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Platelets in Hyperacute Rejection of Heterotopic Cardiac Allografts in Presensitized Dogs

An Experimental Study

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Experiments were carried out to determine whether platelet aggregation plays a primary role in cardiac allograft hyperacute rejection, as has been observed in renal allograft hyperacute rejection. Dogs were presensitized by multiple skin grafts before a cervical heterotopic cardiac allograft was placed. One group of dogs was treated with sulfinpyrazone, a platelet inhibitor, and another was not. In the majority of the untreated dogs, the cardiac transplants were rejected hyperacutely and showed, morphologically, platelet aggregation followed by vascular disruption. In the treated dogs, hyperacute rejection was prevented, but the cardiac transplants were later rejected by primary cellular rejection. These data and the results from the experiments of other researchers lead us to propose that platelets are the "effector" of hyperacute rejection (Am J Pathol 70:155–174, 1973).

KISSMEYER-NEILSON and co-workers [1] described extensive microthrombosis in renal capillaries with subsequent cortical necrosis in hyperacute renal allograft rejection. Sharma *et al* [2] showed platelet aggregation in renal capillaries early in the course of hyperacute rejection before morphologic evidence of vascular injury. Participation of platelets in rejection of transplanted kidneys in dogs presensitized

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with skin grafts has also been reported by Lowenhaupt and Nathan.[3] These reports indicate that intravascular platelet aggregation plays a primary role in hyperacute rejection of the kidney. To test this hypothesis, we have studied the hyperacute rejection in cardiac transplants. To further test the hypothesis we have used sulfinpyrazone (Anturan, Ciba-Geigy Laboratories, Ltd, Toronto, Canada) to inhibit platelet aggregation, to observe the effect on hyperacute rejection. The results indicate that platelets play the primary role as initiators of hyperacute rejection of cardiac allografts.

Materials and Methods

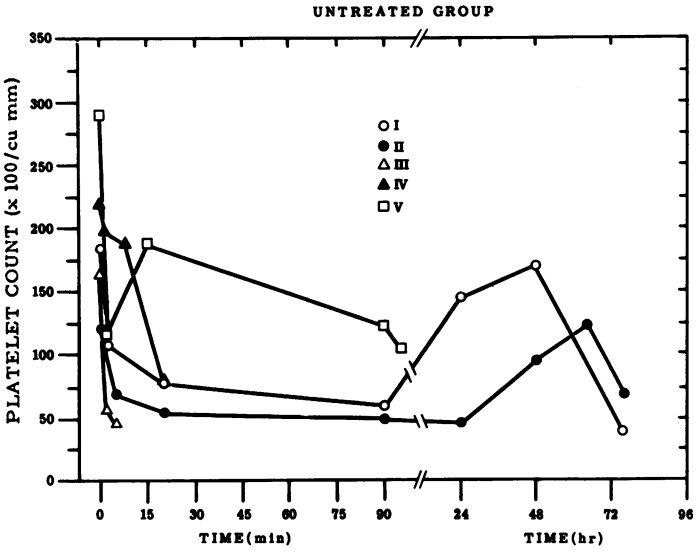
Healthy adult mongrel dogs, weighing between 25 and 30 kg, were used as recipients. The hearts for transplantation were taken from puppies weighing between 8 and 10 kg. The adult dogs were presensitized by three to four full-thickness skin grafts (1 inch × 1 inch) given in succession until the last graft was rejected as a white graft. The white graft rejection was taken as evidence of presensitization. Approximately 8 to 15 days after sensitization, the puppy hearts were transplanted into the neck of adult dogs. The donor heart was held in Ringer's solution at 4 C until transplanted. Before the vascular anastomoses were completed, the left ventricle and aorta were flushed with cold saline to remove trapped air. The anoxia time varied between 20 and 30 minutes. Of the 10 dogs with a heart transplant, 5 were given sulfinpyrazone and are referred to as the treated group, and the remaining 5 were given no drug and are referred to as the untreated group. The treated group of animals were injected intravenously with sulfinpyrazone, 125 mg/kg body weight, 1 hour before transplantation and maintained on a dose of 200 mg every 4 hours until the graft was rejected. The optimal platelet-inhibiting dose of sulfinpyrazone was determined previously.[2] The electric activity of the cardiac allografts was monitored by an electrocardiograph. The transplanted hearts were observed directly for 2 hours for signs of rejection before closure of the surgical wound. Serial determinations of external jugular vein and common carotid artery platelet, white blood cell (WBC) and red blood cell (RBC) counts were made before and up to 2 hours after transplantation. These counts were later done on peripheral venous blood. The donor hearts were biopsied immediately before transplantation and following transplantation at intervals of 1, 5 and 10 minutes, 1 and 2 hours and at the time of rejection for light, phase, immunofluorescent and electron microscopic studies. At rejection of the cardiac allograft, both the recipient and donor hearts were removed and weighed. The extramural coronary arteries were dissected from the epicardium and serially sectioned and examined for thrombosis. Tissue for light microcsopy was fixed in 10% buffered formalin, embedded in paraffin, cut at 4 to 6 μ and stained with hematoxylin and eosin. Tissue for immunofluorescence was rapidly frozen in dry ice and isopentane at −70 C and stored at −70 C until the end of the experiment. Staining was performed for IgG and β1C. Rabbit anticanine IgG (Hyland lot 8138H-002 A) was checked for purity by immunoelectrophoresis and for strength by Ouchterlony technics. Fluorescein isothiocyanate (FITC) tagging was performed and a fluorescein to protein (F:P) ratio of 2.6 was obtained. Rabbit anticanine β1C was prepared and kindly supplied to us by Dr. M. Mandl and Dr. B. Rose, Immunopathology Laboratory, Royal Victoria Hospital, Montreal, Canada. Immunoelectrophoresis, Ouchterlony and tagging were performed and

an F:P ratio of 1:1 was achieved. In view of a low Ouchterlony titer, the β1C antiserum was used full strength, whereas the IgG antiserum was used in dilution of 1:4. All samples obtained from a single animal were processed simultaneously, thus enabling us to compare intensity of staining at different times, before and after transplantation. Staining intensity was graded from negative to 4+. Sections were examined with a Leitz Orthoplan Microscope equipped for fluorescence with HBO-200 light source, using BG 12 exciter (5 mm) filters with Barrier filter K 510. Anscochrome 500 film was used with Orthomat Camera equipment. Tissues for electron microscopic study were fixed in 5 to 6% phosphate-buffered glutaraldehyde, washed in phosphate buffer (pH 7.4), postfixed in 1% osmium tetroxide, dehydrated and embedded in Epon. Toluidine blue-stained sections, 0.5 to 1μ thick, were examined with a phase microscope to select areas for ultrastructural study. Thin sections were cut on a Reichert's ultra microtome Om U_2 and on a Porter-Blum MT-2 microtome, stained with uranyl acetate and lead citrate and examined in a Philips-300 electron microscope.

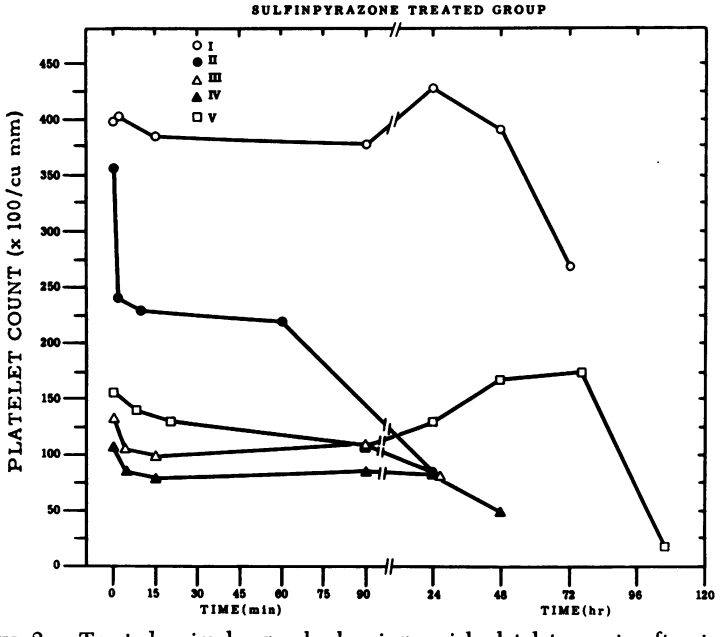
Results

The clamps were removed slowly from the transplanted heart with simultaneous warming, using lukewarm saline. As the coronary circulation improved, the myocardium warmed, and the heart started to beat rhythmically. In an occasional case, one or two electric shocks were necessary to defibrillate the heart. Rejection of the heart occurring while the surgical wound was still open was a dramatic event. The heart became cyanotic and dilated, with a reduced contraction rate, and eventually turned bluish black in color as contraction ceased. On gross examination, the anastomotic sites were patent and extramural coronary arteries were free of thrombi. The transplants in the 5 untreated dogs functioned for varying periods: 5 and 20 minutes and 5½, 76 and 80 hours. The transplants in the 5 treated dogs functioned for the following times: 26, 40, 48, 104 and 110 hours. There was progressive diminution in the platelet counts in the untreated group, except for the 2 dogs where the hearts functioned for 76 and 80 hours, in which there was a tendency for the counts to recover; however, their counts fell terminally (Text-figure 1). In the treated group, there was an insignificant initial platelet-count drop; however, at rejection the platelet counts dropped significantly (Text-figure 2). No significant changes were noted in WBC or RBC counts in either group.

The only morphologic change noted in the heart before transplantation was mild swelling of the mitochondria. The capillary lumens were patent, and there was no evident vascular endothelial damage. Three of the five cardiac transplants in untreated dogs were rejected within 5½ hours; two functioned for 76 and 80 hours. Based on the different function time and anatomical changes, the untreated group could be divided into two subgroups: the three hearts which were rejected within 5½ hours represent hyperacute rejection; two hearts



TEXT-FIG 1—Untreated animals; graph showing serial platelet counts after transplantation. Note the dramatic fall of platelet counts after transplantation. Dog 1 and 2 show recovery of platelet counts but terminally they are also at a lower level. (I–V = No. of dog).



TEXT-FIG 2— Treated animals; graph showing serial platelet counts after transplantation. Note that only a mild initial drop in platelet counts occurred after transplantation, and the major drop in platelet counts at rejection. (I–V = No. of dog).

which were rejected in 76 and 80 hours represent acute rejection. In the treated dogs the morphologic changes were similar to the acute rejection in the untreated group. The recipient hearts examined at the time of rejection of the donor hearts showed no morphologic abnormalities.

Untreated Animals

Hyperacute Rejection

Light microscopic examination of paraffin-embedded tissue showed diffuse interstitial edema and hemorrhage. Plastic-embedded tissue examined by phase microscopy showed platelet aggregates in numerous cardiac capillaries as early as 1 minute posttransplantation. Ultrastructural study of sections taken 1 minute posttransplantation confirmed the phase microscopy observation. At this time there was no morphologic evidence of endothelial damage (Figure 1); however, there was mild swelling of the mitochondria not different from pretransplant mitochrondrial change. Platelets in the aggregates observed 1 minute after transplantation had intact granules. Degranulation was observed first in platelets near the endothelium and later throughout the aggregate (Figure 2). Following degranulation, pseudopods of the platelets were seen penetrating the endothelium and its basement membrane into the perivascular tissue space (Figures 2 and 3). Platelet fragments and free platelet granules were seen outside the vessel wall with red blood cells (Figures 2 and 3). Focal areas of the capillary endothelium were swollen and disrupted. Simultaneous with platelet degranulation and migration, fibrin was evident among the platelets aggregated in capillaries (Figure 3). In capillaries filled with fibrin, platelets often were barely discernable (Figure 4). Occasional sections showed leukocytes in the capillary lumen (Figure 5). Biopsies taken during the course of rejection did not show arteries; arteries were available for study in the hearts only after cessation of function. The arteries at this time showed platelet aggregates and endothelial disruption similar to that in capillaries (Figure 6).

Acute Rejection

Cardiac biopsies taken up to 2 hours posttransplant appeared normal by light microscopy. Electron microscopic examination, however, showed occasional platelet aggregates in capillaries without evidence of endothelial damage. Biopsies taken at rejection, 76 and 80 hours, showed perivascular lymphocyte and plasma cell aggregates and

interstitial hemorrhage, necrosis and edema. The blood vessels in the center of the cellular aggregates showed mural fibrinoid necrosis and lumen thromobosis. Electron microscopic studies showed in addition infiltration of the blood vessel wall with leukocytes (Figure 7).

Treated Animals

Acute Rejection

The morphology of acute rejection in the treated group was the same as that observed in the 2 untreated dogs with transplants surviving for 76 and 80 hours. Figure 8 depicts the occasional capillary observed up to 2 hours after transplantation that had platelet aggregates.

Immunofluorescent Studies

All cardiac biopsies taken before transplantation were negative for IgG and β1C staining. After transplantation in both untreated and treated dogs, IgG staining was positive as early as 5 to 10 minutes posttransplant. The staining was seen as granular deposits on the wall of capillaries and on the sarcolemma. The staining intensity increased in later biopsies. The acute rejection biopsies taken at the time of graft rejection also showed staining in the cytoplasm of the infiltrating plasma cells. β1C staining was negative or faintly positive in the same areas which were positive for IgG, except in the heart of the untreated dog that functioned for 80 hours which showed a definite positive staining reaction. The recipient hearts examined at the time of cessation of function of the donor heart did not show IgG or β1C staining.

Discussion

Allograft rejection in the untreated dogs showed two functional and morphologic patterns. Those dogs rejecting within a few minutes to a few hours were characterized by rapid cessation of function, mottled cyanotic color and flabby consistency of the allograft; this was called hyperacute rejection. In 2 untreated dogs, the allografts functioned for a longer period of time, 76 and 80 hours; this was called acute rejection. In the treated group, all the allografts functioned for more than 24 hours and were called acute rejection. There was a distinct difference in the morphology of hyperacute and acute rejection. Hyperacute rejection was characterized by extensive platelet accumulation in the microvasculature of the heart before vascular endothelial damage was evident. Later, the platelets showed degranulation, pseudopod extension through the vessel with vascular disruption, and hemorrhage. The progressive morphologic changes correlated with the progressive

diminution of the peripheral blood platelet count. Acute rejection was characterized by periarterial lymphocytic and plasma cell infiltration with vascular necrosis and thrombosis. One might infer from the above data that acute rejection is prolongation of the process of hyperacute rejection. This inference is not correct because hyperacute rejection seen after prior sensitization occurs immediately after transplantation, and circulating antibodies directed against the donor antigen can be demonstrated, as measured by lymphocytotoxicity.[4-8] Whereas acute rejection does not involve prior sensitization, does not occur immediately after transplantation and shows a distinctly different morphology.[9-13]

Hyperacute rejection in cardiac transplants is similar to hyperacute rejection of kidney allografts described by Sharma *et al*,[2] Lowenhaupt and Nathan,[3] Lund and Ahrous [14] and Kissmeyer-Nielson *et al.*[1] Evidence of the participation of platelets in hyperacute rejection of renal transplants and the present study with cardiac transplants leads us to propose that the platelet is the "effector" of hyperacute rejection. This proposal is supported by the fact that sulfinpyrazone, a platelet antiaggregator, could prevent hyperacute rejection. A similar observation was made in hyperacute renal allograft rejection which also was prevented by a platelet inhibiting agent, sulfinpyrazone.[2] That platelets accumulate before fibrin formation, as seen in our experiment, is in keeping with the findings of Lowenhaupt *et al*,[15] in which the use of an antifibrinolytic agent prevented fibrin deposition, while platelet accumulation occurred. Their findings suggested that fibrin formation is not an essential step in the early stage of accelerated graft rejection. Similarly, MacDonald *et al*,[16] using Arvin, were unable to prevent hyperacute rejection, even though defibrination occurred. Also, Rosenberg *et al* [17] were unable to prevent hyperacute rejection of renal heterografts by heparinization. These data support the hypothesis that platelet aggregation is the initial morphologic change in hyperacute rejection.

It is very clear from the experiments that platelets aggregate in the process of hyperacute rejection, but the cause for this aggregation is unknown. The recipient in the present experiments was presensitized by multiple skin grafts (as has been done in the experiments with renal transplants)[2] which probably led to formation of circulating antibodies against the vascular membrane HLA antigens. We hypothesize that these circulating antibodies in the recipient form complexes with the vascular antigen of the grafted organ. This hypothesis is supported by granular staining for IgG in the cardiac grafts. These antigen-

antibody complexes then lead to platelet aggregation. That platelets aggregate on antigen-antibody complexes is supported by the experiments of Mustard.[18] Margaretten and McKay [19] also clearly showed the requirement for platelets in the active Arthus reaction in rabbits. There is extensive evidence that γ-globulin, in the form of antigen-antibody complexes on surfaces, will cause platelets to release their constituents.[20–27] The vasoactive substances and lysozymes released from platelets [22,28–33] lead to vascular damage and graft rejection. The platelet-effector hypothesis is supported by our morphologic data, platelet counts and the prevention of hyperacute rejection by sulfinpyrazone, a platelet aggregation inhibitor.

To determine whether complement fixation plays a role in cardiac hyperacute rejection we used β1C antiserum. The results were negative in all dogs, except one representing acute rejection. Reports concerning the role of complement in platelet aggregation that is associated with release of pharmacologically active substances are not conclusive.[34] Studies by Mueller-Eckhardt and Lüscher,[24] using suspensions of washed human platelets, failed to show any evidence that complement was involved in antigen-antibody mediated platelet release reaction. Pariyananda and Mowbray [35] have also reported a series of experiments which indicate that complement is not involved in the interaction of platelets with γ-globulin. There is evidence that complement is present in platelets,[27,36] but it is not known whether it is involved in aggregation and release reactions. It is possible that the reagents we used were not strong enough, or that complement is not required in antigen-antibody mediated platelet aggregation. In this regard, our data are insufficient for the formation of any conclusions. The heart in 2 untreated dogs was not rejected hyperacutely but, on the contrary, showed the features of primary cellular rejection, as has been described by others,[9–13] with lymphocytic and plasma cell periarterial infiltration. The absence of hyperacute rejection in the 2 untreated dogs may be related to low level of cytotoxic antibodies which were not determined in our experiments, or it may be an indication that transplantation antigens responsible for AG-Ab complexes vary in their capacity to cause platelet aggregation. The first explanation seems more likely.

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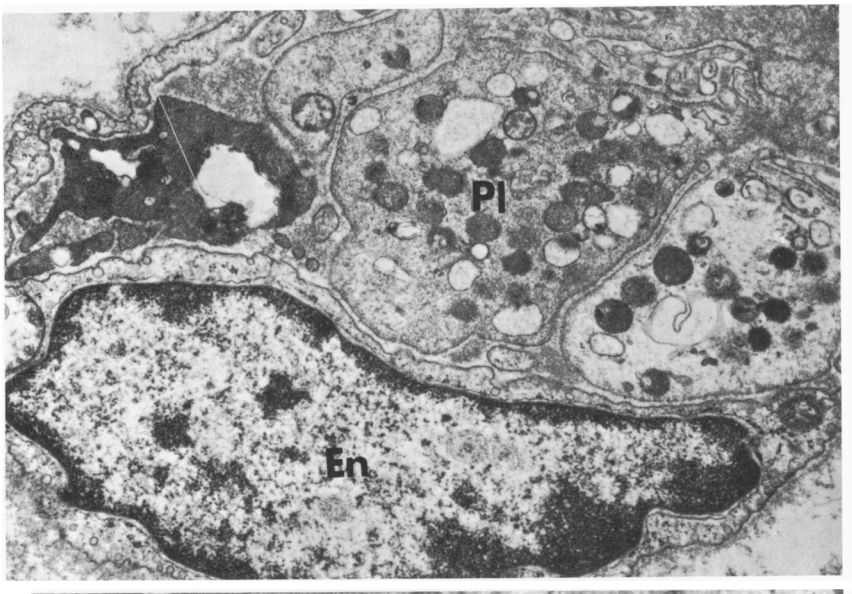
[*Illustrations follow*]

All electron micrographs were taken from sections stained with uranyl acetate and lead citrate.

Fig 1—Untreated animal; cardiac biopsy 1 minute posttransplant, showing platelets *(Pl)* in the capillary lumen. The vascular endothelium *(En)* is intact (× 6647).

Fig 2—Untreated animal; cardiac biopsy 5 minutes posttransplant. Platelets *(Pl)* filling up the capillary lumen with degranulated platelets close to vascular endothelium. See also extension of platelet pseudopod through the capillary wall *(arrow)*. Red blood cells *(RBC)* and degranulated platelets *(Dpl)* lying outside the capillary lumen (× 9400).



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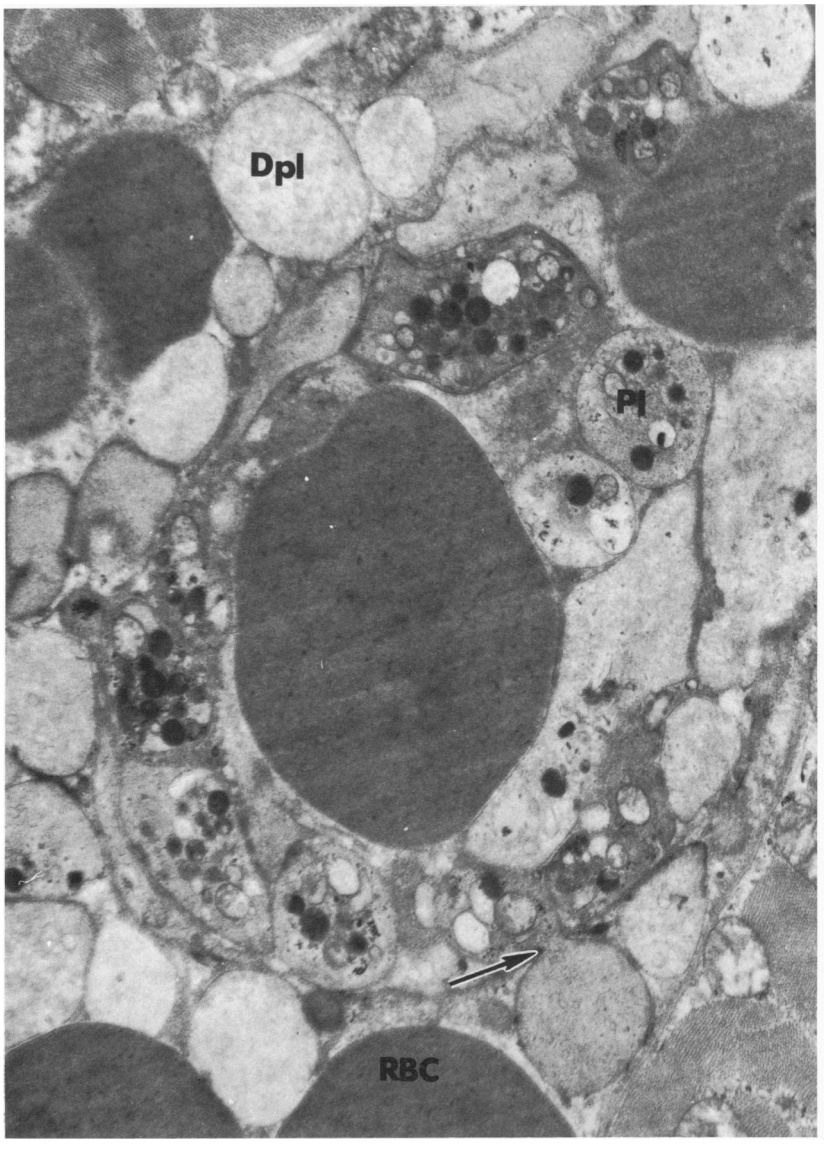

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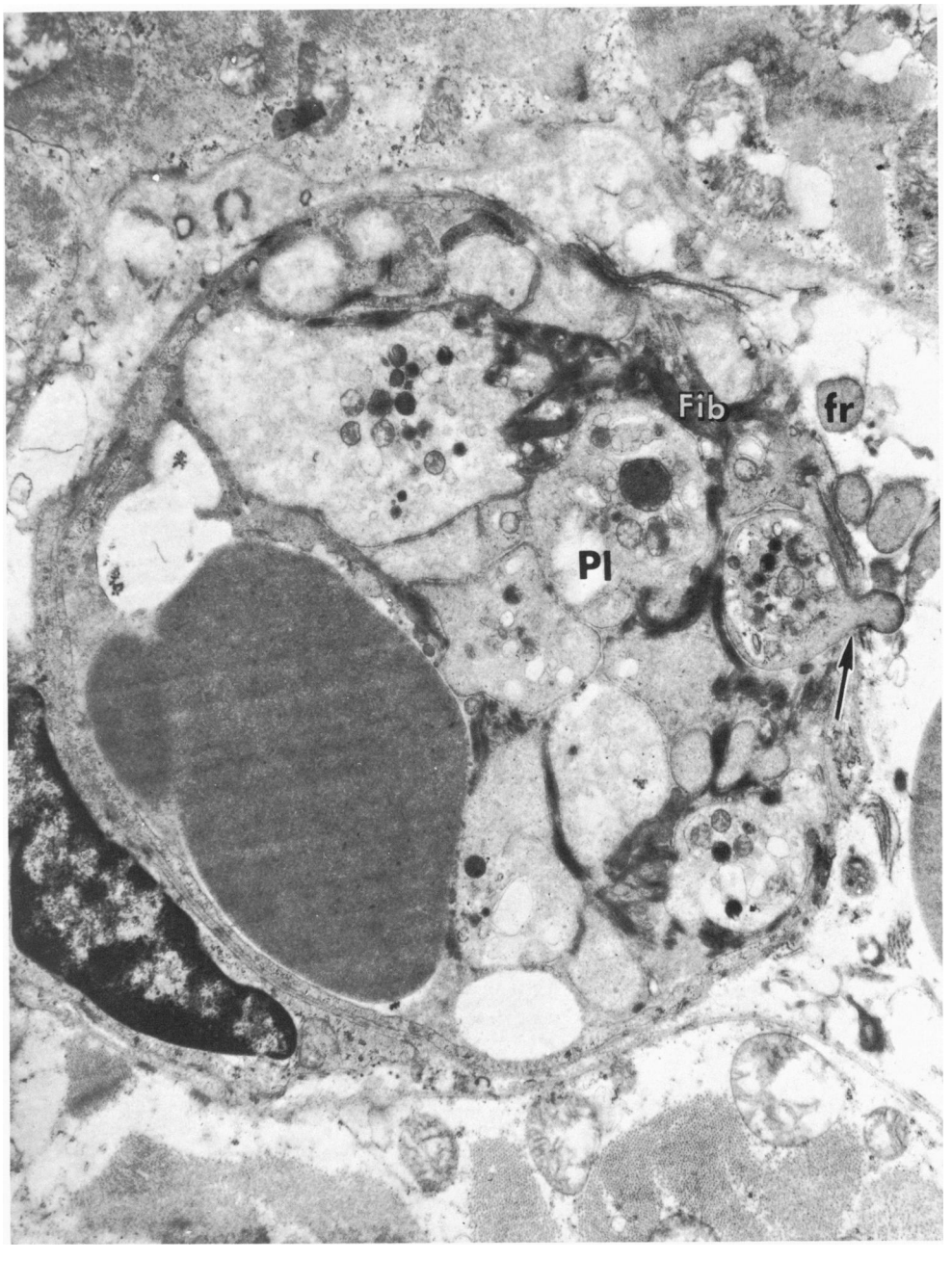

Fig 3—Untreated animal; cardiac biopsy 10 minutes posttransplant, showing platelet clump (*Pl*) and fibrin (*Fib*) in capillary lumen. Platelet pseudopod is extending out through the vessel wall (*arrow*) and few platelet fragments (*fr*) are lying outside capillary (× 10,790).

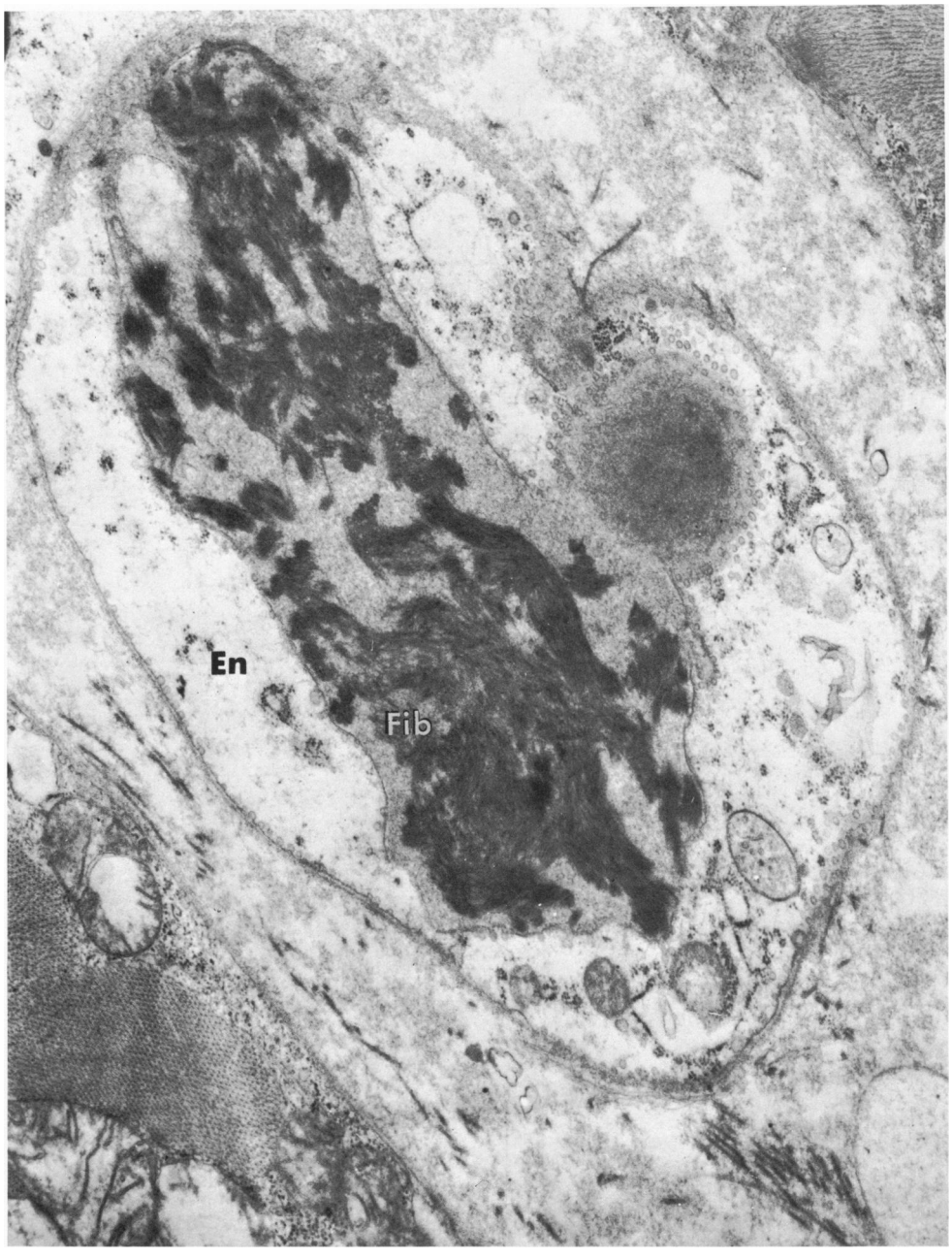

Fig 4—Untreated animal; cardiac biopsy 20 minutes at rejection. Capillary lumen is full of fibrin (*Fib*). See also swollen endothelium (*En*) (× 2188).

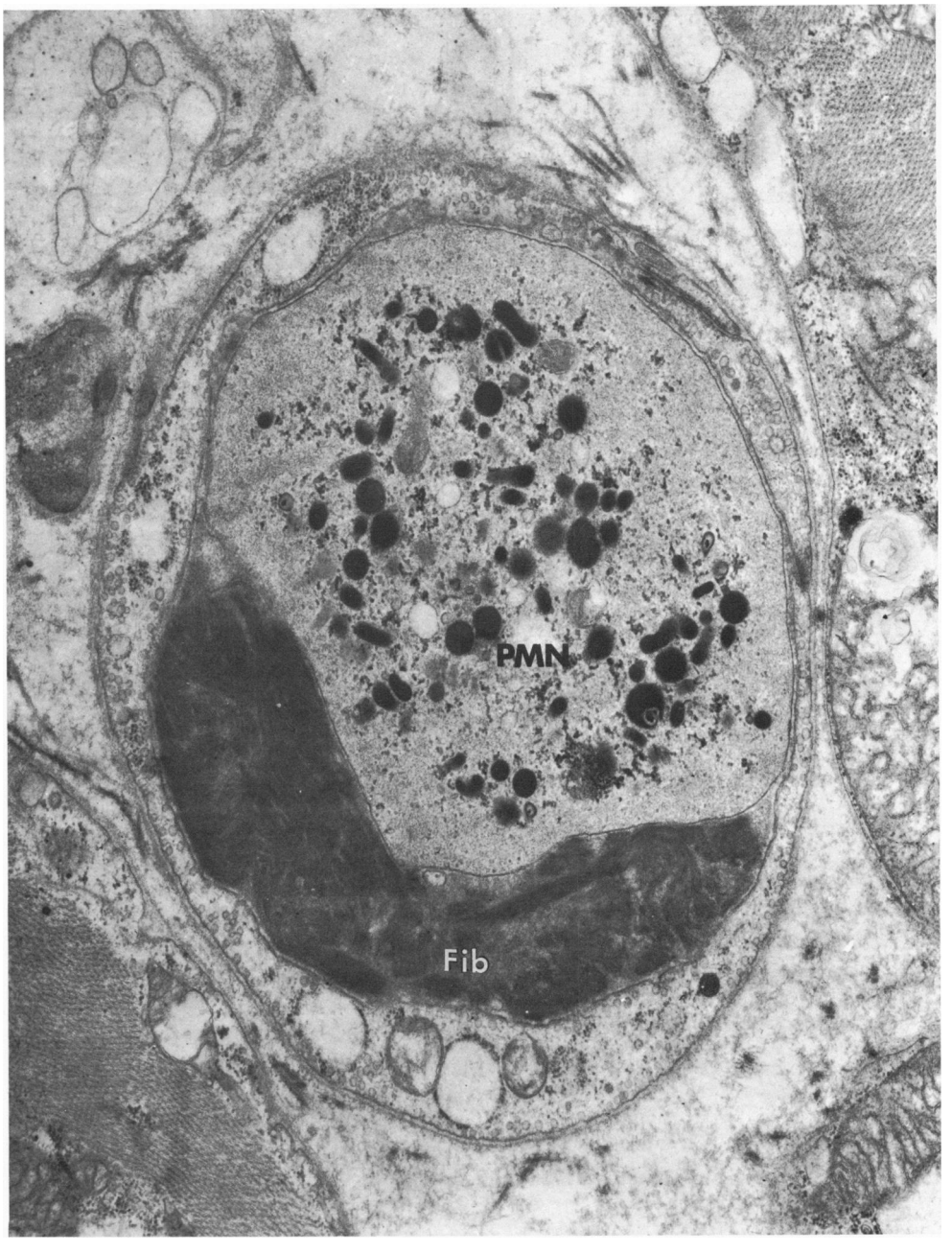

Fig 5—Untreated animal; cardiac capillary at rejection; 20 minutes posttransplantation. Exhibiting extensive fibrin (*Fib*) formation and a leucocyte (*PMN*) in the lumen (× 19,325).

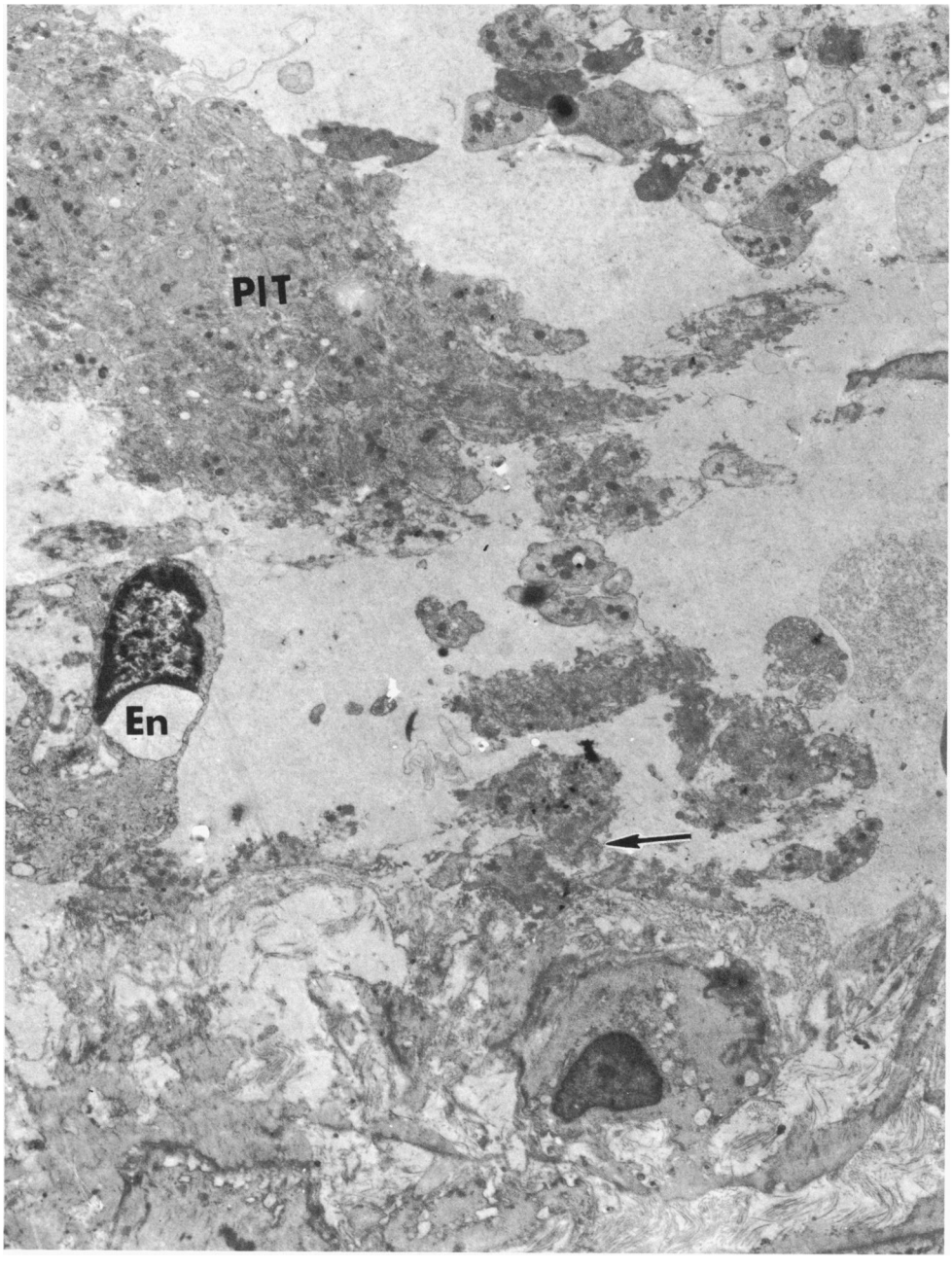

Fig 6—Untreated animal; branch of coronary artery at rejection 5 minutes posttransplantation, showing extensive platelet thrombosis (*PIT*). The vascular endothelium (*En*) is lifted up and the platelets are present on the denuded intimal surface (*arrow*) (× 10,665).

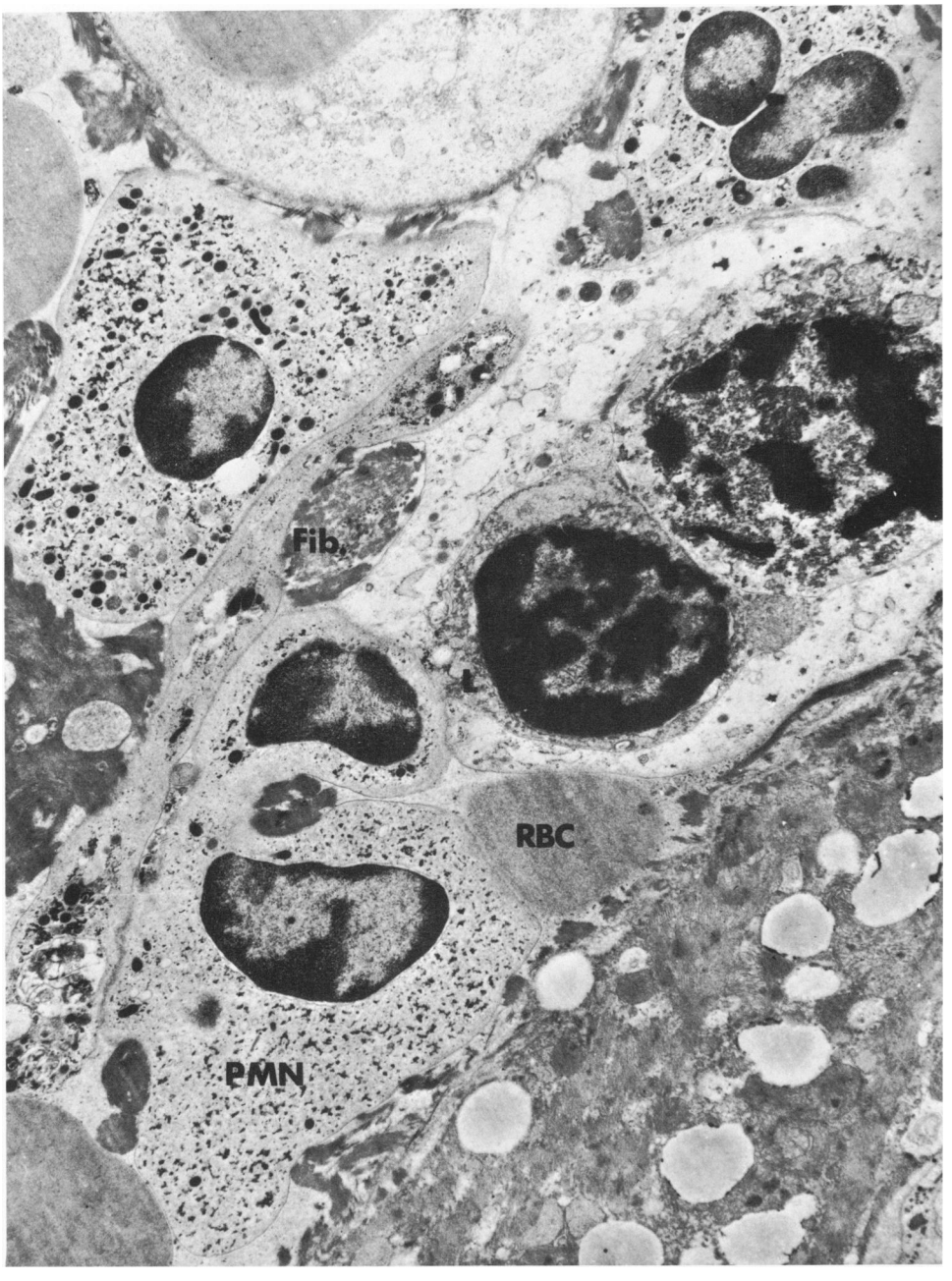

Fig 7—Untreated animal; cardiac tissue at rejection, 6 days posttransplantation; exhibiting vascular disruption with fibrin formation (*Fib*) and presence of polymorphonuclear leucocytes (*PMN*) and lymphocytes (*L*) and hemorrhage; R = red blood cell (× 7571).

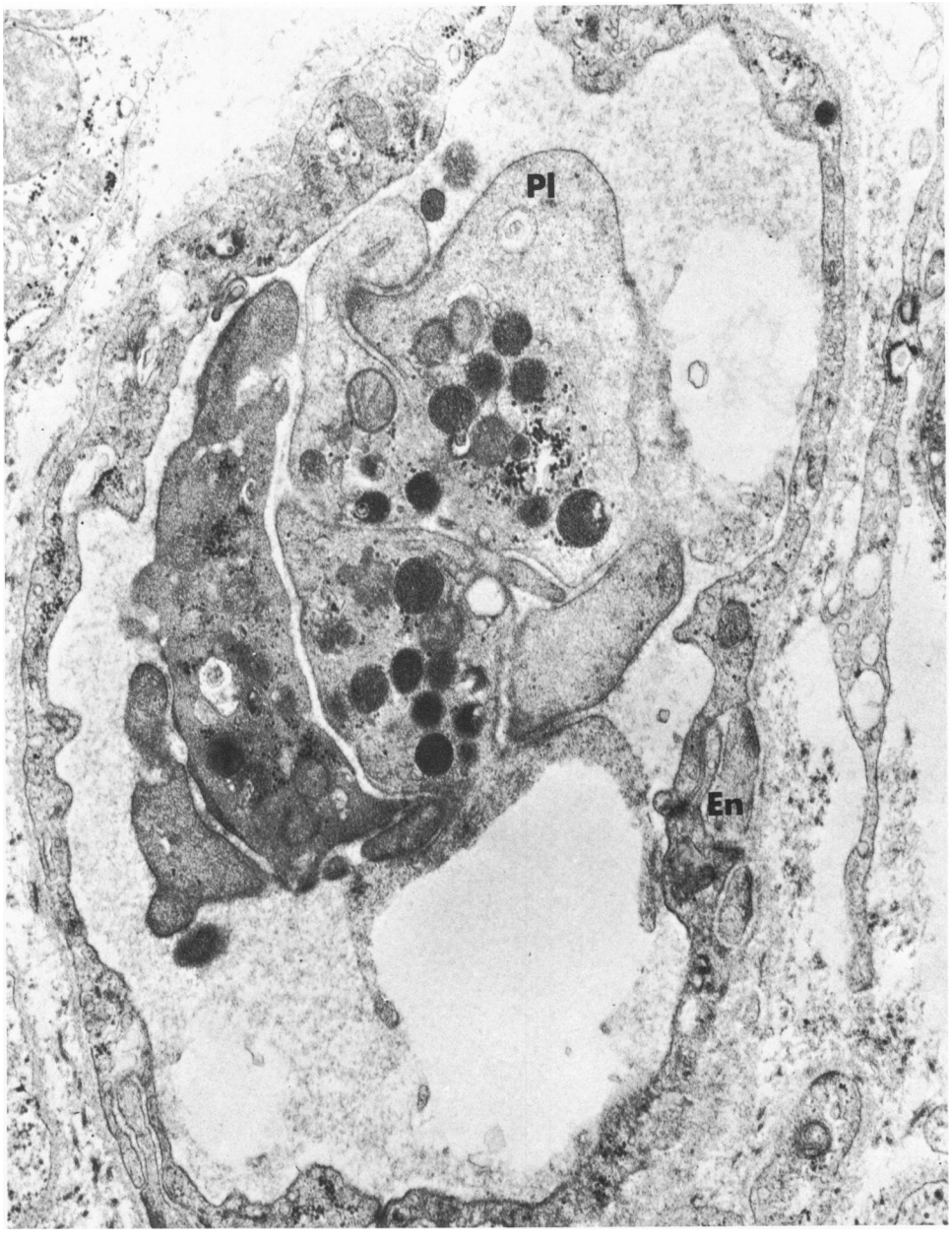

Fig 8—Treated group; cardiac biopsy 2 hours posttransplantation. Capillary lumen exhibiting few platelets (*Pl*); *En* = Endothelium (× 25,000).

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