Phorbol Myristate Acetate

In Vivo *Effects Upon Neutrophils, Platelets, and Lung*

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Phorbol myristate acetate is a potent aggregator of platelets. It was found that it was similarly potent in aggregating neutrophils and in producing striking thrombocytopenia and neutropenia when infused intravenously into rabbits. Aggregation and cytopenia were further correlated in that both types of responses developed abruptly and persisted for more than 90 minutes. Animals infused with 40 μg/kg of the phorbol ester exhibited moderately severe respiratory distress. Their respiratory rate doubled shortly after the infusion, and this tachypnea persisted for more than 2 hours. At necroscopic examination, the lungs of these rabbits contained two outstanding abnormalities: numerous foci of alveolar hemorrhage and extensive intravascular accumulations of platelets and neutrophils. Thus, these animals had evidence of increased permeability and potential occlusion of the pulmonary microvasculature. Increased permeability, occlusion of lung blood vessels, or the occurrence of both processes was further indicated in studies on animals preinfused with the plasma protein marker ^{125}I-albumin: animals infused with the phorbol ester had a significantly increased amount of this label in their lungs in spite of thorough postmortem perfusion of their pulmonary vasculature with saline and fixative. We conclude that phorbol myristate acetate has actions *in vivo* that resemble those of a variety of other platelet (eg, arachidonic acid) and neutrophil (eg, chemotactic factors) aggregating agents that cause cytopenia and lung dysfunction. However, compared with these other agents, the phorbol ester produces respiratory distress of intermediate severity and greater duration. The drug, therefore, induces a syndrome that more closely resembles that seen in a variety of clinical and experimental conditions that associate shocklike states with cytopenia and lung dysfunction. It may serve as a useful tool in the study of the pathophysiology of these states as well as in those produced by other aggregating agents. (Am J Pathol 1980, 101:79-92)

CHEMOTACTIC FACTORS aggregate polymorphonuclear neutrophils (PMNs) *in vitro* and, when infused into animals, cause neutropenia, diffuse sequestration of PMNs in lung capillaries, and pulmonary dysfunction.[1-6] Likewise, infusion of certain platelet (PL) aggregating agents (eg, arachidonic acid, labile arachidonate dirivatives, and platelet-activating factor) produces thrombocytopenia, diffuse sequestration of PL in lung capillaries, and pulmonary dysfunction[7-11] The *in vivo* effects of these agents may result from their aggregating actions: they may stimu-

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late the formation of aggregates that cannot traverse, and therefore lodge within, narrow pulmonary capillaries. Cytopenia and lung dysfunction then ensue.[3,5,7-11]

Phorbol myristate acetate (PMA) aggregates PLs[12-15] and PMNs.[16,17] The PMN-aggregating action of the drug, however, has not been well studied. The present communication extends studies on the phorbol ester's ability to aggregate PMNs and reports on the *in vivo* effects of PMA on PMNs, PLs, and lung function when infused into rabbits.

Materials and Methods

Reagents and Buffers

Glycogen and PMA were purchased from Sigma Chemical Co. (St. Louis, Mo); ^{125}I-albumin was purchased from New England Nuclear Corp. (Boston, Mass.) The PMA was dissolved in dimethylsulfoxide, which, in the final concentrations used here, did not influence PMN function *in vitro* nor kinetics *in vivo*. The buffer was a modified Hanks' balanced salt solution containing (mM): NaCl (130), KCl (5.5), Na_2HPO_4 (0.6), NaH_2PO_4 (0.6), glucose (10), and tris (25). Where indicated, calcium and magnesium were added as the chloride salts.

Neutrophils

Human PMNs were isolated by centrifugation of whole blood (obtained from normal donors) over Ficoll-Hypaque discontinuous gradients. The final preparation contained greater than 97 PMNs and fewer than 2 platelets per 100 leukocytes.[18,19] Rabbit PMNs were obtained from the peritoneum of animals 3–5 hours after instillation of 0.1% (wt/vol) glycogen. This preparation contained more than 95 PMNs and fewer than 5 PLs per 100 leukocytes.[5] Brief hypotonic lysis was employed to remove contaminating erythrocytes.

Aggregation Assay

This assay has been described previously.[5,18] All reagents were made 37 C and pH 7.4 before use. One milliter of a PMN suspension (4600 cells/μl for human and 2600 cells/μl for rabbit PMNs) was placed in a plastic vial, maintained at 37 C, and continuously stirred with a magnetic bar. After 4 minutes, calcium and magnesium were added; 1 minute thereafter, the suspension was treated with PMA. Just before and at 1/4, 1/2, 1, 2, 4, 11, and 15 minutes after this treatment, 20-μl samples were taken, diluted in 10 ml of 37 C Isoton (Coulter Electronics, Hialeah, Fla), and immediately examined for total and large-particle concentrations with a model ZBI Coulter Counter (Coulter Electronics, Hialeah, Fla). Large particles were defined as particles greater than 1.7 times the (volume) size of the unaggregated PMNs. Results are reported as the large-particle percentage (LPP) or maximal change in the LPP (MLPP). The LPP is 100 times the large particle concentration divided by the total particle concentration; the MLPP is the highest LPP found at 1/4, 1/2, 1, 2, 4, 8, 11, or 15 minutes after adding PMA to the suspension minus the LPP found just before the addition. Aggregate formation is reflected by increases in the LPP and MLPP. Cytotoxicity was monitored by assaying release of the cytosolic enzyme lactic acid dehydrogenase. In the concentrations used here, PMA did not increase the level of this enzyme in the fluid phase of PMN suspensions after 20 minutes of incubation.[19]

Cytopenia Assay

In vivo assays were performed on albino white female rabbits weighing 2–4 kg. Substances were infused into a marginal ear vein, and blood samples were obtained from a dif-

ferent ear vein. Leukocyte counts were performed with a Coulter Counter. PL counts were performed by scoring their number in 10 high-power (oil immersion at 1000X) fields of Wright-stained blood smears. This manual method was required because PL counts in PMA-treated animals were frequently too low to be accurately detected by electron- or phase-microscopic examinations.

Lung Content of ^{125}I-Albumin

^{125}I-albumin (10 μCi) was infused into a marginal ear vein of rabbits 20 minutes before further experimental manipulation. Animals were then infused with a test substance and 90 minutes later killed by electrocution. With a midline incision, the animals' thoracic cavity was opened. Their lungs were quickly isolated and immediately perfused via a 19-gauge catheter inserted into the pulmonary artery. The perfusate was collected from another catheter inserted into the left atrium. The perfusion was performed under constant pressure (20 mm Hg) for 15 minutes with isotonic saline and then for 10 minutes with 2% glutaraldenyde (Polysciences, Inc., Warrington, Pa), in saline. Immediately thereafter, the catheters were clamped and the lung vasculature maintained at 20 mm Hg for 10 minute without leakage of the glutaraldehyde. Then, 15 ml of 2% glutaraldehyde was introduced into the trachea and the lungs removed. The lung was partitioned into multiple sections, all of which were counted for ^{125}I-albumin with the use of a gamma well counter (Nuclear of Chicago, Chicago, Ill).

Histologic Examination

The lungs were processed exactly as described above, up to, and including, the 10-minute saline perfusion. At this point, the lungs were further perfused with 3% glutaraldehyde in (300 mOsm) cacodylate buffer (pH 7.2–7.4) for 10 minute and then fixed for 20 hours in the same fluid. Small wedges from upper and lower lobes of both lungs were cut, placed in fresh 2.5% glutaraldehyde–cacodylate buffer for 2 hours, and rinsed twice for 15 minutes in cacodylate buffer. Pieces (1 mm × 1 mm) of the lung wedges were postfixed for 1 hour in osmium tetroxide (1%) and cacodylate buffer, rinsed in cacodylate buffer, dehydrated in graded concentrations of ethanol and embedded in Spurr rapid cure mixture.[20] For light microscopy, 1-μ thick sections were stained with methylene blue–azure II. For electron microscopy (Zeiss EM9S-2 transmission electron microscope) ultrathin (80–100 nm) sections mounted on bare copper grids were stained with uranyl acetate and lead citrate.

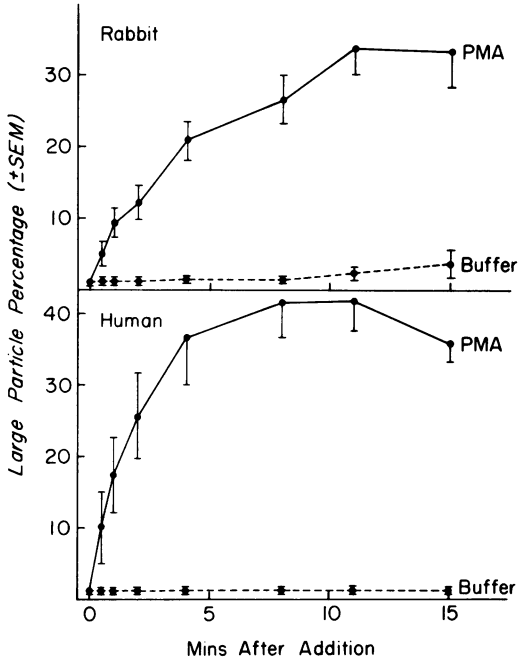
Measurement of ED_{50}

In the aggregation and cytopenia assays, dose-response curves were constructed. The point on these curves that corresponded to a half maximal response was located and extrapolated visually to the dose axis to obtain the amount of drug producing a half maximal response (ED_{50}).

Results

PMN Aggregation

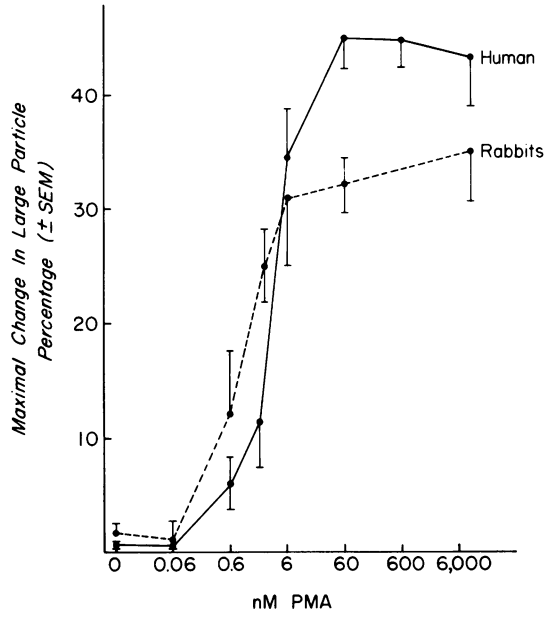
Text-figure 1 shows the LPP of rabbit (upper panel, solid line) and human (lower panel, solid line) PMN suspensions exposed to 6 μM (final concentration) PMA. A rise in the LPP developed almost immediately after exposure and did not reverse during a 15-minute observation interval nor for 75 minutes thereafter (not shown). Indeed, at this PMA concentration, cells became visibly clumped within 8–11 minutes and then settled out of the continuously stirred suspension. In control studies, human PMNs did



TEXT-FIGURE 1—Large particle percentage found after adding 6 μg/ml (final concentration) phorbol myristate acetate to neutrophil suspensions. The drug (*solid lines*) or its buffer (*interrupted lines*) was added to rabbit cells preincubated with 0.2 mM calcium and 0.1 mM magnesium (*upper panel*) or human cells preincubated with 1.4 mM calcium and 0.7 mM magnesium (*lower panel*). Each curve gives the results for at least 4 experiments.

not aggregate spontaneously when treated with 1.4 mM calcium and 0.7 mM magnesium (Text-figure 1, lower panel, interrupted line). However, rabbit peritoneal PMNs aggregated without further treatment. In order to obviate this spontaneous aggregation, studies with rabbit cells were performed with the use of 0.2 mM calcium and 0.1 mM magnesium. Under these conditions, rabbit PMNs showed little tendency to aggregate spontaneously (Text-figure 1, upper panel, interrupted line) yet exhibited prominent responses to PMA (Text-figure 1, upper panel, solid line). Decreasing the bivalent cation concentrations to these levels had little influence on the human PMN response; however, when the cations were omitted entirely from the suspensions, PMNs did not aggregate in response to the drug (not shown).

The effect of PMA was dose-dependent and easily detectable at concentrations as low as 0.6 nM (Text-figure 2). Dose-response curves for PMNs from humans and rabbits were similar and indicated that 1 and 4 nM PMA, respectively, induced a response that was 50% of maximal (ED_{50}). Although not shown by the data in Text-figure 2, the response to all con-



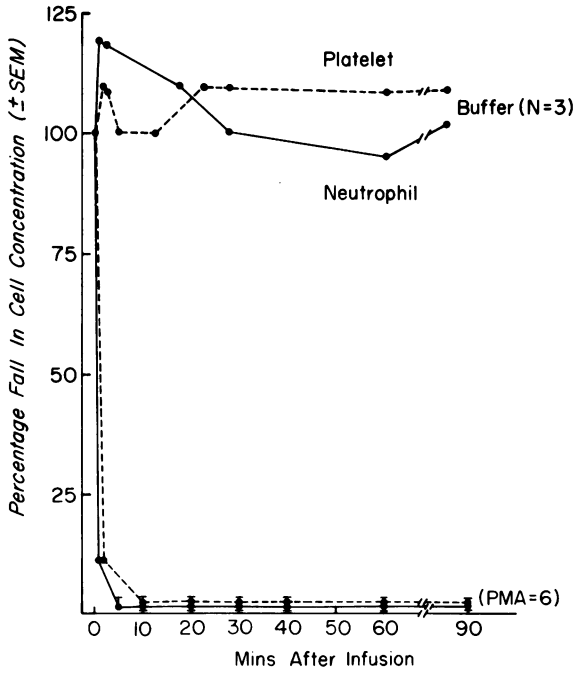
TEXT-FIGURE 2—Maximal change in the large particle percentage of human (*solid line*) and rabbit (*interrupted line*) neutrophil suspensions exposed to varying concentrations of phorbol myristate acetate. Human cells were first preincubated with 1.4 mM calcium and 0.7 mM magnesium; rabbit cells were first preincubated with 0.2 mM calcium and 0.1 mM magnesium. Each point is the mean of at least 4 experiments.

centrations of the drug was irreversible: at 6 nM and higher, PMA caused visible clumps to settle out of the suspension; below 6 nM, the drug produced a delayed response that continued to increase for at least 30 minutes. Thus, under the conditions studied here, PMA is an exceedingly potent inducer of nonreversing PMN aggregation.

Cytopenias

When infused into the ear veins of rabbits, 40 μg/kg PMA produced profound, sustained neutropenia (Text-figure 3, solid line) and thrombocytopenia (Text-figure 3, interrupted line). PMN and PL levels fell to less than 10% of preinfusion values within 1–3 minutes and remained there for at least 90 minutes. In general, PMN and PL levels did not rebound for several hours; they rose to 150–200% of preinfusion values within 18 hours (not shown).

Text-figure 4 illustrates dose-response curves following the infusion of PMA. At all doses, the action of PMA was abrupt, producing a fall in PMN and PL levels that was maximal within 1–5 minutes and persisted during the ensuing 90 minutes. Hence, the PMN and PL counts found 30

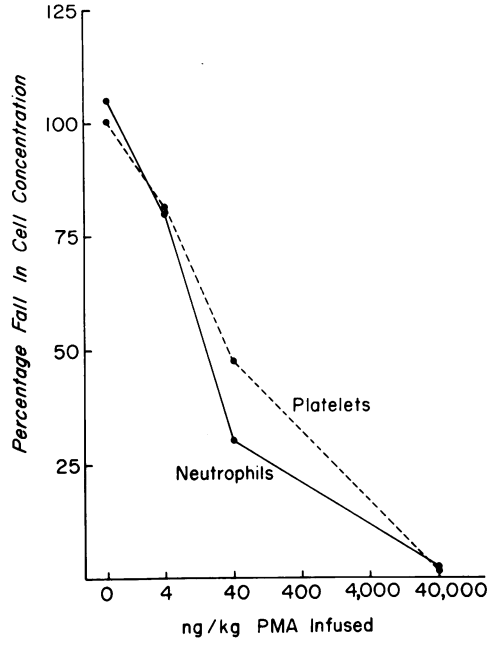


TEXT-FIGURE 3—Percentage of change in neutrophil and platelet concentrations found after intravenous infusion of 40 μg/kg phorbol myristate acetate or its buffer into rabbits. The values in parentheses give the number of experiments performed.

minutes after the infusion are representative of the drug's effect (Text-figure 4). From these data the ED_{50} values for neutropenia and thrombocytopenia are extrapolated to be about 30 and 40 ng/kg, respectively. Assuming a blood volume of 100 ml/kg, no extravasation, and no inactivation, the calculated molar ED_{50} values are about 1 nM, a value close to the ED_{50} for PMN (see previous section) and PL (12) aggregation *in vitro*.

Animal Behavior

Rabbits infused with up to 4 μg/kg PMA appeared well and experienced only moderate increases if any in respiratory rate. However, animals treated with 40 μg/kg PMA developed severe respiratory distress. The respiratory rates (per minute) of these animals were (± SEM): 199± 5, 285±10, 470±13, 355±19, 288±16, 305±18, and 211±13 at 0, 5, 30, 60, 90, 120, and 180 minutes after the infusion. Similar values for animals treated with an equivalent volume of buffer (containing dimethylsulfoxide, which was used to dissolve PMA) were: 172±16, 195±24, 181±30, 161±27, 152±24, 142±2 and 159±16. Except for tachypnea, PMA-treated



TEXT-FIGURE 4—Percentage of change in neutrophil and platelet concentrations found 30 minutes after intravenous infusion of varying concentrations of phorbol myristate acetate into rabbits. Each point is the mean of two or more experiments.

rabbits appeared well for the first 10–15 minutes after infusion. After this time, they began to appear acutely ill. The respiration of some animals was labored, and all exhibited little spontaneous activity, although continuously alert. At 90 minutes, most animals were at the nadir of their respiratory distress and, although tachypnea had partially abated, breathing was most labored and spontaneous activity minimal. During the ensuing several hours, all symptoms eased. Within 8–18 hours, the animals return to fully normal appearance. Of 12 animals infused with this dose of PMA, one died at about 110 minutes after infusion, 6 were sacrificed at 90 minutes, and the remaining 5 recovered fully.

Histologic Study of Lung

The lungs taken from 6 animals 90 minutes afte PMA treatment were abnormal. They had multiple foci of grossly visible hemorrhage throughout all lobes. When viewed by light microscopy, these foci were found to contain all blood elements extravasated into the alveoli (Figure 1, lower panel). Furthermore, all areas of lung examined with the light microscope

appeared hypercellular (not shown). Transmission electron microscopy revealed that this hypercellularity was due to an increase in formed elements within the microvasculature. Figure 2 contains two representative photomicrographs illustrating this increase: in the upper panel are seen two capillaries, one of which contains a mononuclear cell and a grouping of platelets and the other of which appears filled with a PMN; in the lower panel is seen a vessel containing a somewhat amorphous grouping of platelets, a mononuclear cell, a PMN, and a portion of an erythrocyte. In these micrographs, as well as in the remaining portions of the examined lungs, PLs appeared vacuolated and without prominent cytosolic granules; PMNs did not appear abnormal; the endothelium appeared intact; and the interstitial space was not widened. Furthermore, we found no evidence of PMN migration beyond the microvasculature except in areas of hemorrhage. Control animals injected with buffer and dimethylsulfoxide had no abnormal findings on gross examination or by light/electron microscopic examination.

Lung ^{125}I-Albumin

Lung from animals treated with PMA showed a significantly elevated content of the ^{125}I-albumin (Table 1). The lung specimens were assayed only after extensive and careful vascular perfusion for 25 minutes (see Materials and Methods). The effluent perfusate was sequentially counted for label during this time. It was found that the radiolabel in the perfusate declined exponentially to background levels within 5 minutes. After this, the perfusion did not release more label from the lung. This finding suggests that the increase in lung ^{125}I-albumin found here is from label sequestered in a location inaccessible to the perfused vasculature, such as extravascular pulmonary spaces or within permanently occluded pulmonary capillaries.

Table 1—Lung Content of ^{125}I-Albumin After Infusion of Phorbol Myristate Acetate or Its Buffer Into Rabbits*

Agent Infused	Content of ^{125}I-albumin†	
	cpm	Percentage
Buffer	7840 ± 2506‡	0.42 ± 0.15
PMA	31,064 ± 9518§	1.22 ± 0.30§

* Rabbits were infused with ^{125}I-albumin 20 minutes before infusion with PMA or buffer; 90 minutes thereafter the animals were killed by electrocution. Their lung vasculature was perfused with isotonic saline and glutaraldehyde for 25 minutes before the removal of both lungs and assaying of total lung ^{125}I.

† Expressed as counts per minute (cpm) and percentage of infused ^{125}I found in lungs.

‡ Mean value ± SEM for at least 4 animals.

§ Significantly higher ($P < 0.05$, Students unpaired t test) than animals treated with buffer.

Discussion

PMA (Text-figures 1–4) and chemotactic factors [1–6] aggregate PMNs and, in doses that correlate with this effect, produce neutropenia in rabbits. Chemotactins, however, induce only transient responses; the aggregation and neutropenia that they stimulate begin to reverse within 3–5 minutes.[1–6] PMA, on the other hand, causes irreversible aggregation (Text-figure 1) and prolonged neutropenia (Text-figure 3). Thus, the studies reported here complement those of chemotactic factors and lend further support to the notion that aggregation and neutropenia are closely related PMN responses. The *in vitro* and *in vivo* actions of these agents may be consequences of a single event occurring within the stimulated cell. For instance, this event could be the formation of aggregates that cannot traverse and therefore lodge within narrow capillaries.[3,5,18] Or, alternatively, it may be the formation of a hyperadhesive surface membrane that causes PMNs to aggregate *in vitro* and marginate along endothelium *in vivo*.[1,2,4,6,18] Either event would operate to produce a close correlation between the two responses.

PMA also has effects upon PLs. *In vitro*, the phorbol ester induces PL aggregation [12–16] and, at doses which correlate with this effect,[12,13] produces thrombocytopenia *in vivo* (Text-figures 3 and 4). The kinetics (Text-figure 3) and dose-response relationships (Text-figure 4) of thrombocytopenia paralleled those of neutropenia. It may be, therefore, that PLs and PMNs interact in the cytopenic responses. For instance, PMA may cause PMNs to aggregate with PLs or release a platelet-aggregating agent.[10,11] However, electron-microscopic examinations of capillaries did not show excessive attachment of PLs to PMNs. And aggregation assays were performed with essentially homogeneous cellular preparations effectively excluding a PMN–PL interaction in the *in vitro* response. Finally, chemotactic factors neither aggregate PLs [19] nor induce thrombocytopenia,[1–6] suggesting that PMN and PL responses can also occur independently of one another *in vivo*. Thus, it appears more likely that PMA-induced neutropenia and thrombocytopenia result from separate but similar responses of the two cell types. Certain other PL-aggregating agents also induce thrombocytopenia when infused into animals.[7–11] PMA and these other agents, then, may act like PMN-aggregating agents in producing their *in vivo* effects.

The most difficult aspect of studies of the *in vivo* action of aggregating agents is in interpreting the cause of their apparent pulmonary toxicities. For instance, mild, evanescent hypoxemia and increased egress of lung lymphatic fluid occurs concomitantly with neutropenia and pulmonary capillary accumulations of PMNs in animals infused with chemotactic

substances.[1-4] Leukoembolic occlusions of, or endothelial damage to, the pulmonary microvasculature may be responsible for this lung dysfunction, because these same chemotactic substances stimulate PMNs to aggregate and release tissue-injuring enzymes and oxygen radicals *in vitro*.[1-6,18,21-24] Nevertheless, in (unpretreated) PMNs, chemotactins are weak inducers of these responses.[21-24] Furthermore, recent studies in our laboratory show that a surprisingly large number of PMNs persist within pulmonary capillaries long after chemotactic factor-induced lung dysfunction and neutropenia have abated.[25] And, finally, the toxicity of chemotactins has not been disassociated from their anaphylatoxic activities. Thus a recent report shows that various chemotactic preparations contract smooth muscle of lung.[26] Obviously, this action could result in bronchoconstriction and respiratory dysfunction.

Similar difficulties appear in interpreting the pulmonary toxicity of PL-aggregating agents. In relatively high doses, arachidonic acid,[7] labile derivatives of arachidonate,[8,9] and platelet-activating factor [10,11] cause an explosive syndrome of thrombocytopenia, PL accumulation in pulmonary vessels, and apparent respiratory distress. In appropriate concentrations, these agents are rapidly lethal.[7-11] However, in treated animals PL aggregates are clearly widespread and therefore may interfere with blood flow to a variety of vital organs. Furthermore, arachidonic acid aggregates [18] and degranulates [27] PMNs *in vitro*, and platelet-activating factor produces profound leukopenia in concert with thrombocytopenia.[10,11] Thus, the cell type(s) and end organ(s) involved in the toxicity of these agents remain uncertain.

With these considerations in mind, the toxicity of PMA must be interpreted cautiously. Rabbits infused with high doses of the phorbol ester abruptly exhibited respiratory distress and marked tachypnea. Their lungs contained foci of alveolar hemorrhage (Figure 1), accumulations of PMNs and PLs within capillaries (Figure 2), and an increased content of the plasma marker ^{125}I-albumin (Table 1) in spite of extensive postmortem perfusion of their pulmonary vasculature (see Materials and Methods). These animals, therefore, had evidence of increased permeability and possible occlusion of their pulmonary capillaries. Since PMA is a potent stimulator of aggregation (Text-figures 1 and 2), degranulation,[16,17] and generation of reactive species of oxygen [23,28] in (unpretreated) PMNs and of aggregation and degranulation in PLs,[12-16] it is tempting to propose that PMA's toxicity results from its ability to stimulate PMNs and PLs to occlude or injure lung capillaries. However, PMA was not apparently toxic at intermediate doses in spite of significant and prolonged effects on the circulating levels of PMNs and PLs. Furthermore, there was no morpho-

logic evidence of diffuse endothelial disruption or extensive PMN degranulation (Figure 2). And, finally, PMA, although being an exceedingly potent inducer of PL aggregation [12-16] and thrombocytopenia (Text-figures 3 and 4), did not produce a syndrome of toxicity as severe as that produced by other PL-aggregating agents in spite of the appearance of vacuolated, degranulated, and closely apposed PLs within lung capillaries (Figure 2). It is obvious, therefore, that further study is needed for an understanding of the precise events that result in lung dysfunction following the infusion of aggregating agents. These studies appear to be indicated because the various aggregating agents produce models of disease that resemble, at least superficially, certain well-defined clinical conditions that associate cytopenia with lung dysfunction. Thus, with respect to lung dysfunction and cytopenia, chemotactins cause mild, evanescent effects similar in severity and duration to those seen during extracorporal hemodialysis [1-4]; arachidonate, labile arachidonate derivatives, and platelet-activating factor cause precipitous effects similar to those seen during anaphylaxis [7-11]; and phorbol myristate acetate causes intermediate effects that approach in severity and duration those seen during various shock lung syndromes.[29,30] Study of these drugs in animals, therefore, may offer insight into the etiology and pathogenesis of a wide variety of clinical conditions.

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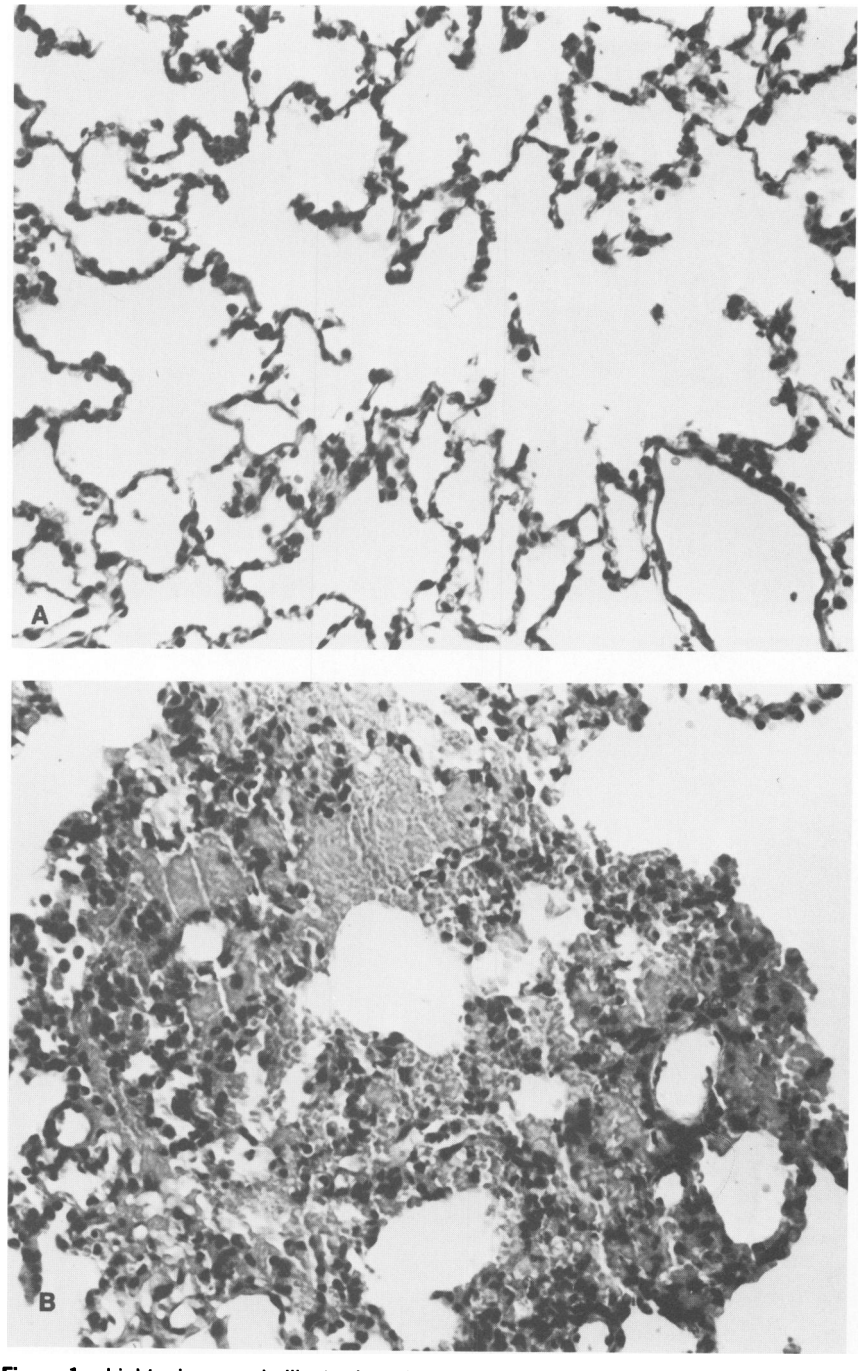

Figure 1—Light microscopic illustration of representative specimens of lung taken from animals 90 minutes after infusion of buffer (**A**) or 40 μg/kg phorbol myristate acetate (**B**).

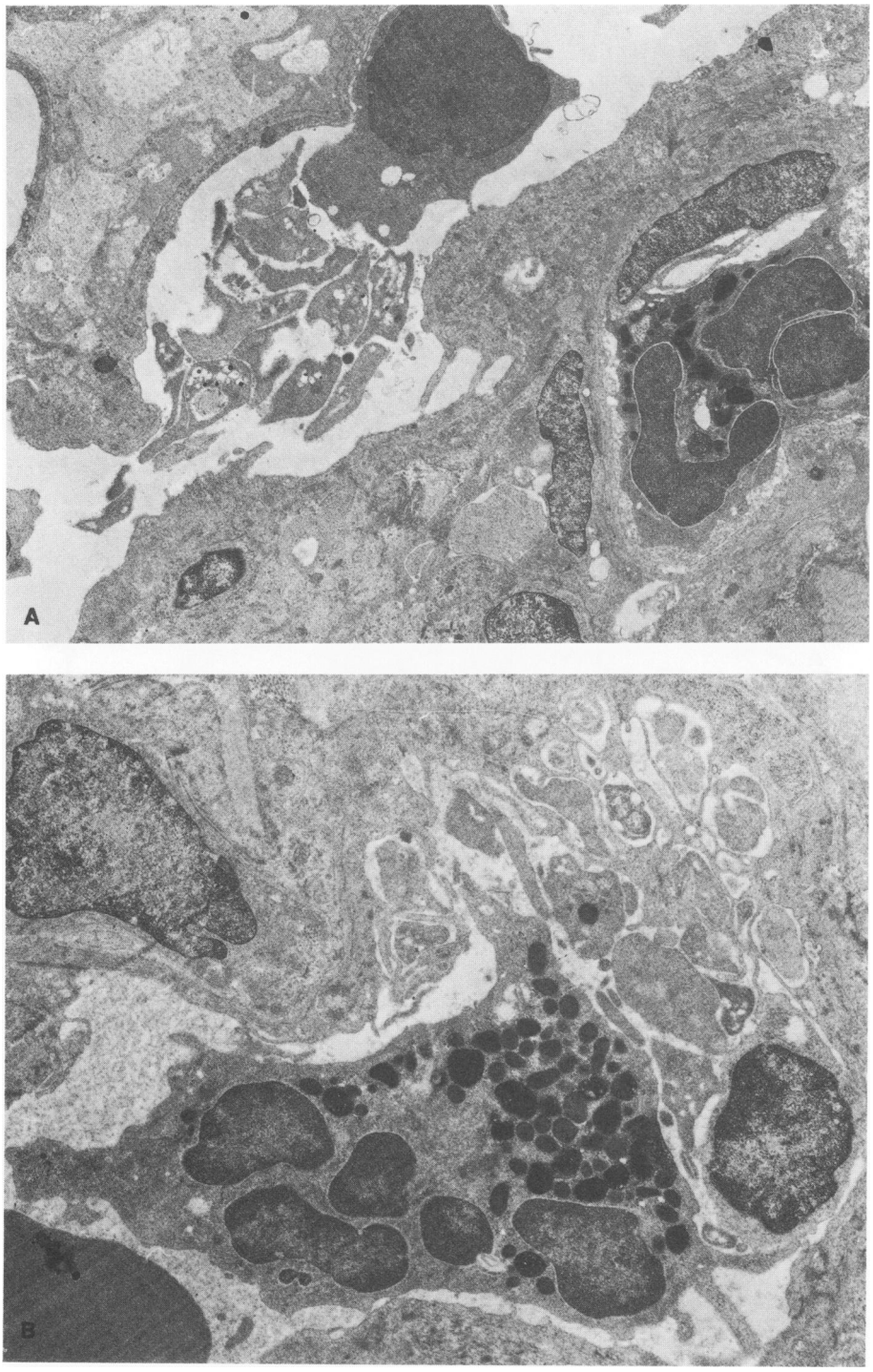

Figure 2—Transmission electron micrographs of lung capillaries taken from two animals infused with 40 μg/kg phorbol myristate acetate. (**A**, ×3620; **B**, ×7740.)