Pulmonary Intravascular Sequestration of Activated Neutrophils

Failure to Induce Light-Microscopic Evidence of Lung Injury in Rabbits

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Rabbits were injected intravenously with glycogen-elicited allogenic peritoneal polymorphonuclear neutrophil leukocytes (PMNs) for the study of the light-microscopic effects of acute and chronic sequestration of PMNs in the pulmonary vascular bed. Infusion of ^{51}Cr-labeled PMNs demonstrated that approximately half of the cells were sequestered in the lung, with no difference observed between PMNs incubated with 10% normal rabbit serum and PMNs incubated with 10% zymosan-activated serum (ZAS) prior to infusion. Quantitative histologic studies demonstrated that the number of ZAS-activated PMNs present in the alveolar walls at 4 hours rapidly declined over the ensuing 20 hours and was back to buffer control values by 48 hours. No PMNs, red cells, or signs of edema were visible in the alveolar spaces. In rabbits injected chronically (twice weekly for 8 weeks) with 2×10^8 PMNs (ZAS-stimulated and unstimulated), no qualitative or quantitative (mean linear intercept) evidence for damage to alveolar walls was observed. These studies indicate that acute and chronic pulmonary sequestration of PMNs activated *in vitro*, infused in the absence of activated serum products, does not cause light-microscopic evidence of lung injury. (Am J Pathol 1982, 108:17–23)

INTRAVASCULAR NEUTROPHIL (PMN) aggregation and sequestration in the pulmonary vascular bed has been observed in patients undergoing hemodialysis,[1,2] filtration leukophoresis,[3] and cardiac bypass.[4] The PMN activation and aggregation appears to be caused by chemotactic fragments generated from complement activation in the plasma induced by artificial surfaces.[1,3]

Studies in humans[1,3] and animals[2,5] have suggested that the resultant plugging of the pulmonary vasculature by PMNs induces lung damage manifested by a fall in the P_{O_2}[5] and enhanced pulmonary capillary permeability.[2] However, recently, the decrease in P_{O_2} in hemodialysis patients has been attributed to changes in CO_2 exchange and the respiratory quotient and not to any deterioration in pulmonary gas exchange itself.[6] Furthermore, since the lung is normally a site of PMN sequestration,[7] the possibility exists that the observed alterations in lung function and permeability could be caused or enhanced by humoral (rather than cellular) elements derived from either complement or coagulation proteins activated by the artificial surfaces. Alternatively, factors in the activated plasma could alter pulmonary vascular permeability primarily, in such a manner that sequestered PMNs could then damage the alveolar wall.

Several studies have demonstrated that PMNs migrating out of the vascular bed into the lung can cause damage to the epithelial and endothelial layers of the alveolar wall.[8,9] In this investigation we sought to de-

Supported in part by Grants HL-23578 and HL-21565 from the National Heart, Lung and Blood Institute.

Accepted for publication February 1, 1982.

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0002-9440/82/0709-0017$00.85

termine whether PMNs activated *in vitro* would cause histologic evidence of lung damage when infused intravenously into rabbits in the absence of activated serum components. Our results revealed that complement-activated PMNs transiently sequester in the rabbit pulmonary vascular bed to the same degree as unactivated PMNs, but that no light-microscopic evidence of acute or chronic lung damage results from this interaction of cells with the pulmonary microvasculature.

Materials and Methods

Animals

New Zealand White rabbits of both sexes, 2.5–3.0 kg in weight, were utilized in the infusion studies.

Peritoneal PMN Harvesting and Stimulation

Rabbits weighing 3.5–4.5 kg were injected intraperitoneally with 150 ml of 0.1% shellfish glycogen dissolved in sterile saline. Four hours later the animals were restrained in a supine position, and the peritoneal cavity was lavaged with 500 ml of sterile saline containing 0.1 unit/ml of heparin. The lavage eluate was chilled and passed through one layer of sterile gauze, and the eluate was centrifuged at 4 C for 10 minutes at 200*g*. The cell pellet was resuspended in Hanks' balanced salt solution with 0.25% bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, Mo) and washed two times. The cells were counted (routinely 80–86% PMNs, 15–20% macrophages) and resuspended at 1×10^7/ml in Hanks' solution containing 10% normal fresh rabbit serum or 10% rabbit serum that had been incubated with washed zymosan (20 mg/ml, Sigma Chemical Co., St. Louis, Mo) for 1 hour at 37 C (ZAS) to activate complement. Zymosan was then removed by centrifugation at 12,000*g*. The PMNs were incubated in this serum-containing solution without agitation for 10 minutes at 37 C. The mixture was chilled and the cells pelleted at 180*g* at 4 C for 8 minutes. The cells were resuspended and washed one time and resuspended in Hanks' buffer containing 2% fresh rabbit plasma at 5×10^7 cells/ml. This suspension was immediately infused into the marginal ear vein of recipient rabbits over 3–5 minutes. In some experiments PMNs in Hanks–BSA were stimulated with 100 μg/ml of *Escherichia coli* 0111:B4 lipopolysaccharide (a butanol extract supplied by Dr. David Morrison, Scripps Clinic and Research Foundation, La Jolla, Calif) for 10 minutes at 37 C before being washed, resuspended, and injected as noted above. Animals occasionally had tachypnea and, rarely, coughing during or immediately after the infusion. No deaths related to PMN infusion occurred.

Verification of zymosan generation of chemotactic fragments in rabbit serum was obtained with the use of the modified Boyden chamber chemotactic assay of Ward et al.[10] Employing 2.5×10^6/ml peritoneal PMNs in Hanks' buffer in the upper compartment and Hanks' with 10% normal rabbit serum or 10% ZAS in the lower compartment separated by a 0.65 μM millipore filter, 140 ± 15 versus 771 ± 56 (cells/5 high-power fields, mean ± standard error of the mean (SEM) for four experiments) PMNs migrated into the filter over 90 minutes in response to normal rabbit serum or ZAS, respectively.

^{51}Cr-Labeled PMNs

Peritoneal PMNs were suspended in Hanks'–BSA at 1×10^7 cells/ml and incubated for 1 hour at 37 C with 0.25 mCi/ml of ^{51}Cr (450 Ci/g, New England Nuclear, Cambridge, Mass). The cells were pelleted at 4 C and washed two times in Hanks'–BSA and resuspended in Hanks'–10% normal rabbit serum or 10% ZAS and activated as noted above. Following this, the cells were washed and resuspended in Hanks' 2% normal rabbit serum at 5×10^7/ml. A 10-μl aliquot was removed for counting and the remainder immediately infused in the ear vein of rabbits at 5×10^7 PMNs/rabbit. Twenty-five minutes later, the rabbits were injected intravenously with 2 mg of ^{125}I-BSA. Five minutes later, the rabbits were sacrificed by an overdose of pentobarbital and samples of blood, lung, liver, spleen, and kidney weighed and counted for ^{51}Cr and ^{125}I radioactivity in a gamma counter. Sequestration of ^{51}Cr-PMNs was expressed as a percentage of the total PMNs injected per organ, after correction for ^{125}I-BSA blood volume,[11] based on the radioactivity and weight of 5 samples from each organ. Examination of heparinized blood samples taken at sacrifice revealed that less than 5% of blood ^{51}Cr radioactivity was present in plasma.

Intratracheal C5 Fragment Injections

Pulmonary inflammation was induced in 4 rabbits out of 2 groups utilized for the infusion experiments. These rabbits were given intratracheal injections of C5 fragments prepared from yeast-activated serum exactly by methods described previously. After 4 hours, the animals were sacrificed and examined by light microscopy.

Histologic Studies

The animals were sacrificed at the appropriate time points after injection of PMNs with an overdose of pentobarbital. The lungs were removed, and the left mainstem bronchus was occluded with a ligature. The left lung was removed and immersed in phosphate-buffered 10% formalin. The right lung was inflated at 25 cm H_2O pressure with buffered formalin for 48 hours. Blocks from each lobe were cut and embedded in paraffin, and 5 μM sections were cut and stained with hematoxylin and eosin.

Mean linear intercepts (L_m) were determined on 5 blocks of each right lung (20 measurements each at 100×) by the technique of Thurlbeck.[12]

We performed quantitative histologic assessment of PMN sequestration by counting the number of PMN nuclei and number of total alveolar wall nuclei in 10 randomly selected 400× fields in one section from each lobe of each rabbit. The mean ± SEM for the number of PMN nuclei per alveolar wall nuclei was calculated for all 5 lobes for each rabbit at each experimental time point examined.

Results

Isolated rabbit peripheral blood PMNs labeled with ^{51}Cr were stimulated with 10% ZAS on fresh normal rabbit serum for 10 minutes at 37 C, rapidly washed and resuspended in Hanks' buffer, and immediately infused into the marginal ear vein of rabbits. At sacrifice 30 minutes later, approximately half of the injected ^{51}Cr-PMNs were found in the lung, and there were no significant differences between animals that received ZAS-stimulated PMNs and those that received unstimulated PMNs (Figure 1). The liver and spleen contained 12–15% and 2–3%, respectively, of the injected ^{51}Cr radioactivity. Only 7–9% of the radioactivity was present in blood at sacrifice. Recovery of ^{51}Cr radioactivity in the four organs and blood at sacrifice equaled 79 ± 2.5% (mean ± SEM) of injected ^{51}Cr. Sequestration appeared to be nearly complete during the first pass through the circulation, because arterial blood samples taken 30 seconds after the completion of the infusion always revealed less than 20% of the ^{51}Cr-PMNs remaining in the circulation.

In order to assess the temporal aspects of PMN sequestration in lungs, rabbits were injected with ZAS-stimulated peritoneal PMNs (1 × 10^8/animal) and sacrificed 4, 24, and 48 hours later. Histologic examination revealed PMNs sequestered in pulmonary arterioles and alveolar wall capillaries at 4 hours (Figure 2A), while 48-hour sections (Figure 2B) revealed only rare PMNs in the alveolar wall. No PMNs, red cells, or edema in alveolar spaces were found in any of the 8 animals infused with peritoneal PMNs (0.2–2 × 10^8 PMNs/animal) and sacrificed within 4 hours. Light-microscopic sections revealed that PMNs remained in the pulmonary arterioles or alveolar wall (Figure 2A). These findings were in contrast to the intraalveolar accumulation of PMNs, red cells, and edema fluid seen in each of 4 rabbits given intratracheal injections of 1 mg protein of C5 fragments.[9]

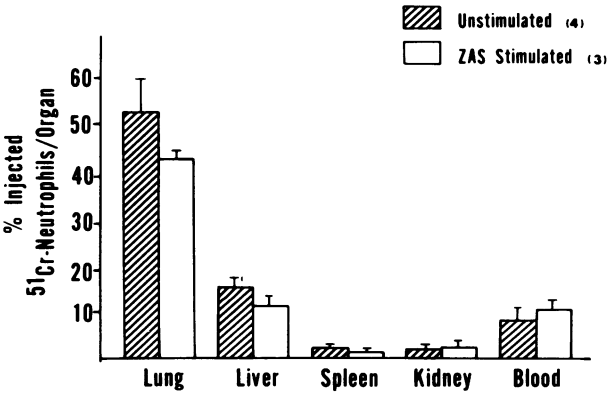

Figure 1—Sequestration of intravenously injected ^{51}Cr-labeled PMNs in the vasculature of various rabbit organs. Glycogen-elicited rabbit peritoneal PMNs were labeled with ^{51}Cr and incubated with zymosan-activated (ZAS) or normal rabbit serum. After washing, cells (5 × 10^7) were infused into the marginal ear vein. Percent ^{51}Cr-PMNs sequestered in each organ at sacrifice 30 minutes later was determined as described in Methods. The numbers in parentheses are the number of rabbits in each group. *Bars* are the mean ± SEM for each group.

When the time course of disappearance of ZAS-stimulated PMNs (2 × 10^8/animal) for the pulmonary vascular bed was examined quantitatively, 75% of the PMNs present in the lungs at 4 hours had disappeared by 24 hours (Figure 3). At 48 hours the number of PMNs visible in histologic sections had returned to saline control levels. Rabbits injected with PMNs stimulated with 100 μg/ml of *E coli* 0111:B4 lipopolysaccharide demonstrated a similar time course for PMN sequestration in the pulmonary vasculature.

Although no light-microscopic evidence of lung injury (alveolar edema and hemorrhage) was evident acutely, the possibility existed that submicroscopic alveolar capillary wall damage was occurring and might become evident after recurrent episodes of intravascular PMN sequestration, as noted previously in primates given injections of endotoxin.[12] Hence, we gave rabbits twice weekly injections for 8 weeks with 2 × 10^8 peritoneal PMNs per injection per animal. At sacrifice 24 hours after the last PMN injection, both uninflated and inflated (Figure 4) lung

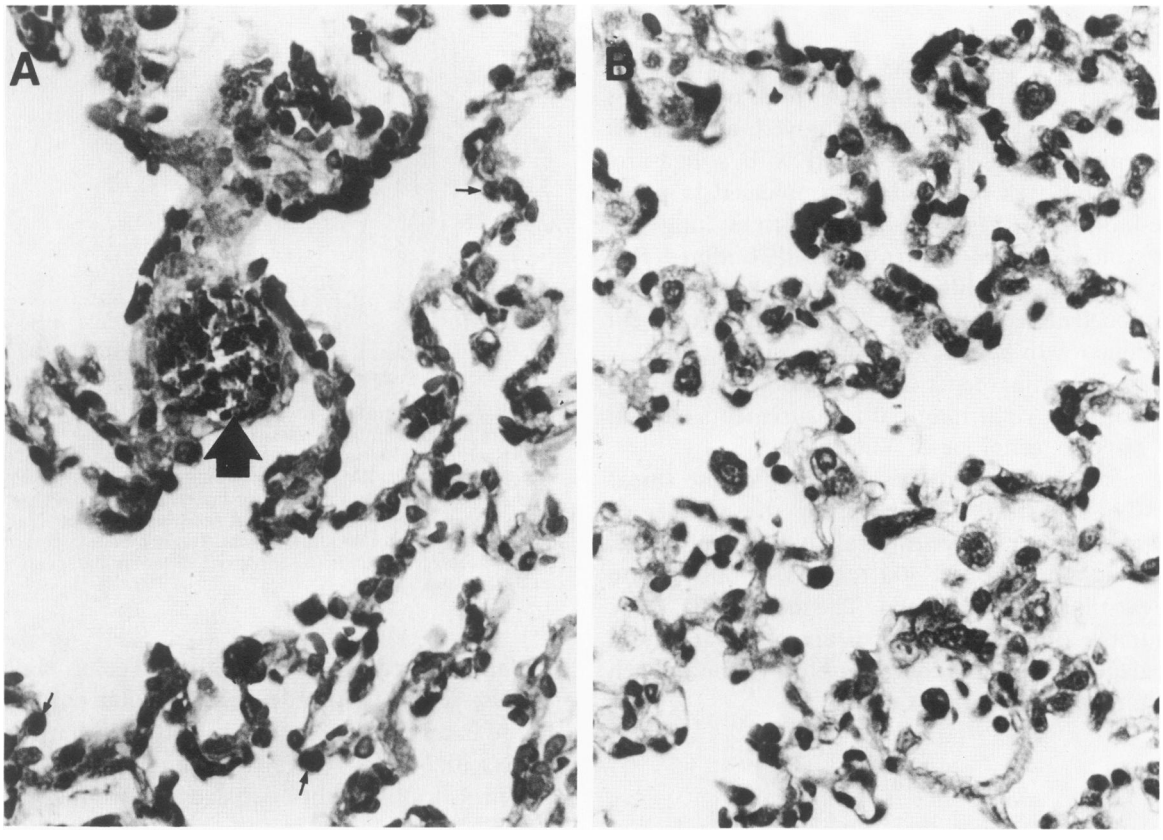

Figure 2—Light-microscopic findings in rabbits injected with ZAS-stimulated PMNs. High-power (400×) views of rabbits 4 hours (**A**) and 48 hours (**B**) after PMN (2×10^8) injection show an absence of edema fluid and cells in the alveolar spaces. The view at 4 hours (**A**) shows the presence of PMNs in a pulmonary arteriole (*large arrow*) and alveolar wall capillaries (*small arrows*). Forty-eight hours later (**B**) very few PMNs still remain in the alveolar wall. (H&E, ×400)

sections failed to show any qualitative changes between animals given buffer (Figure 4A) and those given unstimulated PMNs (Figure 4B) or ZAS-stimulated PMNs (Figure 4C). No evidence of alveolar wall thickening or septal loss was noted when micrographs from groups given PMNs and groups given buffer were compared. The lack of alveolar wall destruction with chronic PMN sequestration in the pulmonary vasculature was confirmed by statistically similar L_m determinations in groups given PMNs and groups given buffer (Table 1).

Discussion

We designed the experiments reported here to examine the injurious effects of infused allogenic PMNs, stimulated by complement-derived fragments *in vitro* and washed free of stimulants prior to injection. In this manner potential injury to the pulmonary vascular bed caused by humoral meditators generated in ZAS was avoided by the removal of the activated serum prior to cell infusion. Previous

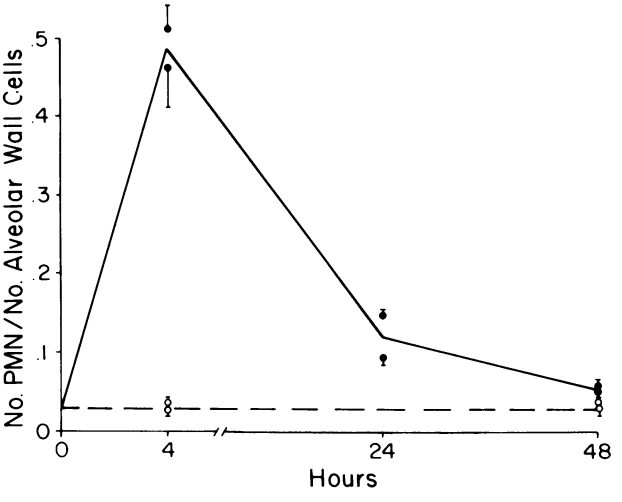

Figure 3—Time course of PMN sequestration in pulmonary vasculature. ZAS-stimulated glycogen-elicited PMNs (*solid line*) or buffer (*broken line*) were injected intravenously in rabbits, and at designated time points two animals each were sacrificed and the lungs were fixed as described in Methods. Each point represents the mean ± SEM of the ratio of PMN nuclei to total alveolar wall nuclei from 10 randomly selected 400× fields in each of 5 lobes for each rabbit, determined as described in Methods.

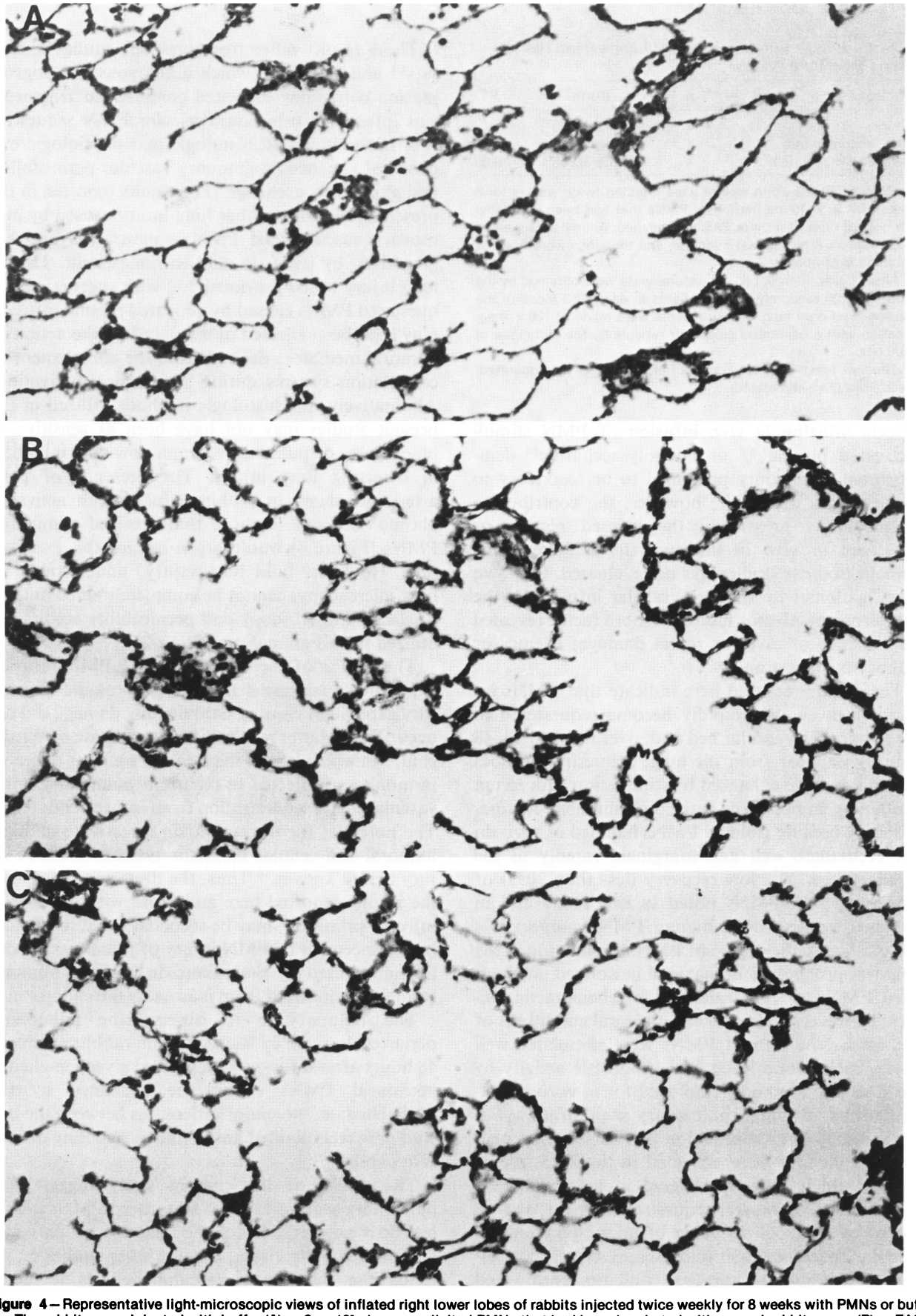

Figure 4—Representative light-microscopic views of inflated right lower lobes of rabbits injected twice weekly for 8 weeks with PMNs or buffer. The rabbits were injected with buffer (**A**) or 2 × 10^8 glycogen-elicited PMNs that had been incubated with normal rabbit serum (**B**) or ZAS (**C**). Inflation fixation was performed with buffered formalin at 25 cm H_2O pressure for all sections. Note the similar alveolar wall thickness and alveolar space size and absence of intraalveolar cells and edema in all three groups. (H&E, × 100)

Table 1—Linear Intercepts (L_m) of Lungs From Rabbits Given PMNs for 8 Weeks

Injection*	n	L_m (mm)†	P‡
Buffer	4	0.075 ± 0.002	–
PMNs (unstimulated)	4	0.069 ± 0.003	>0.05
PMNs (ZAS-stimulated)	4	0.075 ± 0.003	>0.05

* Rabbits 2.0–2.5 kg in weight were injected twice weekly for 8 weeks with 2 × 10^6/ml peritoneal PMNs that had been incubated with normal rabbit serum or ZAS and washed. Animals were sacrificed 24 hours after the last injection, and the lungs were fixed as described in Methods.

† Mean linear intercept (L_m) measurements were obtained by the counting of 20 randomly selected fields in each of 3 lobes of the inflation-fixed right lung. Measurements were made at 100× magnification with a calibrated cross-hair reticule by the technique of Thurlbeck.

‡ Student *t*-test—PMNs versus buffer and PMNs (unstimulated) versus PMN (ZAS-stimulated).

reports utilizing *in vivo* infusion of PMN stimuli (activated plasma[1,2,5] or lipopolysaccharide[13] demonstrated lung injury presumed to be secondary to sequestration of PMNs; however, the contribution of mediators present in the infused plasma or generated *in vivo* in inducing the observed lung damage in those studies was not evaluated. Our own investigations with the intravascular infusion of the complement activator molecule cobra factor revealed no evidence of alveolar septal damage, edema, or leukocyte emigration.[14]

The results reported here indicate that PMNs infused intravenously rapidly become sequestered in the pulmonary vascular bed and, over the next 24–48 hours, disappear from the lung. Sequestration does not appear to be enhanced by stimulation with serum containing complement-derived chemotactic factors, probably because isolated PMNs handled *in vitro* do not recirculate well and marginate rapidly in the capillary bed. The low recovery (less than 20%) of circulating ^{51}Cr-PMNs noted in this study and in previous studies[15] with human PMNs support this notion. Hence the degree of PMN margination in the lung was probably near maximal in isolated unstimulated PMNs and not enhanced by chemotactic factors. However, under the experimental conditions of this study, the infused PMNs were stimulated well above background levels of chemotactic activity by the ZAS (see Methods) employed for *in vitro* activation. Thus, although pulmonary sequestration was unaltered by ZAS stimulation of PMNs, other neutrophil functions were activated in the ZAS-treated PMNs, which were sequestered in the pulmonary vascular bed. However, sequestration is not accompanied by histologic evidence of migration from the vascular space nor light-microscopic evidence of injury to the alveolar capillary wall (ie, edema and hemorrhage).

These results differ from previous studies in rabbits[2,5] and sheep,[1] in which autologous or allogenic plasma containing activated complement fragments was infused, causing intravascular PMN sequestration in the lung and histologic and physiologic evidence of enhanced pulmonary vascular permeability and altered gas exchange. The results reported in the present study suggest that lung injury caused by pulmonary vascular bed PMN sequestration and aggregation, by itself, is mild to nonexistent. Hence, lung injury noted previously[1,2,5] with aggregated, sequestered PMNs caused by activated plasma infusion may have been effected or enhanced by the action of humoral mediators derived from the complement or coagulation systems during complement activation. Alternatively, the histologic methods utilized in the present studies may not have been as sensitive as blood gases or pulmonary lymph flow determination in detecting lung injury. The presence of periarteriolar edema in rabbits infused with activated plasma[1] but not in those that received stimulated PMNs (Figure 4) would argue against this explanation. However, mild lung injury, undetectable by light microscopy, cannot be completely ruled out until electron microscopy and permeability studies are utilized in this animal model.

The results of the 8-week chronic PMN infusion experiments suggested that a subhistologic lung injury capable of causing chronic lung damage did not occur. These latter results differ from those of Wittles et al,[13] in which loss of the alveolar wall was observed in monkeys subjected to recurrent pulmonary intravascular PMN sequestration from infused endotoxin. The potential for the endotoxin to activate multiple humoral and cellular mediator systems of tissue injury is well known.[16] Thus, the discrepancy between the results reported here and those with endotoxin-injected primates[13] may be secondary to direct injury or enhancement of PMN injury of pulmonary endothelium caused by the endotoxin itself or humoral mediators generated from plasma by the endotoxin.[16]

The failure here to observe the pulmonary periarteritis noted by Richerson[17] in rabbits sacrificed 48 hours after infusion with washed glycogen-elicited peritoneal PMNs cannot be explained by the available data. No major differences between the two studies in technique of harvesting or handling of cells is apparent.

The results of this present study suggest that pulmonary sequestration of stimulated PMNs in rabbits does not cause histologic lung injury. This suggestion is not surprising, because early studies established the pulmonary vascular bed as a major repository for margination of PMNs in blood.[7] If in-

jury is to occur, some other mediator must alter the pulmonary vascular bed. Studies by Webster et al[14] in rabbits given injections of cobra venom factor support this notion. Alternatively, if sequestered or circulating PMNs are stimulated, by chemoattractants, to migrate out of the vascular bed into the interstitium and alveolar space, edema and hemorrhage do occur.[9] Hence, the potential of PMNs to injure alveolar walls appears dependent on the ability of the PMN itself or its toxic products to cross the pulmonary capillary endothelium.

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