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Quantitation of Platelets in the Microcirculation. Measurement of Indium-111 in Microthrombi Induced in Rabbits by Inflammatory Lesions and Related Phenomena[1] (41002)

BRIAN J. JEYNES, ANDREW C. ISSEKUTZ,[2] THOMAS B. ISSEKUTZ,[3] AND HENRY Z. MOVAT[4]

Division of Experimental Pathology, Department of Pathology, Medical Sciences Building, University of Toronto, Toronto, Ontario, M5S 1A8, Canada, and, The Izaak Walton Killam Hospital for Children, Dalhousie University, Halifax, N.S., Canada B3J 3G9

Abstract. The use of platelets labeled with ^{111}In-oxine for *in vivo* studies of thrombosis was examined. Platelets were separated from the blood of rabbits, labeled *in vitro* with ^{111}In, and returned to the animals by intravenous injection. In some animals repeated blood samples showed that the ^{111}In platelets gradually declined in the blood reaching half their initial concentration in 48 hr. In other rabbits, inflammatory lesions were produced in the skin of the back by the intradermal injection of killed *Escherichia coli,* or endotoxin, producing a local Shwartzman reaction. One hour after the iv injection of ^{111}In platelets the animal was killed, the inflammatory lesions were removed, and the radioactivity in them was determined. ^{111}Indium accumulated in *E. coli* lesions within 1 hr after the injection of bacteria. The maximum rate of accumulation occurred in lesions which were 3–4 hr old, but some ^{111}In platelets continued to accumulate in lesions that were 8–24 hr of age. The local Shwartzman reaction produced extensive thrombosis. This was demonstrated by a large accumulation of ^{111}In-labeled platelets, and by the observation of numerous platelet-containing thrombi in histological sections of the lesions. The findings demonstrate that ^{111}In can be used to label platelets for the *in vivo* quantitation of thrombosis in inflammatory tissue.

Indium-111 oxine, a lipid-soluble complex, has been shown to be useful for labeling of leukocytes and platelets. It is superior to ^{51}Cr as a radiolabel because of its γ-emission characteristics and high cell labeling efficiency (1–6). Upon decay ^{111}In emits γ-photons at two energy levels, 173 keV and 247 keV, with 84 and 94% abundance, respectively. This allows easy external detection by either γ-camera or rectilinear scintiographic techniques (1, 4, 5). Its half-life of 67 hr is ideally suited to kinetic and distribution studies. In general ^{111}In has been found to be nontoxic both in terms of the rad dose in the labeled cells (6–10), and in terms of the effects of the oxine and ethanol involved in the labeling procedure (6, 8). There may be some toxicity, however, associated with chelated metal ions (8). Further, high labeling efficiency can be achieved (1, 2, 5, 8, 11, 12) and both low elution (2, 5, 11–13) and maintenance of cell function (2, 4–6, 11, 12) are consistently reported, at acceptable experimental doses. ^{111}In-labeled platelets have already been used to study the presence and distribution of venous (3, 4), cerebral (14), and coronary (15) arterial thrombosis, as well as platelet kinetic studies (4).

It is now possible to study the kinetics of the acute inflammatory reaction (16), by quantitating the infiltration of neutrophils (17, 18), the amount of hemorrhage (19), the increase in vascular permeability (20), and alternations in blood flow in the microcirculation (21). However, one of the complications of an inflammatory reaction is

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[3] Postdoctoral Fellow of the MRC of Canada.

[4] Career Investigator of the MRC of Canada.

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thrombosis of the microcirculation. Hemorrhage is often associated with bacterial inflammatory lesions and with lesions such as the local Shwartzman reaction (19). Thrombi in the microcirculation may also be present in both these reactions. Iodine-125-labeled fibrinogen could be used to measure thrombosis but fibrinogen is deposited extravascularly as well as intravascularly. Therefore we attempted to label platelets with ^{111}In and measure thrombi within the microcirculation during the development of various inflammatory lesions.

Materials and methods. In vitro platelet labeling. Blood (30–60 ml) was collected from the central ear artery of rabbits into buffered 3.8% sodium citrate (pH 6.5). The blood was sedimented first at 300*g* for 10 min at 4° and the platelet-rich plasma was removed. After recentrifugation at 2400*g* for 2½ min additional platelet-rich plasma was separated and added to that obtained with the first centrifugation (M. A. Packham—personal communication). The platelet-rich plasma was recentrifuged at 1000*g* for 10 min, the plasma was removed and set aside, and the pellet of platelets was resuspended in pyrogen-free Tyrode solution, free of Ca^{2+} and Mg^{2+} and containing 20% platelet-poor plasma. This was incubated with 10 μCi of pyrogen-free ^{111}In–oxine (Diagnostic Isotopes, Inc., Bloomfield, N.J.) for 10 min at room temperature. The platelets were again centrifuged at 1000*g*, resuspended in the original platelet-poor plasma (which binds avidly free ^{111}In), and centrifuged at 1000*g*. The pellet of platelets was resuspended in 2 ml Ca^{2+} and Mg^{2+}-free Tyrode's and injected intravenously into rabbits.

In vivo sampling. Platelets collected from 30 ml of blood were labeled with ^{111}In as described above, and injected into the marginal ear veins of four rabbits. At various intervals, samples of 4.5 ml blood were taken from ear veins, 2 ml (in duplicate) were placed into polypropylene tubes (75 × 11 mm), centrifuged at 2500*g*, and the plasma was separated from the formed elements. Radioactivity was determined in a Nuclear Chicago Mark II γ-spectrometer at a window setting of 70–500 keV. All the samples and aliquots from the injected labeled platelets were counted together at the end of each experiment, and the results were corrected for the isotope decay back to the time of labeling of the platelets.

Elicitation of skin lesions. Two types of leisons were elicited. Inflammatory lesions were produced as described before (16). Briefly, the hair of the back of 2–2.5 kg New Zealand white rabbits was clipped and 6×10^8 Formalin-killed *Escherichia coli* (BAM strain, Medical Teaching Laboratories, University of Toronto, Toronto, Ontario, Canada) were injected intradermally (0.2 ml) at the following times prior to sacrifice: 24, 8, 6, 4, 3, 2, and 1 hr. One hour before sacrifice the animals received a suspension of ^{111}In-labeled platelets as described above. The fate of the platelets in the circulation was followed by taking blood samples at 5 and 50 min after the infusion of the platelet suspension. After killing the rabbits with an overdose of sodium pentobarbital, the skin of the back was removed, the visible blood was expressed manually from the large vessels (19), and the lesions were punched out with a cork borer (1.5 cm in diameter). The disks of skin were counted in the γ-scaler. Four to five saline-injected sites were also punched out and counted.

Local Shwartzman reactions were elicited as follows: Rabbits were given 20, 10, 5, 2.5, and 1.25 μg of *Serratia marcescens* endotoxin (3130-25B, Difco, Detroit, Mich.) intradermally into four to five sites per dose and 18 hr later were challenged iv with 100 μg of the same endotoxin. They were killed 6 hr later with an overdose of sodium pentobarbital. As with the *E. coli* lesions, the lesions were punched out with a standard cork borer and discs of skin and appropriate controls counted for radioactivity.

Results and Discussion. Figure 1 shows the concentrations of ^{111}In in the blood over a period of 48 hr following a single injection of labeled platelets. The plasma contained approximately 4–6% of the radioactivity in the blood, with the remaining 94–96% being associated with platelets. Based on the estimated blood volume of the rabbits, greater than 90% of the ^{111}In-labeled platelets were circulating 1 hr after injection. There was a gradual disappearance in

the labeled platelets from the blood, that reached one-half of the immediate postinjection value at about 48 hr. These findings are in agreement with those previously reported for platelets in the rabbit using ^{51}Cr (1, 22). Although it should be pointed out, that due to the small error (5%) in measuring the radioactivity in the blood, it was impossible to determine whether the disappearance of ^{111}In platelets in the first 48 hr was exponential with a $t½ = 48$ hr or linear.

In a group of three animals thrombosis in response to the id injection of *E. coli* was measured. As described above, bacteria were injected at various times and 1 hr before sacrifice the rabbit received iv ^{111}In-labeled platelets. Because each lesion regardless of age is exposed to labeled platelets for only 1 hr, the radioactivity in any given lesion approximates the rate of thrombus formation, rather than the total platelet accumulation up to that time.

The results of a typical experiment are shown in Fig. 2. Detectable platelet accumulation was measurable by 1 hr after the injection of *E. coli*. The maximum rate of platelet accumulation occurred in the 3- to 4-hr-old lesions. In addition, even in lesions that were nearly 24 hr old at the time the rabbit was labeled, there was still ongoing platelet accumulation. Grossly, the lesions older than 3–4 hr showed redness due to hemorrhage (19).

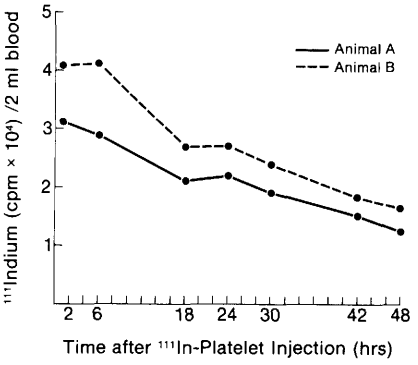

FIG. 1. Disappearance of labeled platelets from rabbit blood. Platelets from 30 ml of blood were labeled with 2.8×10^6 cpm and injected iv at zero time. Samples of 4.5 ml of blood were taken at the times indicated and two aliquots of 2 ml each were centrifuged and the radioactivity was determined in the plasma and the formed elements. The ordinate (cpm of ^{111}In) refers to the formed elements in 2 ml of blood.

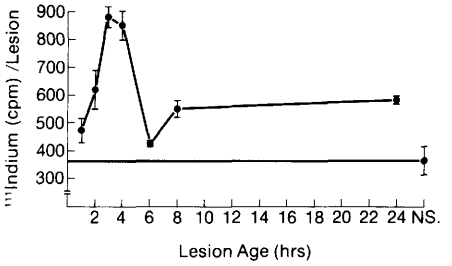

FIG. 2. Aggregation of platelets induced by injecting killed *Escherichia coli* intradermally as described under Materials and Methods. The ordinate (cpm of ^{111}In) refers to the radioactivity in discs of skin punched out immediately after the death of the animals. NS represents normal saline injected sites.

When labeled platelets were given not as a pulse 1 hr before sacrifice, but at the beginning of the experiment and lesions examined up to 24 hr, there was a gradual increase of ^{111}In in the lesions up to 8 hr, followed by a plateau thereafter (not shown). However, the increase in the number of counts was steeper between 1- and 3-hr than between 4- and 8-hr-old lesions.

Platelet accumulation within an inflammatory site may occur either with the formation of large fixed thrombi within the microcirculation, or platelets may transiently adhere in the inflammatory site to plug small gaps between endothelial cells, later detaching and reentering the blood. The accumulation of ^{111}In platelets in the lesions could occur by either or both of these mechanisms. Microscopic examination of the lesions demonstrated substantial thrombus formation, so it would seem reasonable to conclude that during the time of maximum platelet accumulation (3–4 hr) ^{111}In platelets were incorporated into thrombi. Furthermore, in experiments reported elsewhere (23) lesions produced by a local Shwartzman reaction were studied simultaneously with ^{111}In platelets, ^{125}I-fibrinogen, and ^{131}I-albumin. It was found that ^{111}In platelets accumulated together with ^{125}I-fibrogen, while ^{131}I albumin did not accumulate. This also supports the view,

that ^{111}In-labeled platelets accumulated when microthrombi developed. The pulse experiments indicate that ^{111}In platelets were sticking in the microcirculation of 8- to 24-hr-old lesions. However, the cumulative studies demonstrated no further accumulation of ^{111}In at that late time. These results could be explained by the transient adherence of platelets followed by detachment, and do not necessarily indicate ongoing thrombosis.

It is interesting to compare these findings with the previously reported kinetics of the inflammatory response in *E. coli* lesions (16). The maximum rate of platelet accumulation occurred between 2 and 4 hr which coincides with the period when maximum PMN emigration, hyperemia, and increased vascular permeability take place. Hemorrhage characteristically occurrred somewhat later; after 4 hr. In addition, all of these changes had virtually subsided by 24 hr, while there was still evidence of platelet accumulation or turnover at this late stage in the inflammation.

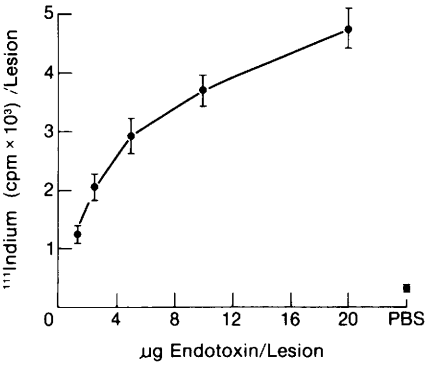

FIG. 3. Aggregation of platelets in local Shwartzman reactions induced with *S. marcescens* endotoxin. The cpm of ^{111}In refer to skin lesions (see Fig. 2 and Materials and Methods).

Of the 15,000–20,000 cpm of ^{111}In/ml of blood, about 500–1000 cpm were "free"

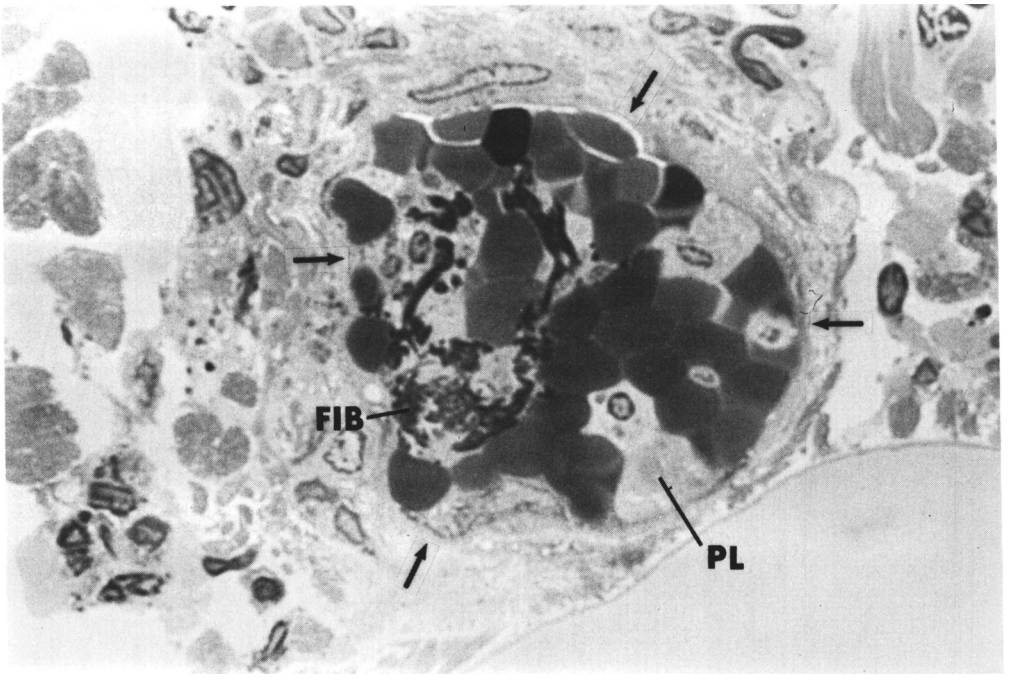

FIG. 4. Light microscopic appearance of a dermal venule in local Shwartzman reaction. The arrows outline the limits of the vessel. Fibrin (FIB) is present in the lumen together with numerous red blood cells and a mass of platelets (PL) recognized at the ultrastructural level (see Fig. 6). The homogeneous structure in the right lower corner is part of a fat cell. ×1600.

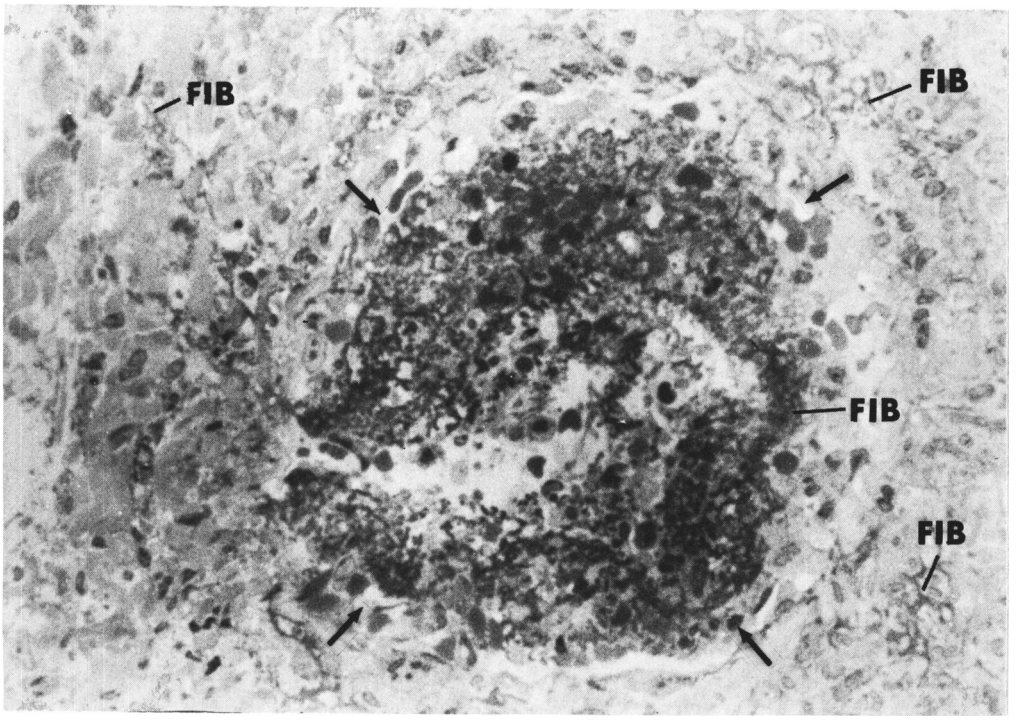

FIG. 5. Light microscopic appearance of a dermal venule showing marked alterations. The wall is no longer recognizable, but its approximate limits are outlined by arrows. A mass of fibrin (FIB) seen in the lumen is continuous with similar material in the wall. Some fibrin is seen also in the surrounding connective tissue. ×640.

and presumably, as shown previously (8), bound to plasma transferrin. Because of this we entertained the possibility that the ^{111}In accumulation was due to the increase in vascular permeability. However, studies employing ^{125}I-albumin have shown that in 2-hr-old lesions the maximum accumulation of albumin is equivalent to about 0.1 ml of plasma radioactivity. This could only account for 50–100 cpm of the observed 900 cpm in these lesions.

Using a 1 hr pulse injection of ^{111}In-labeled platelets, thrombosis produced by the Shwartzman reaction was examined. Figure 3 shows the dose–response curve obtained with increasing amounts of endotoxin. As little as 1.25 μg of endotoxin caused readily measurable platelet accumulation and there was a plateau in the response to doses higher than 20 μg (not shown).

Grossly, marked hemorrhagic spots and areas of thrombosis were noted when the skin was examined from the undersurface. The thrombosed vessels appeared dark purple and the blood could not be expressed from them. Both from quantitation using ^{111}In-labeled platelets and from morphology, it was evident that the Shwartzman reaction produced far more extensive thrombosis than the inflammation resulting from *E. coli*.

Histological sections (0.5–1 μm thick) stained with Azure II showed thrombi in the vessel (Figs. 4 and 5). The most prominent feature in these venules was fibrin which could also be seen extravascularly. Electron microscopy confirmed the presence of platelets in the microthrombi (Figs. 6 and 7). Many of these were degranulated (Fig. 6). In other thrombi there was abundant fibrin (Fig. 7).

The findings presented here demonstrate that ^{111}In-oxine can be used to label platelets for *in vivo* studies. The labeled platelets are able to aggregate and take part

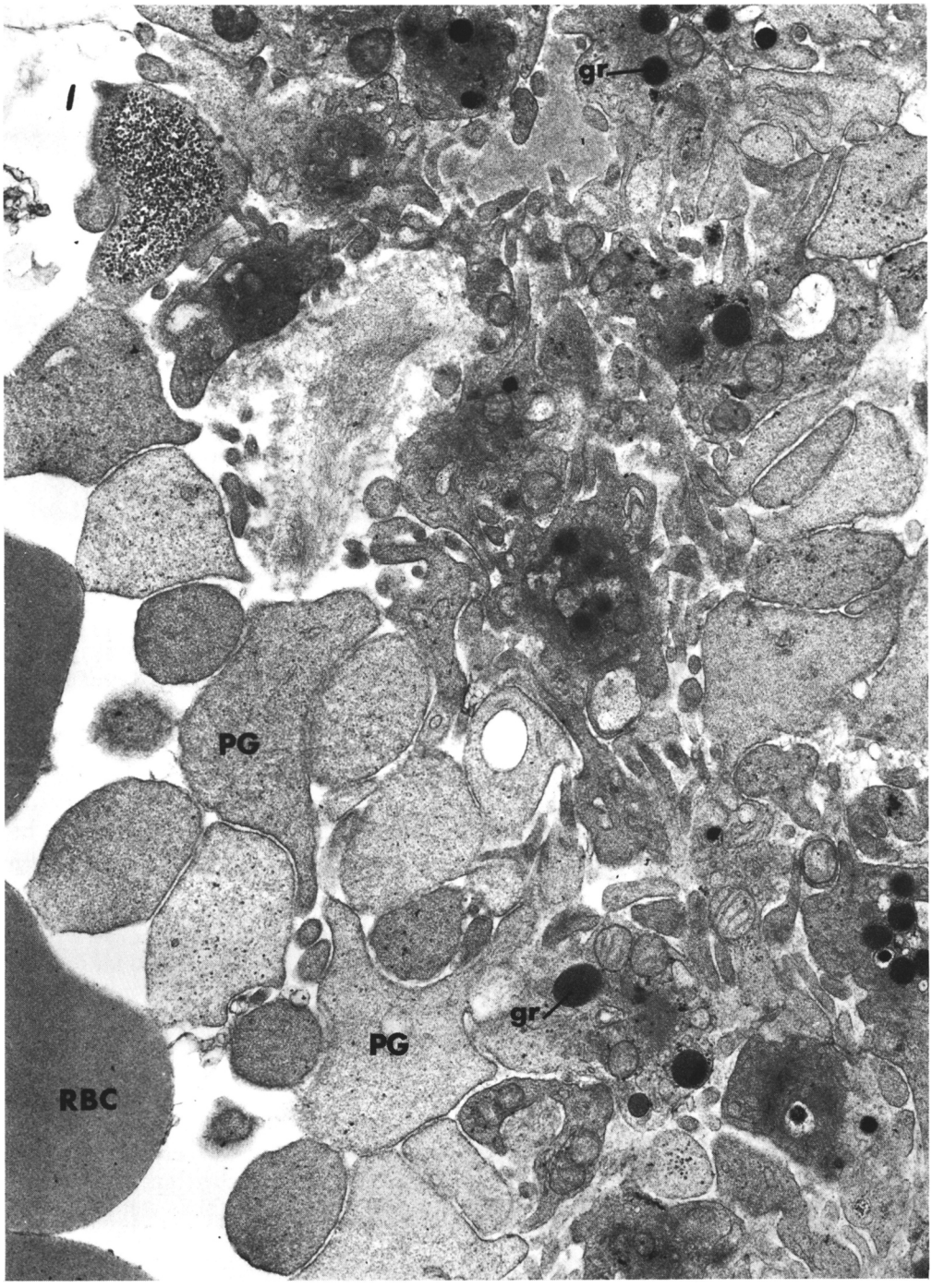

FIG. 6. Electron micrograph of platelets seen as a homogeneous mass in Fig. 4. Some of the platelets contain granules (gr), other structures represent platelet ghosts (PG). ×17,500.

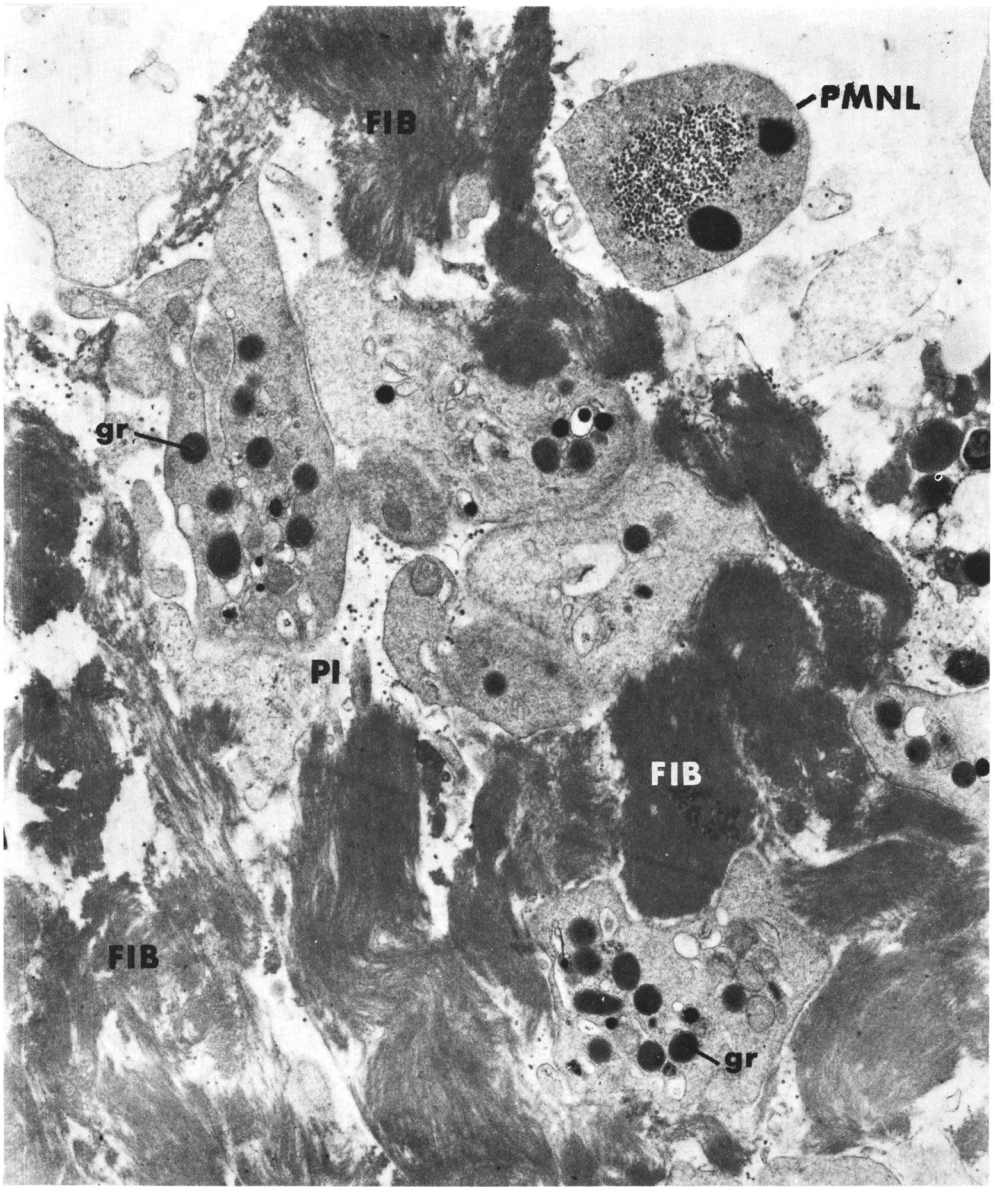

FIG. 7. Electron micrograph of thrombus shown in Fig. 5. Platelets (PL) are seen between strands of fibrin (FIB). gr = platelet granule; PMNL = part of a polymorphonuclear leukocyte. ×16,400.

in thrombus formation. This approach provides a sensitive method for the quantitation of thrombosis within the microcirculation and can be used to describe the kinetics of these events. ^{125}I-labeled fibrinogen could also be used but a considerable amount of fibrin is found extravascularly (Fig. 5). Finally, because ^{111}In has a half-life of 67 hr it can be used simultaneously with ^{51}Cr ($t_{1/2}$ = 27 days) in the same rabbit. By counting the samples twice a few days apart, one can determine the amount of each isotope in a given sample. This makes it possible to measure both platelet accumulation with ^{111}In and PMN–leukocyte migration with ^{51}Cr concurrently.

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