Radiation-Induced Cardiomyopathy

II. An Electron Microscopic Study of Myocardial Microvasculature

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Sequential ultrastructural lesions of rabbit myocardial microvasculature after a single dose (1008 or 1300 rads) of local x-irradiation are described. Vascular permeability status was assessed through use of ferritin and colloidal carbon. Endothelial cell swelling and increased vascular permeability were the most conspicuous lesions during the first week following irradiation. Increased vascular permeability, as indicated by the observed ferritin and carbon distribution, appears to be a result of altered pinocytotic transport and widening of endothelial junctional gaps. These lesions, on day 14 and later, were followed by basement membrane thickening, endothelial cell extrusions and bleb formation, platelet sequestration, abnormal endothelial cell phagocytosis and appearance of myelin-like figures within the endothelial cells (Am J Pathol 74:125–136, 1974).

RADIATION-INDUCED HEART DISEASE is a well-recognized entity, both in humans exposed to therapeutic irradiation and in experimental animals. Myocardial sensitivity to x-irradiation in general and relative sensitivity of its tissue components in particular remain controversial.[1,2] Most of the experimental studies on morphologic aspects of radiation-induced cardiomyopathy have been at the light microscopic level. Recently we undertook a sequential study of radiation-induced cardiomyopathy at the ultrastructural level in order to further explore the nature of lesions involving the various tissue components of myocardium, their pathogenesis and the relative roles that they play in evolution of radiation-induced cardiomyopathy. We have described the sequential changes in myocardial myocytes previously.[3] This paper describes the changes in the microvasculature of myocardium and reflects on their pathogenesis and relative importance in radiation-induced cardiomyo-

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pathy. Vascular compromise has generally been regarded as the main underlying factor, and significant experimental work reported to date is related to lesions of the coronary arteries and their main branches.[4] Because of the lack of ultrastructural studies to date, morphologic changes in microvasculature have remained unexplored and poorly understood.

Materials and Methods

Forty male New Zealand white rabbits weighing 1.5 to 2 kg, under intravenous pentobarbital sodium (Nembutal®) anesthesia (30 mg/kg), were subjected to a single cardiac dose of 1008 rads (10 animals) or 1300 rads (30 animals) of x-irradiation calculated at 1.5 cm depth from surface of the skin. Using a precardial approach, radiation was delivered from source of 250 keV (Ma 15, HVL 2.2 mm Cu, TSD 15 cm, air dose 66 rads/min) and through an x-ray cone 4 × 5 cm. Before irradiation, cardiac localization was verified by a chest x-ray; precardial skin was either shaved or depilated with depilatory paste, Nair (Carter Products). An additional 12 animals subjected to sham irradiation served as controls. Animals were housed in air conditioned facilities and served the usual laboratory food and water *ad libitum*.

Following irradiation, animals were selected at random in batches of 3 or 4 along with a control and, under intravenous pentobarbital sodium anesthesia, were killed at 24 hours, 72 hours, 7 days and at predetermined weekly or biweekly intervals thereafter for 4 months. The thoracic cavity was opened by splitting the sternum, and the pericardial cavity was incised. A block of muscle approximately 3 mm thick was quickly excised from left ventricular wall and minced with a razor blade to approximately 1-mm cubes and placed in ice cold 2.5% phosphate-buffered gluteraldehyde at 4 C for 2 hours. Sampling site was constant in all animals. To study the vascular permeability status, 2 treated animals at each killing up through 14 days were injected intravenously, one with ferritin (1 cc/kg body weight) and the other with carbon suspension (1 cc/kg body weight), 15 minutes before killing, as were selected controls. Two additional animals, 1 each at 48 hours and 72 hours postirradiation, injected with carbon, were killed 1 hour after injection. The aqueous solution of ferritin (100 mg/ml) was twice crystallized and was cadmium free (Polysciences Inc, Warrington, Pa); the colloidal suspension of carbon was batch No. C1-1143-1A made by Guenther-Wagner Pelikan-Werke, Hanover, Germany.

Glutaraldehyde-fixed tissue was washed thoroughly in 1 M phosphate buffer (pH 7.3) and postfixed in 1% phosphate-buffered osmium tetraoxide for 1 hour at 4 C. Tissue was dehydrated and embedded in Epon 812 according to Luft's method.[5] One-micron sections were cut with a glass knife on an LKB ultramicrotome III and stained with toluidine blue. Thin sections, cut with a diamond knife and supported on 250-mesh copper grids, were stained with uranyl acetate followed by lead citrate.[6] Sections were examined and photographed in RCA EMU-3 electron microscope.

Results

A variety of ultrastructural changes in myocardial microvasculature was present as early as 24 hours when, excepting some congestion and

focal capillary hemorrhage, no lesions were recognizable by light microscopy. No significant difference in the lesions in animals receiving 1008 and 1300 rads of x-irradiation was discerned.

Twenty-four hours to 7 days postirradiation, capillaries showed progressive endothelial cell swelling which varied from capillary to capillary and even in the same capillary. In many instances the swollen endothelial cells almost completely obliterated the lumen. There was sparsity of organelles, as well as dilated membrane-bound vesicles and clumping of nuclear chromatin (Figure 1). Besides the swelling there was some increase in pinocytotic activity with comparable increase in ferritin transport across the endothelial cells. Endothelial junctional gaps showed some widening, but ferritin was seen only within the endothelial vesicles. Perivascular spaces were enlarged, contained ferritin particles and electron-dense debris resembling intravascular plasma. Ferritin particles were seen even in the subsarcolemmal position of myocardial fibers (Figure 2). In irradiated animals injected with colloidal carbon 15 minutes before killing, carbon particles were seen free in the extravascular space. No carbon was seen in actual transit through either endothelial junctional gaps or pinocytotic vesicles (Figure 3). In treated animals administered colloidal carbon 1 hour before sacrifice, carbon particles were seen phagocytized by interstitial phagocytic cells confirming the *in vivo* vascular leakage. There was suggestion of more pronounced widening of endothelial junctional gaps (Figure 4).

On day 14 and through 4 weeks postirradiation endothelial cells showed variable but progressive plasmalemmal projection, blebbing and extrusion; some extrusions lay free within the lumen. The basement membrane of some capillaries showed focal thickening (Figure 5). An occasional capillary exhibited plugging by sequestered platelets, with clumping and partial degranulation (Figure 6). The capillary endothelium during this period also showed abnormal phagocytic activity with evidence of carbon and erythrophagocytosis (Figure 7). This was not observed in controls at any time. At 6 weeks postirradiation, endothelial cells showed many myelin-like figures and vacuolation with increase in perivascular collagen fibers. Occasional endothelial cell contained Palade bodies (Figure 8).

In sham-irradiated controls, capillary ultrastructure was essentially within normal limits. Semiquantitative estimates of ferritin distribution within the endothelial cells and in extracapillary sites indicated lower density of particles as compared to that of treated animals. Furthermore in contrast to the irradiated animals, controls showed no evidence of endothelial cell phagocytosis or perivascular leakage of carbon.

Discussion

The occurence of radiation-induced vascular changes at the level of light microscopy and their role in radiation pathology is well accepted.[4] The present ultrastructural study demonstrates a sequence of lesions of the myocardial microvasculature and its altered permeability status from a relatively small single dose of external x-irradiation.

Stearner *et al*[7] and Keyeux *et al*[8] demonstrated functional circulatory disturbances as early and late consequences of x-irradiation by cinemicrographic and radiotracer studies respectively. Our findings indicate that increased permeability following irradiation occurs through: a) increased pinocytotic transport across the endothelium, as demonstrated by ferritin distribution and b) intercellular junctional gaps or disruption of endothelial cell sheets, as indicated by leakage of carbon particles.

Pinocytosis seems to be one of the primary responses of endothelial cells to many diverse stimuli. Marchesi[9] suggests this to be a specific adaptation of cell membrane for transport of large molecules of the size of ferritin. Marchesi and Barrnett[10] have demonstrated increased ATPase reactivity associated with pinocytosis and it is conceivable that radiation in common with other injurious insults, acts through activation of this enzyme. Bruns and Palade[11] have described in detail the organization of blood capillaries in muscle and the nature of pinocytic vesicles. They regard these endothelial cell vesicles as a structural equivalent of the large pore system and their studies have indicated that ferritin is restricted to these vesicles while in transit through the endothelium.[12] Our studies also tend to support their conclusions. During this period, endothelial junctional gaps of the type induced by histamine and serotonin, as demonstrated by Majno and Palade,[13] were notably rare. The presence of carbon particles within the extravascular space and within the perivascular phagocytic cells, as demonstrated by our study, strongly favours the abnormal vascular leakage.[14] However, at no time did we observe colloidal carbon particles in actual process of leakage through either the intercellular junctional gaps or pinocytic vesicles.[15] This could merely represent the sampling limitation of this study. Besides the altered permeability of capillaries, endothelial cells exhibited many changes of their cytoplasmic organelles and clumping of nuclear chromatin in the earlier phase following x-irradiation. These were followed by marked extrusions and blebbing of endothelial cells, similar to those found following ischemia of the skin,[16] and administration of injurious agents such as vasoactive amines and mechanical injury.[17] Basement membranes showed focal areas of thickening, sup-

porting the previously reported findings by McDonald and Hayes.[18]

This study, besides demonstrating endothelial phagocytosis of carbon particles of minor degree, also demonstrated endothelial cell erythrophagocytosis. Bari and Sorenson [19] have shown increased phagocytosis by splenic endothelial cells following irradiation, and Teneff and Stoppani [20] have demonstrated radiation-induced increase in phagocytic activity to be dose related. The subject of endothelial cell phagocytosis outside the reticuloenothelial system, under normal circumstances, has remained controversial and when present is extremely uncommon and insignificant. Cotran [21] and Benacerraf *et al* [22] have shown considerable phagocytosis by endothelial cell under a variety of circumstances such as overload of reticuloendothelial system, inflammation and injection of histamine, endotoxins and antigen-antibody complexes. Following x-irradiation, the enhanced phagocytic activity of endothelial cells may in some yet unknown way be related to a direct effect of radiation on endothelial cells or it may be an indirect result of inhibition or overloading of reticuloenothelial system by radiation-induced toxic cellular breakdown product.

Platelet sequestration within the microvasculature following irradiation may follow a number of pathways. It could result from endothelial cell injury,[23] release of various thromboplastin-like substances or tissue breakdown products,[24] and finally it may be initiated by radiation-induced release of endogenous catecholamines and serotonin-like substances. We have observed significant changes in sympathetic nerve endings in irradiated animals, and these are the subject of presently continuing studies. It is, however, more likely that some combination of these factors may be responsible for platelet sequestration. Besides endothelial cell swelling and altered vascular permeability, the platelet sequestration and clumping may represent yet another pathogenic mechanism of microvascular reaction. Jorgenson *et al* [25] have shown ischemic changes following platelet sequestration.

Radiation injury, once initiated, seems to be a continuous process involving complex chain reactions and interactions of events from injuries to individual tissue components, sustained individually or simultaneously and in sequence or out of sequence, associated with release of toxic cellular breakdown products and concomitant functional disturbances. These complex and interreacting events ultimately result in obsolescence of microvasculature and myocardial fibrosis.[3] Changes within the microvasculature play a central and much more important role than previously recognized, in the final radiation-induced morphologic changes. Delayed radiation-dependent tissue necrosis, a recurring

theme in literature related to radiation pathology,[26] seems to result from fibrinoid necrosis of microvasculature,[18,27] which in turn seems to result from various toxic agents released by radiation injury[28] long after the exposure to radiation.

To conclude, this sequential ultrastructural study demonstrates a spectrum of microvascular lesions. During the first week following irradiation these lesions are characterized by endothelial cell swelling and altered vascular permeability. These are followed in later stages, by basement membrane thickening, endothelial cell extrusions, platelet sequestration and abnormal endothelial cell phagocytic activity.

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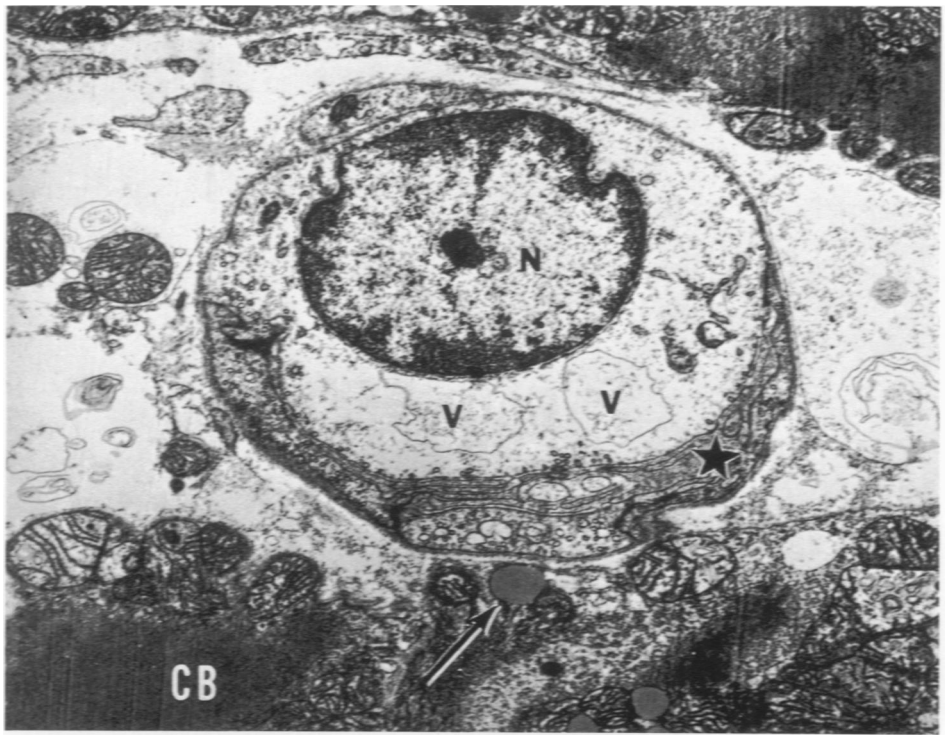
[*Illustrations follow*]

Legends for Figures

Fig 1—Myocardial capillary 24 hours postirradiation (1300 rads). Capillary showing a markedly swollen endothelial cell with sparsity of organelles, membrane-bound vesicles (*V*) and nucleus (*N*) with condensation of nuclear chromatin. Blood vessel lumen (*star*) is significantly obliterated. Lipid bodies (*arrow*) and contraction bands (*CB*) of myocardium are seen (Uranyl acetate and lead citrate, × 10,500).

Fig 2—Myocardium 24 hours postirradiation (1300 rads) and 15 minutes after intravenous ferritin injection. Capillary endothelium with small and large membrane-bound vesicles'(V), ferritin particles within the pinocytic vesicles (*arrows*), wide endothelial junctional cleft (*arrowhead*) and electron-dense proteinaceous debris and ferritin particles in the enlarged extravascular space (*E*). Ferritin particles are also seen within the subsarcolemmal space of adjacent myofiber (*curved arrow*). *R* = red blood cell within the lumen (Uranyl acetate and lead citrate, × 30,000).



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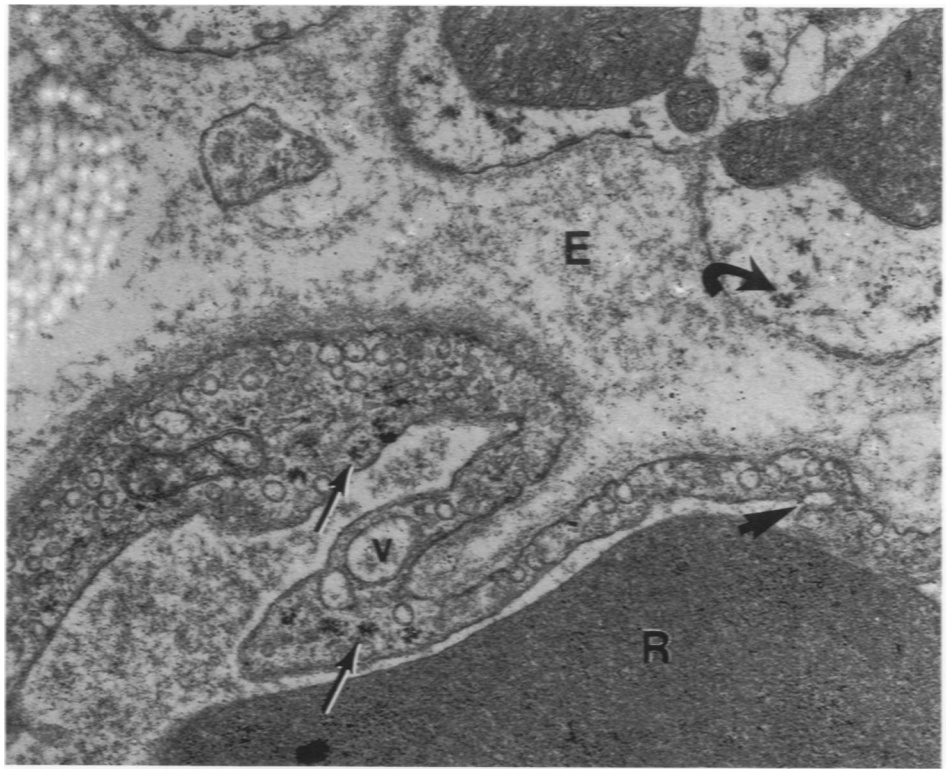

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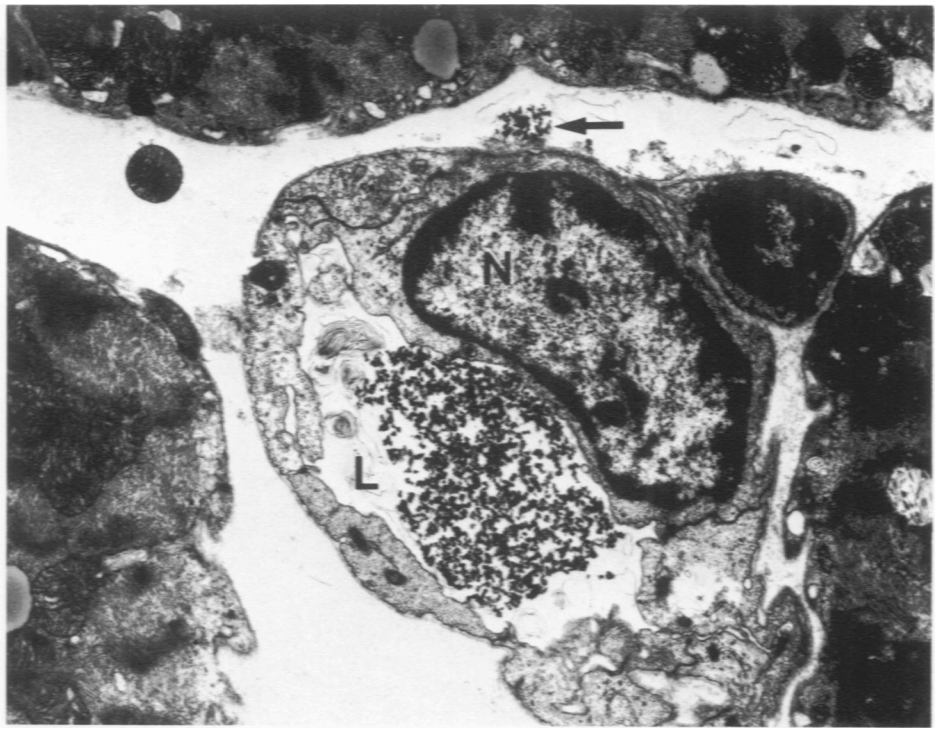

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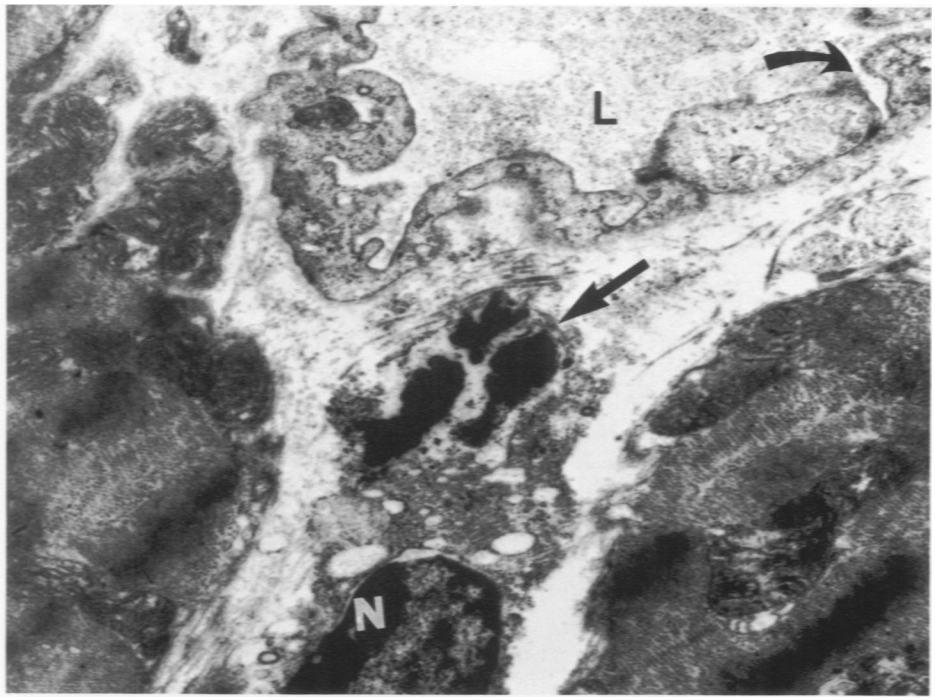

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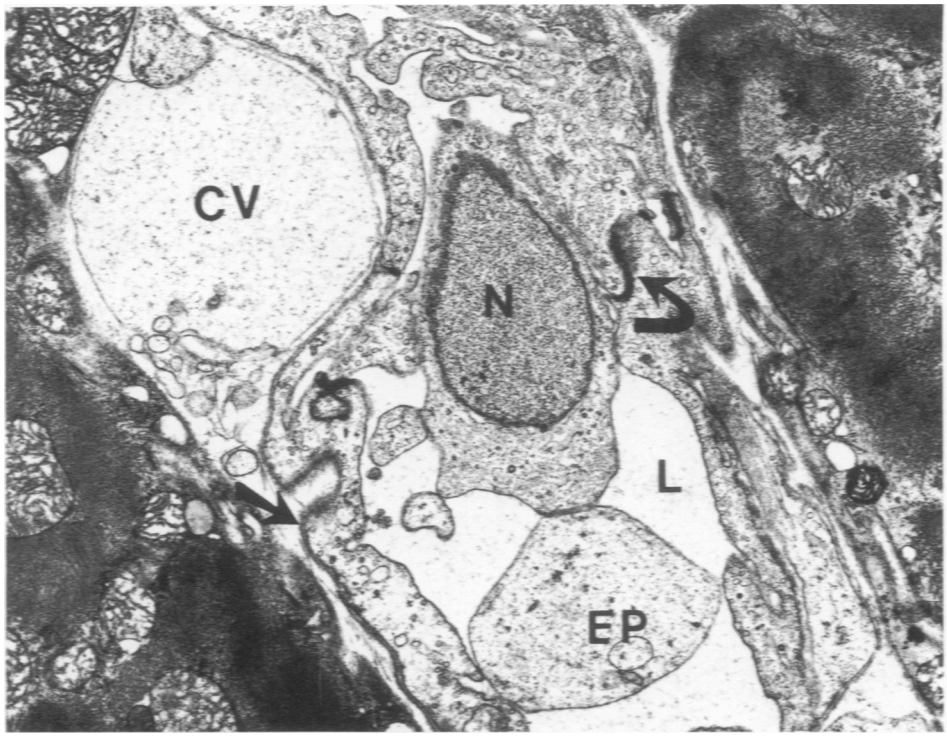
Fig 3—Myocardium 24 hours postirradiation (1300 rads) and 15 minutes after intravenous colloidal carbon injection. Carbon particles are seen within the lumen (*L*) and free in adjacent extravascular space (*arrow*). Nucleus (*N*) of an endothelial cell with condensation of chromatin and apparently intact interendothelial cell junctions (Uranyl acetate and lead citrate, × 12,000). **Fig 4**—Myocardium 72 hours postirradiation (1300 rads) and 1 hour after intravenous colloidal carbon injection. A perivascular phagocytic cell with nucleus (*N*) showing phagocytosed carbon (*arrow*). An adjacent capillary with lumen (*L*) shows a wide endothelial junction gap (*curved arrow*) (Uranyl acetate and lead citrate, × 10,500).



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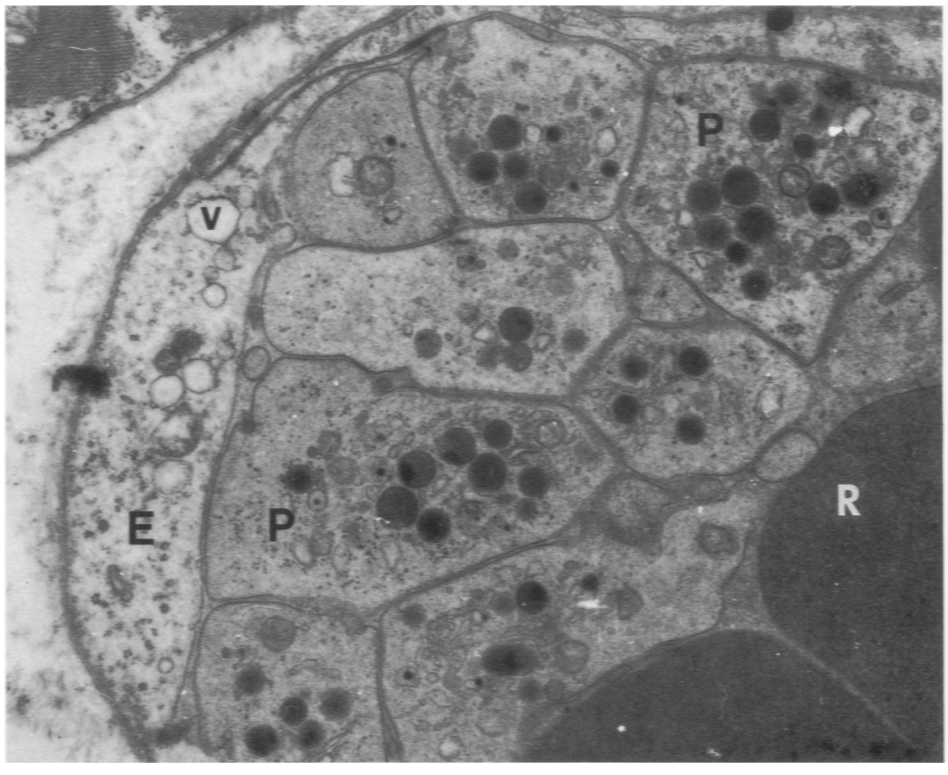

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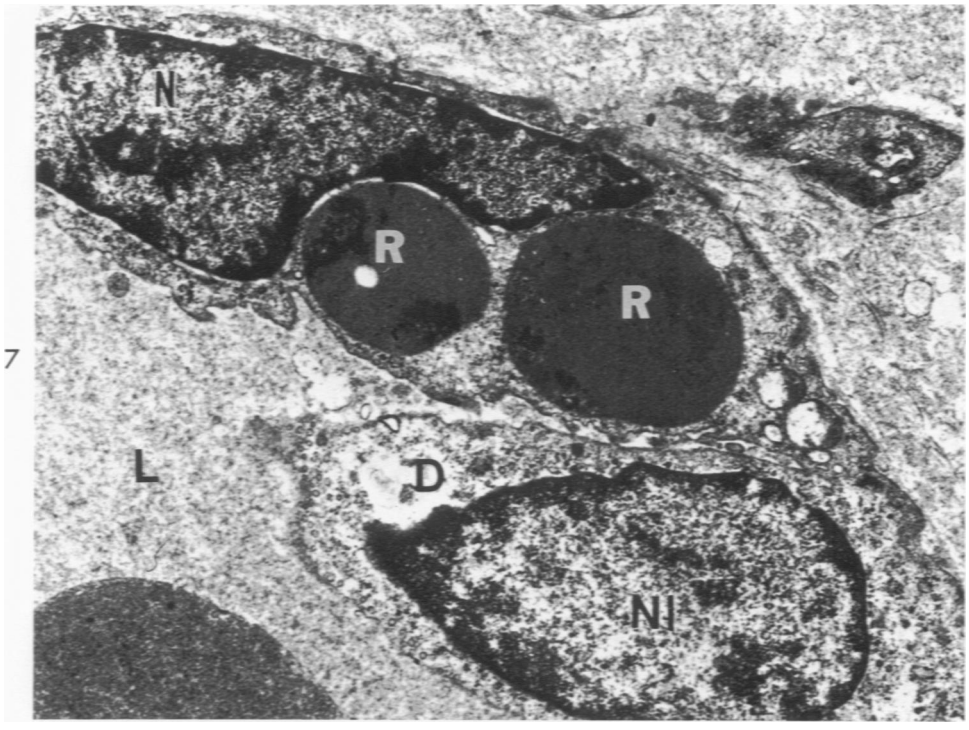
Fig 5—Myocardium 14 days postirradiation (1300 rads) showing capillary with focally thickened, densely staining basement membrane (*arrow*), endothelial cells with rich content of small membrane-bound vesicles, and marked endothelial cell projections and extrusions (*EP*) some of which are seen free within the lumen (*L*) along with a mononuclear cell with nucleus (*N*). An interendothelial junctional complex (*curved arrow*) shows thickening with increased electron density and focal pocketing. A swollen membrane-bound complex vesicular structure (*CV*) is also seen outside the blood vessel (Uranyl acetate and lead citrate, × 9500). **Fig 6**—Myocardial capillary 3 weeks postirradiation (1300 rads) showing red blood cells (*R*) and large number of partially degranulated, sequestered and clumped platelets (*P*) in its lumen. Endothelial cell (*E*) with dilated membrane-bound vesicles (*V*) (Uranyl acetate and lead citrate, × 12,500).



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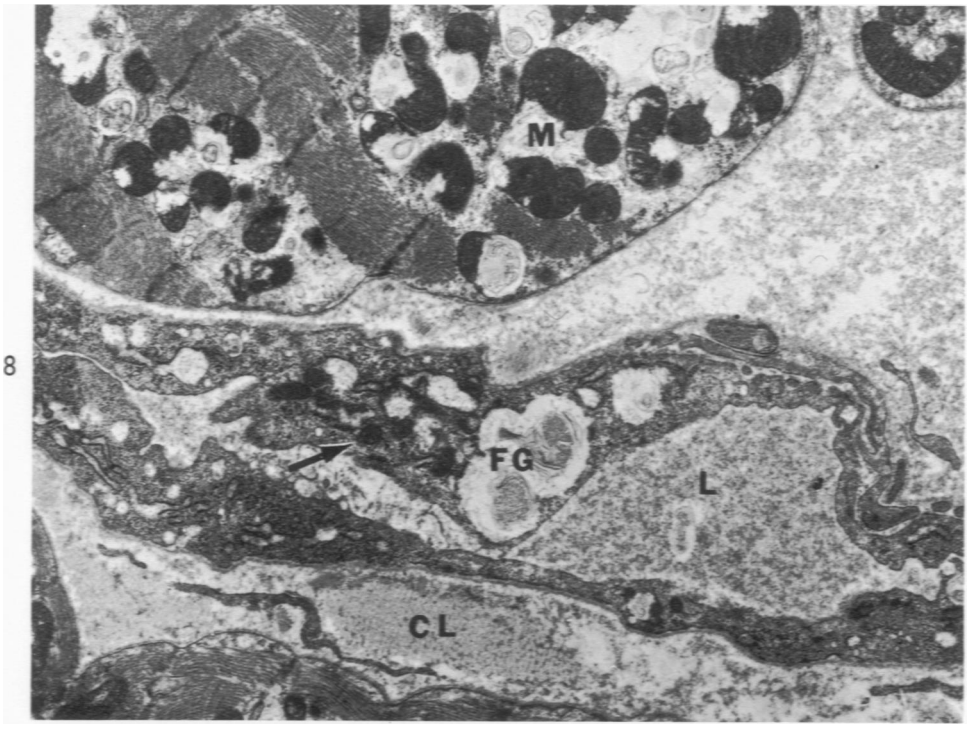

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Fig 7—Capillary from myocardium 4 weeks postirradiation showing erythrophagocytosis by an endothelial cell with nucleus (*N*) Adjacent endothelial cell with nucleus (*NI*) shows cytoplasmic vacuolation (*D*). *L*=lumen of the capillary (Uranyl acetate and lead citrate, × 9000). **Fig 8**—Myocardium 6 weeks postirradiation showing a capillary with lumen (*L*) and endothelial cell with myelin-like figures (*FG*) and palade bodies (*arrow*). Pericapil lary space contains collagen bundles (*CL*). Adjacent myocardial fibers show vacuolated mitochondria (*M*) and myofibrillar degeneration (Uranyl acetate and lead citrate, × 9400).