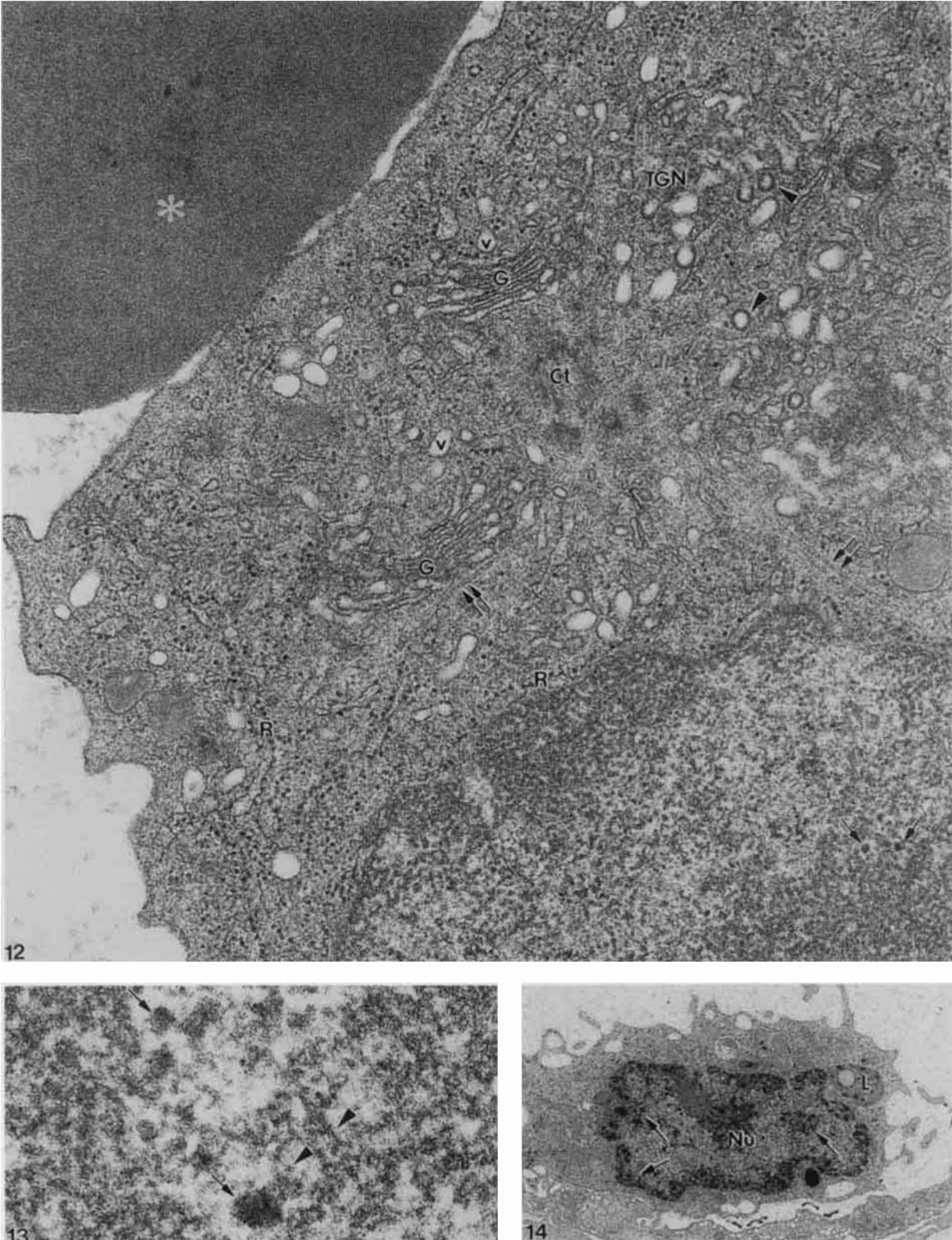
This report is the first to describe the effects of IR, LMW and HMW radiation on the pig pulmonary vascular cells, with specific focus on a resident population of mononuclear phagocytes called PIMs. Ultrastructural and cytochemical data demonstrate that the

Fig. 8. Low microwave treatment. Part of a PIM, attached to the endothelium (En), shows globule-carrying endosomes (E), scattered rough endoplasmic reticulum (R), normal-looking mitochondria (m), and a lysosome (L). A neutrophil (Nt) is devoid of intracellular globules. $\times 30,000$.

Fig. 9. Low microwave treatment. A micrograph of a part of a PIM attached to the endothelium (En) depicting extensive profiles of rough endoplasmic reticulum (R), a large phagolysosome (PL), and plasma membrane ruffles (single arrows). Et, elastin. $\times 17,500$.

Fig. 10. Low microwave treatment. A PIM aligned along the endothelium (En) shows many AcPase reactive lysosomes (L), but a monocyte (Mo) does not. $\times 17,500$.

Fig. 11. Low microwave treatment. This micrograph shows electron-dense material in the dilated lumen of rough endoplasmic reticulum (R). Rough endoplasmic reticulum and elongated mitochondria (M) are closely related to microtubules (single arrows). Small arrows indicate microtubules cut in cross section. $\times 30,000$.



Figs. 12-14.

LMW treatment of pigs induced proliferation of RER along with other structural signs of synthetic activity such as prominent Golgi complexes and secretory vesicles in the PIMs in contrast with those exposed to the IR or HMW radiation. High-MW-exposed lungs had free acid hydrolases in their pulmonary parenchyma along with damage to extracellular matrix.

The PIMs of control pigs had characteristics of fully differentiated macrophages, as described by Winkler (1988). These cells showed attachment to capillary endothelium, presence of fuzzy material in the endosomes, and lysosomes. A layer of surface coat globules, as described in the PIMs of sheep, cattle, goat, and horses by Atwal and colleagues, was not observed in the present study, although PIMs of IR and LMW-exposed pigs contained intracellular globules (Atwal et al., 1992; Singh et al., 1995). The PIMs of 4-week-old pigs may not have a globular surface coat.

Low-MW-exposed PIMs looked strikingly different from those in the control, IR, and HMW-treated PIMs. Nuclear interchromatin granules and perichromatin fibrils, numerous RER profiles, prominent Golgi complexes, secretory vesicles, microtubules, and centrioles were consistent features of these PIMs. Using ultrastructural immunocytochemical, autoradiographic, and molecular techniques, the interchromatin granules have been shown to contain mRNA splicing factors and nascent mRNA species (Fakan and Puivon, 1980; Spector, 1993; Baskin, 1995). These granules are seen prominently in secretory cells, for instance, hepatocytes, and in tumor cells (Spector, 1993), and their presence in the PIMs may be a sign of stimulated transcription following LMW exposure. Acid phosphatase reactivity of nuclear heterochromatin of the PIMs in the present study could be due to exposure of phosphate groups of nucleic acids during transcription. Recently, a relationship between AcPase expression and increased glycoprotein processing has been demonstrated (Thomopoulos et al., 1992). A similar nuclear cytochemical reaction in conjunction with other signs of secretory activity was reported in the PIMs of heparin and *Escherichia coli* lipopolysaccharide-treated sheep (Singh and Atwal, 1994; Singh et al., 1995).

The RER as a cell compartment is integral to proper protein folding and degradation of poorly constructed proteins prior to further processing and onward journey through the Golgi complex (Mellman and Simons, 1992; Sitia and Meldolesi, 1992; van Meer, 1993). In the LMW-treated pigs, RER with prominent ribosomes

Fig. 12. Low microwave treatment. A magnified view of a PIM showing active Golgi complex (G) associated with centriole (Ct) and microtubules (double arrows). The *trans*-Golgi network (TGN) is surrounded by uncoated (v) and coated (arrowhead) secretory vesicles. Rough endoplasmic reticulum (R) is spread profusely in the PIM. Single arrows indicate interchromatin granules in the nucleus. Asterisk, red blood cell. $\times 42,500$.

Fig. 13. Low microwave treatment. This micrograph depicts interchromatin granules (single arrows) and perichromatin fibrils (arrowheads) in the nuclear heterochromatin of a PIM. $\times 150,000$.

Fig. 14. Low microwave treatment. A PIM showing granular AcPase reactivity (arrows) in heterochromatin of nucleus (Nu) and lysosome (L). $\times 10,000$.

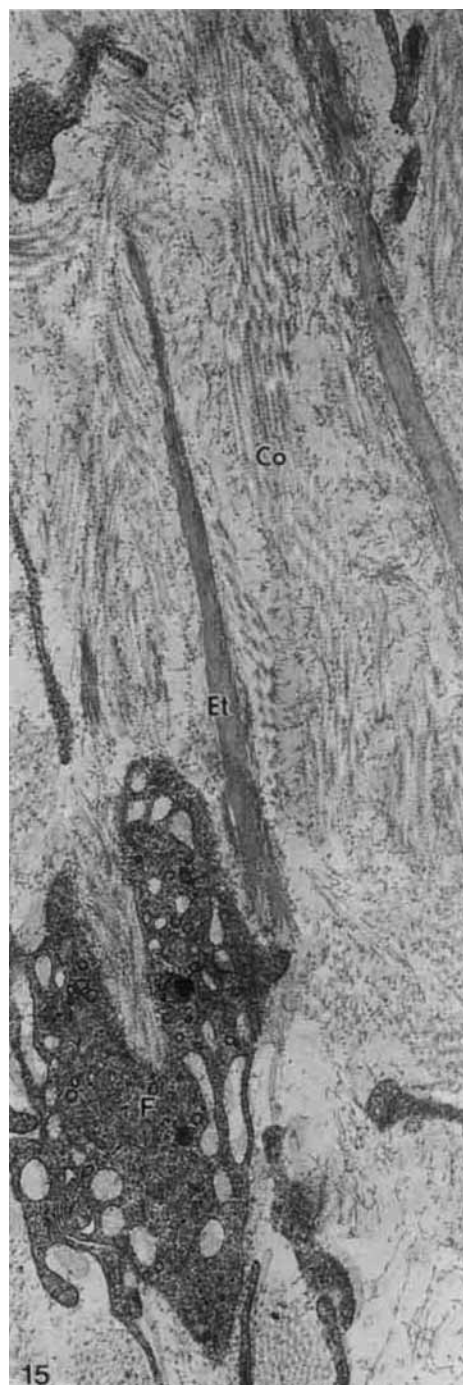


Fig. 15. Low microwave treatment. A micrograph demonstrating unaltered typical organization of interstitial matrix with collagen (Co), elastin (Et), and fibroblast (F). $\times 12,000$.

was observed in association with microtubules, and its lumen was dilated with electron-dense material, possibly a consequence of biosynthetic activity in this organelle. Numerical counts suggested a higher RER:cytoplasm ratio in the PIMs of LMW-treated pigs than that in those of other groups. Role of centriole and mi-

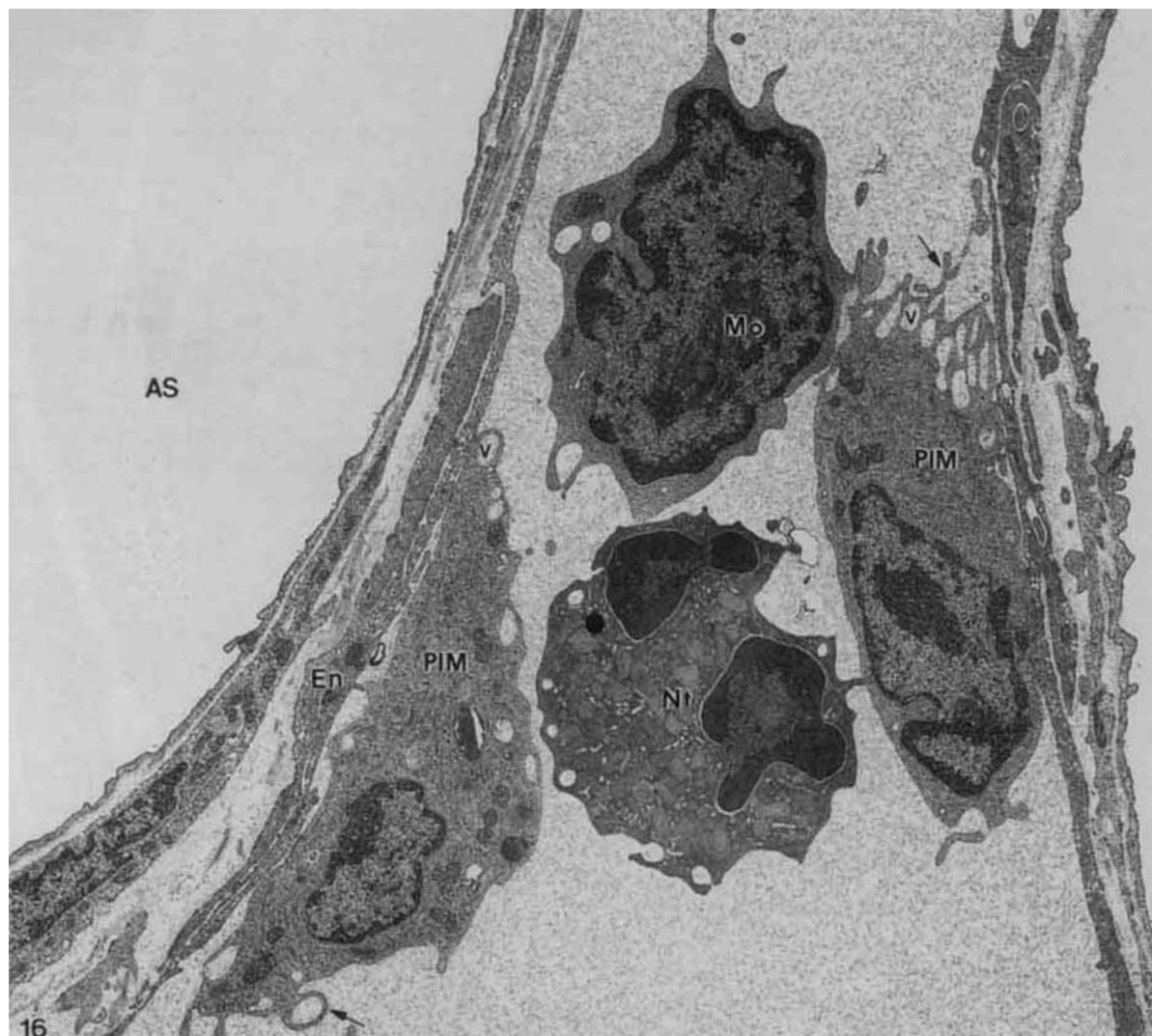
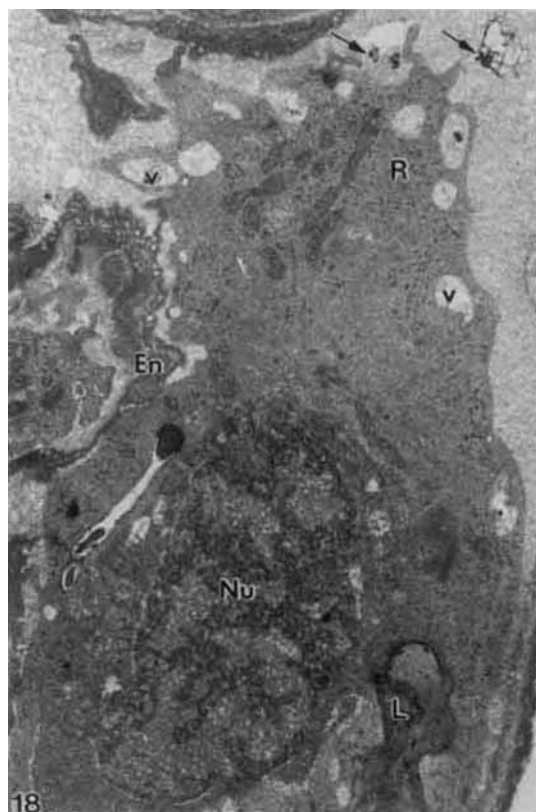
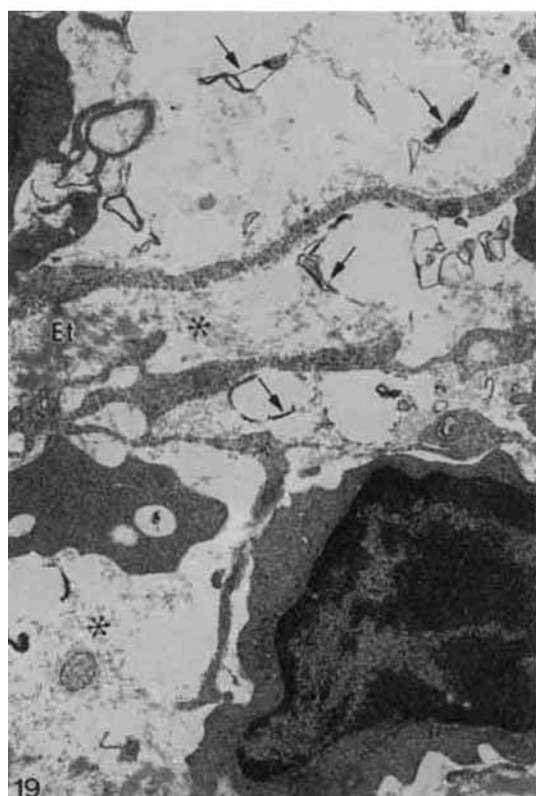


Fig. 16. High microwave treatment. A view of pulmonary capillary showing pulmonary intravascular macrophages (PIM) attached to endothelium (En), monocyte (Mo), and a neutrophil (Nt). PIMs show many empty vacuoles (v) and membrane ruffles (single arrows). Note the absence of intracellular globules as compared with the PIMs of IR and LMW-treated pigs. AS, alveolar space. $\times 10,000$.

Fig. 17. High microwave treatment. A PIM shows multiple lysosomes (L) and elongated mitochondria (m) and sparsely distributed rough endoplasmic reticulum (arrowheads). Note absence of endosomal globules. En, endothelium; AS, alveolar space. $\times 17,500$.



Figs. 18 and 19.

cro tubules in the spatial arrangement of cell organelles, for instance, RER and Golgi complex, targeting of secretory products during activation or cell movement is also well established (Debora and Sheetz, 1988; Ho et al., 1989; Kelly, 1990; Teraski, 1990; Mizuno and Singer, 1994).

Golgi complex-associated transport vesicles are crucial to the flow of secretory products between different cell compartments (Mellman and Simons, 1992). Accumulation of coated and uncoated transport vesicles in the vicinity of Golgi complexes, as noticed in the PIMs of LMW exposed-pigs, is accepted as one of the features of enhanced cellular secretory activity (Rothman and Orci, 1992). Microwave treatment may increase ADP-ribosylation in the rats (Singh et al., 1994). The ADP-ribosylation factor, a small guanine-binding protein, plays an important role in intracellular signaling and in coatomer complex formation during vesicle budding from the Golgi cisternae (Donaldson et al., 1992). Recently, we showed secretory responses in the PIMs of sheep and horses following a variety of stimuli such as endocytosis of vascular tracers, exposure to halothane, and administration of *E. coli* LPS and heparin (Atwal et al., 1992, 1994; Singh and Atwal, 1994; Singh et al., 1995). Present observations extend this list by the addition of LMW radiation as an important cell stimulus.

These morphological features of the biosynthetic response of PIMs correlate with the previous reports of MW-induced nonspecific activation of macrophages and other lymphoid cells (Norton et al., 1990; Liu and Olszewski, 1993). Linear and pulsing low-frequency electromagnetic fields (1.5–72 Hz) alter protein synthesis in salivary glands in a manner independent of heat shock effects (Goodman et al., 1983; Goodman and Henderson, 1988). However, the intensities of MW radiation in this study, 6.1 mW cm^{-2} and 11.4 mW cm^{-2} , have higher heating capacity than the low-frequency electromagnetic radiation and have been used to re-warm hypothermic pigs and other species of domestic animals by raising their core body temperature (Braithwaite et al., 1991, 1994). An increase in the rectal temperature, used as an indicator of core body temperature, was also reported in rabbits, rhesus monkeys, and rats following MW radiation (Phillips et al., 1975; de Lorge, 1978). Therefore, the secretory activation of PIMs may be attributed to a combined effect of temporarily increased cell temperatures and the influence of enhanced kinetic energy of plasma membrane molecules due to MW radiation. Recently, exposure to increased environmental temperature has been shown to induce transcription of the heat shock proteins that are believed to have protective cellular effects (Mori-moto, 1993; Flanagan et al., 1995). We can only speculate on the possibility of a similar modulatory effect of the LMW treatment on the PIMs of pigs.

Fig. 18. High microwave treatment. A PIM attached to endothelium (En) shows empty vacuoles (v), rough endoplasmic reticulum (R), lysosome (L), and AcPase reaction product (single arrows) in the capillary lumen. Nucleus (Nu) does not show AcPase reactivity. $\times 12,500$.

Fig. 19. High microwave treatment. A view of pulmonary interstitium showing AcPase precipitates (single arrows), loss of collagen (asterisks), and very spotty elastin (Et). $\times 12,500$.

The lungs of pigs following IR or LMW-radiation did not show signs of damage. However, HMW treatment resulted in structural disorganization of pulmonary extracellular matrix. These interstitial alterations by the HMW radiation could be an outcome of damage to the extracellular matrix caused by the abnormal release of acid hydrolases as a consequence of enhanced cell heating in contrast with the IR and LMW treatment. Such a release of acid hydrolases by the cells is not uncommon because it has also been noticed in the sheep PIMs following heparin injection (Singh et al., 1995). The increased expression of secretory organelles of PIMs following LMW treatment may reflect a preinjury response. However, the absence of damage to pulmonary parenchyma and of extracellular hydrolases even after 24 hr of continuous LMW exposure, would suggest it to be a physiological modulation of the cell activity. Based on this study, the use of HMW versus LMW may not be a good method to provide supplementary heat to the neonatal pigs. However, further intensity titration should determine the optimal level of MW radiation to maximize its beneficial effects.

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