Prevention by Granulocyte Depletion of Increased Vascular Permeability of Sheep Lung following Endotoxemia

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ABSTRACT To see whether circulating granulocytes are necessary for the lung vascular reaction to endotoxin, we measured the endotoxin response in chronically instrumented sheep before and after granulocyte depletion with hydroxyurea. Granulocyte depletion did not affect the pulmonary hypertension caused by endotoxin (peak mean pulmonary artery pressures = 38±2 cm H_2O before depletion and 42±2 after depletion, P = NS). The late phase increase in lung lymph flow after endotoxin was significantly lower in the granulocytopenic animals as reflected by lung lymph flow (mean steady state lymph flow before depletion = 30.6±2.0 SE ml/h; mean steady state lymph flow after granulocyte depletion = 15.4±1.0; $P < 0.01$) even though late phase pulmonary vascular pressures were similar before and after granulocyte depletion. Lung lymph protein clearance (lymph flow × lymph/plasma protein concentration) was also significantly lower after granulocyte depletion (mean steady state before depletion = 21.4±1.4 SE ml/h; and after depletion = 10.4±1.0; $P < 0.01$). We conclude that circulating granulocytes are necessary for the development of increased lung vascular permeability to fluid and protein following endotoxin. The pulmonary vasopressor effects of endotoxin in sheep are independent of granulocytes.

INTRODUCTION

Infusing *Escherichia coli* endotoxin into unanesthetized sheep causes transient severe pulmonary hypertension followed by a long period of increased lung vascular permeability to fluid and protein (1). During the early part of this reaction leukocytes, primarily granulocytes, are sequestered in pulmonary vessels (2–8).

Previous studies attempting to implicate granulocytes as mediators of the pulmonary vascular reaction to endotoxin have been inconclusive (2–4). We showed, in sheep, that granulocyte depletion prevented increased pulmonary vascular permeability after autologous complement-activated plasma infusion (9). Since endotoxin activates complement (5), and activated complement infusion also causes pulmonary leukostasis, we measured pulmonary vascular responses to endotoxin in unanesthetized sheep before and after granulocyte depletion with hydroxyurea. Pulmonary hypertension following endotoxin infusion was not affected by granulocyte depletion, but in the absence of granulocytes, the increase in lung vascular permeability was diminished. We conclude that circulating granulocytes are required for the endotoxin induced increase in lung vascular permeability, but that the pulmonary hypertension is mediated in some other way.

METHODS

Experimental preparation. We prepared yearling sheep as previously described for collecting lung lymph and measuring vascular pressures (10–14). Briefly, through three thoracotomies we put catheters into the left atrium, pulmonary artery, and the efferent duct from the caudal mediastinal lymph node. We ligated the tail of the node at the lower margin of the inferior pulmonary ligament to eliminate nonpulmonary lymph (12). We also put catheters through neck vessels into the superior vena cava and aortic arch. The initial experiments were performed 3–5 d after recovery from surgery when the animals were fully recovered and there was a stable flow of blood free lymph.

Experimental protocol. All sheep were unanesthetized and standing throughout each experiment. We continuously recorded mean pulmonary arterial, left atrial, and aortic pressures using strain gauges (Micron Instruments, Inc., Los Angeles, Calif.) and an electronic recorder (Hewlett-Packard Co., Palo Alto, Calif.). The zero reference was the estimated level of the left atrium. We measured lung lymph flow by

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measuring the amount accumulated in a heparinized graduated centrifuge tube each 15 min. We collected heparinized aortic blood each hour and pooled lymph each 0.5 h and measured their protein concentrations after centrifuging out the formed elements.

In all experiments, we used the same lot of endotoxin prepared according to the Westphal method by Difco Laboratories from *E. coli 0127:B8*. We dissolved endotoxin in 20 ml sterile pyrogen free 0.89% NaCl for each series of experiments. Between experiments the endotoxin was stored in a sterile syringe at 4°C.

Each experiment was identical. Before infusing endotoxin we measured all variables during a 1 and 1.5–2-h base-line period. *E. coli* endotoxin (0.5 μg/kg of body wt) was then infused over 20 min into the superior vena cava catheter by a constant infusion pump (Harvard Apparatus Co., Inc., Millis, Mass.) and the course of the reaction was followed for 4–5.5 h.

Granulocyte depletion. We depleted sheep of granulocytes using hydroxyurea (Squibb & Sons, Inc., E. R., Princeton, N. J.). We dissolved 4.0 g of pure hydroxyurea in 60 ml of 0.89% pyrogen free NaCl solution which was then filtered through a millipore filter. Three sheep, after the initial endotoxin experiment, were given 4 g of hydroxyurea intravenously each day until the peripheral blood granulocyte count decreased to <10% of the base-line value or to an absolute count of <300 cells/mm^3 and then a second endotoxin experiment was done. In two sheep hydroxyurea was started 2 d before the initial endotoxin study, and the second study was again performed once the peripheral blood granulocyte count decreased to <10% of the base-line value. In one sheep a third endotoxin study was performed once the peripheral blood granulocyte count returned to normal.

Other methods. White blood counts were done by hand using a hemocytometer. Differential counts were performed on Wright stained smears. We counted total leukocytes and granulocytes in pulmonary arterial blood samples before each endotoxin infusion and in simultaneously collected pulmonary arterial and aortic blood at 0.5-h intervals during each endotoxin experiment. Similar counts were made in pulmonary arterial blood samples daily after hydroxyurea was started. Peripheral blood platelet counts were done before each endotoxin infusion.

Protein concentrations in blood and lymph samples were determined by the Biuret method (15) with an automated system (AutoAnalyzer, Technicon Instruments, Tarrytown, N. Y.). Duplicate determinations differed by <5%.

Statistics. We compared variables measured in the same animals before and after granulocyte depletion using a paired t test (16). We interpreted $P < 0.05$ as significant.

RESULTS

Granulocyte depletion. Hydroxyurea primarily depleted granulocytes; granulocyte depletion required 4–11 d. The effects of hydroxyurea on blood leukocyte and platelet counts are shown in Fig. 1. Total leukocyte counts fell by 72% while granulocyte counts decreased by 94% and mononuclear cell counts decreased only 35%. Platelet counts decreased an average of 21% but the change from baseline was not significant.

In the two sheep given hydroxyurea for 2 d before the initial endotoxin study the leukocyte counts on the day of the first endotoxin study were decreased by 54% (from 10,013 to 4,562 cells/mm^3) and the granulocytes by 61% (from 6,586 to 2,583).

Endotoxin response. Fig. 2 shows the time-course of the fall in pulmonary arterial blood total leukocyte and granulocyte counts after endotoxin in the experiments done before granulocyte depletion. Within 15 min after beginning the endotoxin infusion, both total leukocyte and granulocyte counts fell rapidly, reaching a nadir at 1 h with granulocyte counts remaining <10% of baseline for several hours. Average granulocyte counts were lower in left atrial than in pul-

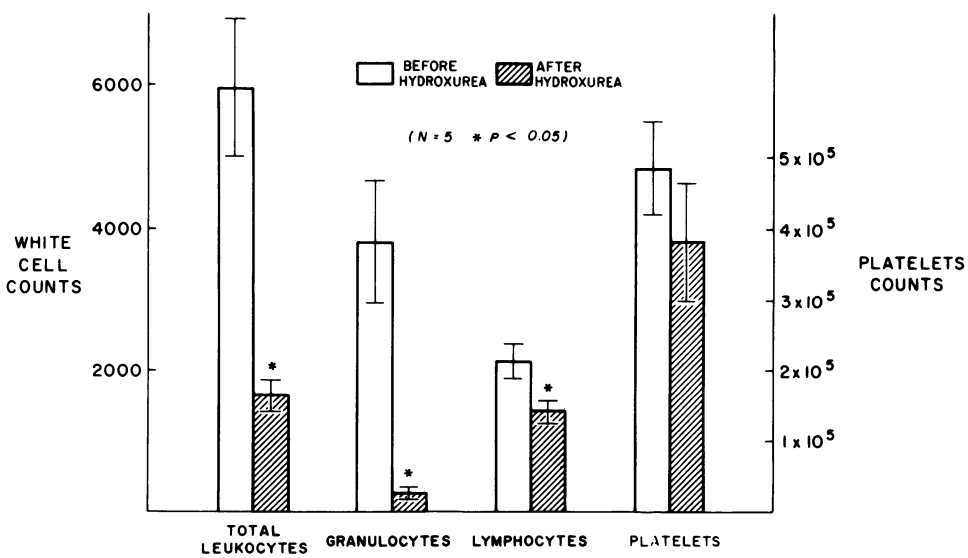

FIGURE 1 Effects of hydroxyurea on blood leukocyte and platelet counts in unanesthetized sheep (see text for hydroxyurea treatment protocol).

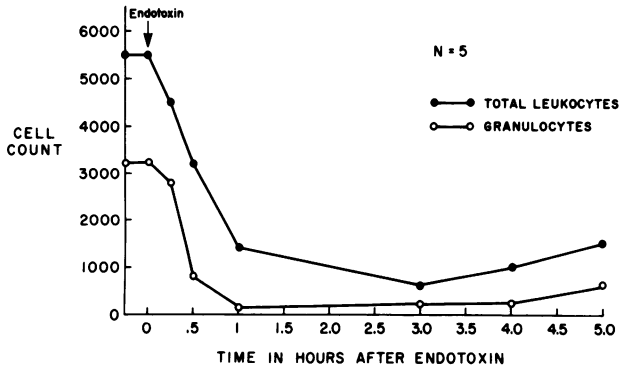

FIGURE 2 Effects of endotoxin on blood total leukocyte and granulocyte counts in unanesthetized sheep.

monary arterial blood at 15 min (pulmonary artery = 2,867 cells/mm^3, left atrium = 2,418 cells/mm^3) and 30 min (pulmonary artery = 837 cells/mm^3, left atrium = 531 cells/mm^3) after endotoxin, suggesting pulmonary sequestration.

Table I summarizes the endotoxin responses in the presence and absence of granulocyte depletion. Baseline pulmonary vascular pressures, lung lymph flows and lymph and plasma protein concentrations were unaffected by granulocyte depletion with hydroxyurea. Effects of endotoxin on pulmonary vascular pressures were the same whether or not circulating granulocytes were present, that is, the early marked increase in pulmonary artery pressure occurred to a similar degree in both situations. In contrast, the late phase steady-state increase in lung lymph flow was much less when animals were granulocyte depleted, even though pulmonary vascular pressures were similar in the two situations.

The time-course of the pulmonary arterial pressure and lung lymph flow responses to endotoxin with and without granulocyte depletion are compared in Fig. 3. Pulmonary arterial pressure increased a similar amount in the two situations and the time-course of the response was also similar. Lung lymph flow increased less during granulocyte depletion and the differences were significant throughout the late phase steady-state portion of the response.

Fig. 4 shows lung lymph protein clearance (lymph flow × lymph/plasma concentration) over the course of the endotoxin reaction before and after granulocyte depletion. Clearance combines lymph flow, which reflects transvascular fluid movement and the lymph to plasma protein concentration ratio that reflects transvascular solute movement. Effects of endotoxin on lung lymph protein clearance were significantly less after granulocyte depletion throughout most of the reaction. Before depletion steady-state lung lymph protein clearance increased threefold but after depletion protein clearance increased to less than one and one half times baseline.

Fig. 5 shows the average late phase steady-state values for pulmonary artery pressures and lymph flow from three endotoxin experiments performed in one animal. One study was done before granulocyte depletion, another after depletion, and a third after the peripheral blood granulocyte count had returned to baseline. In the initial study steady-state pulmonary artery pressures after endotoxin were on the average 45% above baseline, in the depletion study 30% above baseline and in the recovery study 12% above baseline. Even though the mean pulmonary artery pressures were lower in the recovery study, the steady-state lung

TABLE I

*Summary of Pulmonary Vascular Responses to Endotoxin before and after Granulocyte Depletion**

Experimental group	N	Mean pressure: Pulmonary artery	Mean pressure: Left atrium	Lung lymph flow	Protein concentration: Plasma	Protein concentration: Lymph	Protein concentration: Lymph/plasma
		cmH_2O		*ml/h*		*g/dl*	
Normal granulocytes	5						
Baseline		23±1	6±1	8.0±0.8	5.7±0.2	3.9±0.2	0.67±0.02
Endotoxin							
Phase I		38±2‡	3±2*	25.8±3.4‡	6.0±0.2	3.4±0.2	0.56±0.02
Phase II		30±1‡	3±1*	30.6±2.0‡	5.3±0.1	3.7±0.2	0.71±0.02‡
Granulocyte depleted	5						
Baseline		26±1	4±2	6.6±0.8	6.0±0.2	3.8±0.2	0.64±0.02
Endotoxin							
Phase I		42±2‡	1±1‡	18.0±1.8‡§	5.9±0.1	3.5±0.1	0.58±0.01
Phase II		32±1‡	3±1	15.4±1.0‡§	5.7±0.1	3.8±0.1	0.69±0.01‡

* Data represent mean±SEM

‡ Significantly different from baseline ($P < 0.05$)

§ Significantly different from group with normal granulocyte counts ($P < 0.05$)

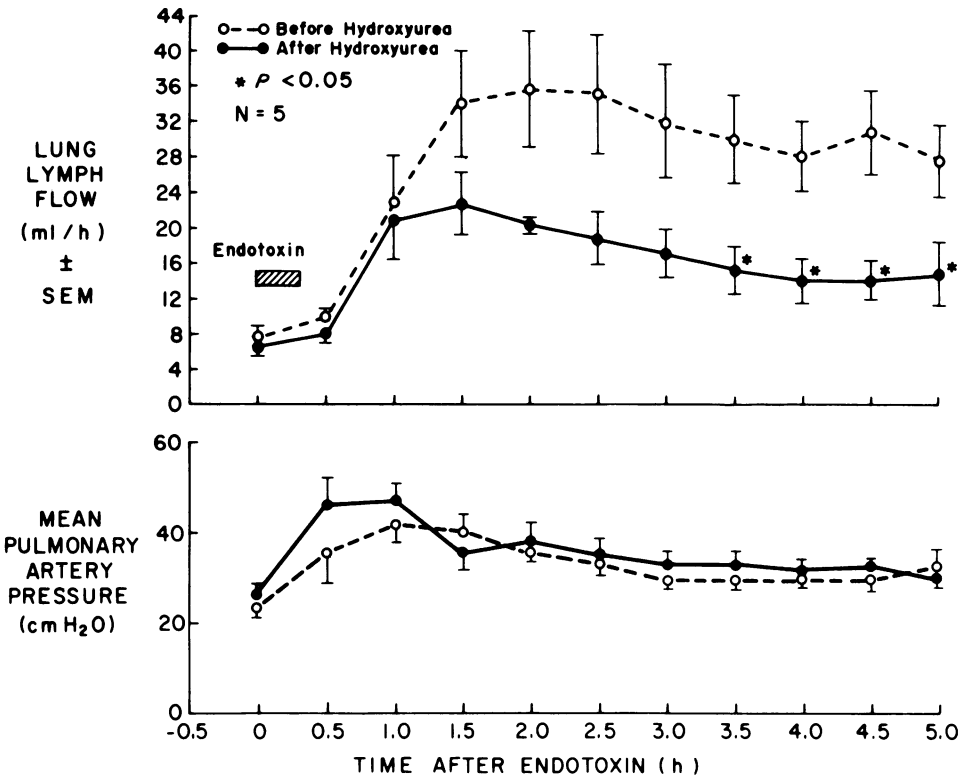

FIGURE 3 Effects of endotoxin on lung lymph flow and pulmonary artery pressure in sheep before and after granulocyte depletion with hydroxyurea. The zero time values are averaged over 1–2 h steady-state base-line period.

lymph flow approached the level of the initial (before depletion) study. The steady-state lymph flow response was also higher than steady-state lymph flow in the granulocyte depleted study, even though, again, the pulmonary artery pressures in the recovery study were lower.

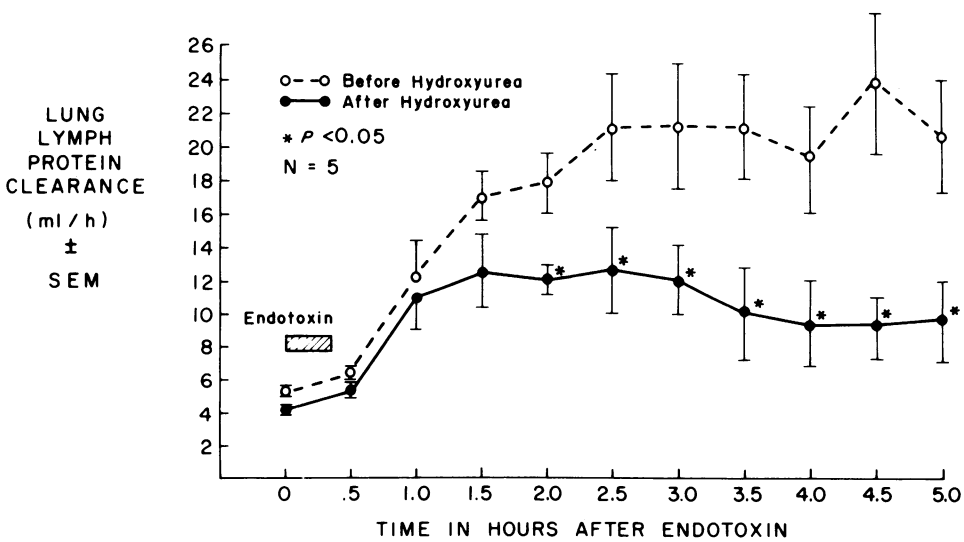

FIGURE 4 Effects of endotoxin on lung lymph protein clearance (flow × lymph/plasma concentration) in sheep before and after granulocyte depletion with hydroxyurea. The zero time values are averaged over 1–2 h steady-state base-line period.

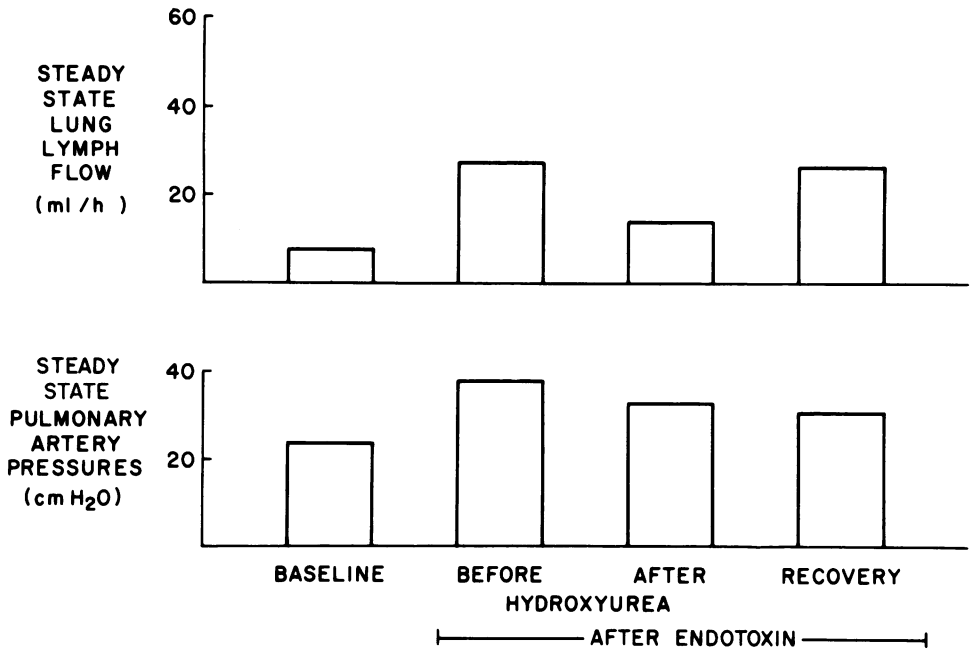

FIGURE 5 Average steady-state pulmonary artery pressure and lung lymph flow during baseline and following endotoxin infusion in three studies in the same sheep. Baseline data were similar in all three studies and are averaged together. Experimental values are averaged over 2-h steady-state during the late phase of the endotoxin reaction. Endotoxin responses before and after granulocyte depletion with hydroxyurea and again following return of blood leukocyte count to baseline values are shown.

The initial response to endotoxin in the two animals treated with hydroxyurea for 2 d before the initial endotoxin study was similar to the first endotoxin study in the three animals that had received no hydroxyurea. In the two animals that had received hydroxyurea before their first endotoxin infusion (they were not yet granulocyte depleted), mean pulmonary artery pressure was 32±2 SE cm H_2O, and lung lymph flow was 16.8±0.9 SE ml/h during the late steady-state phase of the endotoxin reaction; in the three animals that had not received hydroxyurea pulmonary artery pressure = 30±1 SE cm H_2O and lung lymph flow = 14.3±1.6 SE ml/h during the same period after endotoxin.

DISCUSSION

When *E. coli* endotoxin is infused intravenously into unanesthetized sheep it initially causes pulmonary hypertension followed in several hours by a prolonged late phase of high flow of protein rich lung lymph. The reaction is dose-related and reproducible for up to three studies in a given sheep (1). There is a sizeable body of data supporting the assumption that lung lymph flow and protein concentrations under steady-state conditions reflect the flow and protein concentration of transmicrovascular filtrate in the lung (17–19). Therefore the high flow of protein rich lymph seen during the steady-state late phase of the endotoxin reaction is interpreted as increased lung vascular permeability to fluid and protein (1). Similar changes might also be expected with increased exchanging vessel surface area.

Our present study shows that lung lymph flow and lung lymph protein clearance during the late phase of increased vascular permeability after endotoxin are significantly lower in granulocyte depleted animals than in the same animals with normal blood granulocyte counts. In agreement with other reports in the literature (2, 7), pulmonary hypertension following endotoxin was not altered by granulocyte depletion. Since pulmonary artery pressures were equivalent with and without granulocyte depletion the lower lung lymph flow and lung lymph protein clearance in the granulocytopenic sheep is possibly due to lower lung vascular permeability. Other possible effects which could account for lower lymph flow and protein clearance would include lower vascular surface area, depressed lymphatic function, and possibly alterations in the lung interstitium.

We do not believe that our results can be explained by endotoxin tolerance. We have previously shown that giving endotoxin every other day over an 8-d period to sheep does not diminish the increased lung vascular permeability response (1). In four of the five sheep in the present study, the second endotoxin study (during

granulocyte depletion) was performed within 5 d of the initial study. The one sheep in which the granulocyte depleted study was done more than 8 d after the initial endotoxin study was the animal in which endotoxin was given a third time after the granulocyte count had returned to normal. At that time, the lung lymph response to endotoxin was similar to that in the initial study (Fig. 5). Tolerance to the lung vascular permeability effects of endotoxin did not occur.

We also do not believe that moderation of the increased permeability response to endotoxin in the granulocytopenic animals resulted from an effect of hydroxyurea other than granulocyte depletion. In two sheep, hydroxyurea was begun 2 d before the initial endotoxin study. At the time of the initial study, there was an obvious hydroxyurea effect evidenced by a 50% reduction in granulocytes. However, the endotoxin response in those animals was similar to that in the animals which received no hydroxyurea before their initial endotoxin study.

Hydroxyurea caused relatively specific granulocyte depletion. Blood lymphocyte counts fell by only 30% and blood platelet counts did not change significantly at a time when blood granulocyte counts had decreased to <10% of the baseline value. We conclude, therefore, that the attenuated response to endotoxin after hydroxyurea is most logically attributed to granulocyte depletion.

Jacob and associates (9) have shown in vitro that activated complement (more specifically the C5a cleaved polypeptide) causes endothelial cell injury only when granulocytes are present. In addition, they found that endothelial cell injury was significantly decreased in the presence of superoxide dismutase and catalase and concluded that endothelial cells were injured by superoxides (and possibly other radicals) released by granulocytes in the presence of activated complement. Lysozymes did not appear to cause the injury, although they are released by complement activation and may cause vascular injury (21–23). Jacob and associates have proposed a similar mechanism in increased myocardial capillary permeability caused by cholesterol embolization (24).

We have previously shown a fall in the blood hemolytic complement with gram-negative bacteremia in sheep with a transpulmonary gradient indicating complement consumption in the lungs. The gradient developed coincident with the pulmonary sequestration of leukocytes in the pulmonary vasculature (25). Endotoxin activates complement in vitro (20) and in humans with gram-negative sepsis complement is activated by the alternate pathway (5). These data combined with ours demonstrating that granulocytes are required for the increased lung vascular permeability response to endotoxin support the hypothesis that circulating endotoxin activates complement causing granulocytes to adhere to pulmonary endothelium where local production of superoxides and possibly other free radicals damage endothelium causing increased vascular permeability.

These findings do not rule out possible involvement of other mediators in the lung vascular response to endotoxin. We have shown that H_1 antihistamines reduce the degree of permeability change following endotoxin suggesting some role for histamine as a mediator (26), although the effects of antihistamines are much less than the effects of granulocyte depletion. Also, pulmonary hypertension following endotoxin was not prevented by granulocyte depletion (2, 7), but is prevented by inhibiting prostaglandin synthesis (27). Prostaglandin inhibition also exaggerates the increase in permeability (27). Pulmonary hypertension following endotoxemia does not appear to involve granulocytes and some of the increase in lung vascular permeability may result from events independent of pulmonary leukostasis, but granulocytes do appear to play a major role in the permeability change.

The scientific literature concerning the role of granulocytes in the pulmonary vascular response to endotoxin is controversial. In 1957, Hinshaw (6), using isolated perfused lungs, demonstrated that the increase in arterial resistance, venous resistance, and lung weight following endotoxin infusion, was not seen when the infusate was free of formed elements. There was no significant reaction seen when infusates composed of gelatin, dextran, or plasma were used. Addition of the buffy coat to the perfusate resulted in changes in resistance and lung weights equivalent to those seen with whole blood (28).

Using an isolated in vivo lobe preparation, Kux (2), came to the opposite conclusion. "Blood uptake," arterial resistance, and morphological changes after endotoxin infusion were equivalent with both leukocyte filtered blood and unfiltered blood. Pingleton also showed that after endotoxemia there was no difference in arterial blood partial pressure of oxygen, alveolar-arterial oxygen gradient, and morphological changes in the lungs in Rhesus monkeys rendered leukopenic by radiation compared to normals (4).

These negative results can be explained by considering several of the facts now known about the endotoxin reaction. As seen in our study, the increase in lung lymph flow is greatly reduced, but not totally prevented, by depletion of >90% of the granulocytes. This is not surprising for several reasons. Complete granulocyte depletion was not obtained, therefore, some cells were present to participate in the reaction. The pulmonary artery hypertension (which is not affected by granulocyte depletion) would cause an increase in lung lymph flow independent of any increases in permeability. Also, prostaglandins, complement fragments, platelets, mononuclear cells (generating superoxides,

lysozymes or lymphokines), or even direct cellular effects of endotoxin may contribute to the reaction. Studies measuring only changes in arterial blood gases, lung weights and histological appearance after large doses of endotoxin may not be sensitive enough to detect the substantially lower permeability effect of endotoxin in the absence of granulocytes.

The findings of several investigators are consistent with our data. Craddock and coworkers (9) showed that the increases in lung vascular permeability following infusion of autologous complement-activated plasma in sheep was prevented by granulocyte depletion. These same investigators have also shown that complement-mediated granulocyte aggregation can be inhibited in vitro and in vivo (rat) by pharmacological doses of steroids (29) and previous work from our laboratory using the sheep model has shown that increased capillary permeability following endotoxin is prevented by high doses of methylprednisolone (30).

There is recent evidence that the increase in lung vascular permeability that follows several different insults may require circulating granulocytes. Flick and associates (31) prevented increased lung vascular permeability following air emboli in sheep by granulocyte depletion. Johnson and Malik found less pulmonary edema after glass bead pulmonary embolization in granulocytopenic dogs than in dogs with normal granulocyte counts (32). Recent evidence also implicates granulocytes in the pathogenesis of pulmonary oxygen toxicity (33), a disease in which pulmonary edema, apparently resulting from increased lung vascular permeability, is a prominent finding. These studies of others together with our data suggest that interaction of circulating granulocytes with the lung microcirculation may be a common pathogenic event in responses to a variety of insults that cause pulmonary edema.

In summary, we have shown that the increase in pulmonary vascular permeability following endotoxin infusion in unanesthetized sheep is significantly diminished by granulocyte depletion but that granulocyte depletion has no effect on the pulmonary hypertensive response. We conclude that circulating granulocytes are obligate participants in the sequence of events leading to increased lung vascular permeability with endotoxemia, but that pulmonary vasoconstriction is mediated some other way.

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