Volutrauma activates the clotting cascade in the newborn but not adult rat

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Chan, Anthony, Kusala Jayasuriya, Leslie Berry, Matthias Roth-Kleiner, Martin Post, and Jaques Belik. Volutrauma activates the clotting cascade in the newborn but not adult rat. Am J Physiol Lung Cell Mol Physiol 290: L754–L760, 2006. First published December 2, 2005; doi:10.1152/ajplung.00339.2005.—Coagulopathy and alveolar fibrin deposition are common in sick neonates and attributed to the primary disease, as opposed to their ventilatory support. Hypothesizing that high tidal volume ventilation activates the extrinsic coagulation pathway, we air ventilated newborn and adult rats at low (10 ml/kg) or high (30 ml/kg) tidal volume and compared them with age-matched nonventilated controls. Blood was collected at the end of the experiment for measurement of clot time, tissue factor, and other coagulation factor content. Similar measurements were obtained from lung lavage material. The newborn clot time (44 ± 1) was lower and plasma tissue factor content higher (103.4 ± 0.4 nM; P < 0.01) than adults (88 ± 4 s and 26.6 ± 1.4 units; P < 0.01). High, but not low, tidal volume ventilation of newborns for as little as 15 min significantly reduced clot time and increased plasma tissue factor content (P < 0.01). High volume ventilation increased plasma factor Xa (0.1 ± 0.1 to 1.6 ± 0.4 nM; P < 0.01) and thrombin (1.3 ± 0.2 to 2.2 ± 0.4 nM; P < 0.05) and decreased antithrombin (0.12 ± 0.01 to 0.05 ± 0.01; P < 0.01) in the newborn. Lung lavage material of high-volume-ventilated newborns showed increased (P < 0.01) factor Xa and thrombin. No changes in these parameters were observed in adult rats that were high volume ventilated for up to 90 min. Compared with adults, newborn rats have a greater propensity for volutrauma-activated intravascular coagulation. These data suggest that mechanical ventilation promotes neonatal thrombosis via lung tissue factor release.

THE MAINTENANCE OF ADEQUATE blood flow to small-diameter vessels, prevention of clot formation, and hemostasis hinge on the fine balance between pro- and anticoagulant factors. Clot formation can be triggered by activation of the intrinsic and/or extrinsic pathways. Activation of the extrinsic pathway is observed after tissue injury, and the extent of clot formation is dependent on the ratio of thrombin production and plasma antithrombin (AT) inhibition.

Although uncommon, thrombosis in children is predominately diagnosed in the neonatal period and possibly related to the significantly lower AT plasma levels immediately after birth (16) and the higher vascular potential for tissue factor (TF) production (41). Among others, respiratory distress syndrome (31), shock (32), AT consumption, and impairment of thrombin inhibition (36) are the most important factors predisposing sick newborns to thrombosis. Although most often these conditions present in association with respiratory failure, these coagulopathies are believed to be induced by the primary disease and not the ventilatory support provided to the infants.

Mechanical ventilation can induce lung tissue stretch and injury as part of a process known as volutrauma (5). Although clinically unintended, volutrauma can occur in situations such as the initial newborn resuscitation in the delivery room where monitoring of tidal volume is often unavailable. Such injury is known to result in local alterations in lung compliance and pulmonary edema secondary to capillary leak and are important contributing factors in the pathogenesis of chronic lung disease in neonates (9, 13). Fibrin deposition and the formation of hyaline membranes are commonly found in neonates with surfactant deficiency and are believed to be related to the primary lung condition. Yet, we (10, 11) and others (43) have shown that volutrauma is associated with a rise in lung tissue expression and circulating blood levels of proinflammatory cytokines. The possible causal association between ventilation and thrombosis is supported by the fact that TF can be produced by several lung cells after proinflammatory cytokine (26) and mechanical stretch stimulation (8).

In this study we hypothesized that high tidal volume ventilation may induce the release of lung TF, resulting in activation of the coagulation cascade. Therefore, we studied high and low tidal volume ventilated and nonventilated control newborn rats and measured plasma clot formation time and markers of activation of the extrinsic pathway. To explore whether such a phenomenon was unique to the newborn, we evaluated adult rats subjected to a similar high tidal volume ventilation protocol. We documented volutrauma-induced activation of the intravascular coagulation cascade in the newborn, but not adult, animals.

MATERIALS AND METHODS

Institutional review. All procedures involving animals were conducted according to criteria established by the Canadian Council for Animal Care. Approval for the study was obtained from the Animal Care Review Committee of the Hospital for Sick Children Research Institute.

Animal preparation. Newborns (5–8 days, average body wt 15 g) that were kept with their dam until the day of the experiment and adult Sprague-Dawley rats with an average weight of 300 g were studied. The animals were sedated with an intraperitoneal injection of a mixture of xylazine and ketamine (8 and 80 mg/kg, respectively.) To avoid any further lung injury related to out-of-synchrony spontaneous breathing activity after tracheal cannulation and initiation of mechanical ventilation, the animals were paralyzed with pancuronium bromide (0.1 mg/kg im). In pilot experiments, we have confirmed that...

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this dose of anesthesia is sufficient to maintain the animals pain free for the maximum 60-min duration of the experiments.

Newborn rat ventilation. After a small neck incision, a plastic cannula (22 gauge, 2.5 cm in length) was placed in the trachea and firmly secured with a 4.0 silk suture to prevent air leaks. Chest electrodes were placed, and the animal’s electrocardiogram signal was acquired continuously (Hewlett Packard, Palo Alto, CA) to monitor for cardiovascular stability. The animal was kept on a warm circulating water pad to maintain the body temperature constant at 37°C.

The animals were randomized to receive 60 min of air ventilation with either 10 (low; n = 6) or 30 (high; n = 23) ml/kg tidal volume and 1:1 inspiratory/expiratory ratio. The mechanical ventilatory parameters utilized are shown in Table 1. The different mechanical ventilator rates for the high and low tidal volume groups were chosen to avoid hypocapnia in the former. Nonventilated newborn animals served as controls (n = 12). To evaluate the effect of high tidal volume ventilation duration on clot time formation, animals were also ventilated for 15 (n = 6) and 30 (n = 13) min at the same settings described above. We have previously shown that the higher tidal volume ventilation strategy results in lung injury in the newborn and adult rat (10, 11, 23).

A custom-made, previously described mechanical ventilator was used (44). Briefly, the ventilator incorporated a 24-cm-long capillary tube, with a very small internal diameter (0.025 cm), that was placed between a high-pressure gas source and the animal. The animal was connected to the ventilator via a miniature Y piece (dead space 0.02 ml) and plastic tubes of low compliance. One tube was for inspiration, the second one was for expiration, and the third was for connection to a pressure transducer in the ventilator that monitored airway opening pressure. The expiratory circuit was approximately linear in the flow ranges used, and expired volume could be measured by integrating expiratory flow.

In pilot experiments, mixed arterial/venous blood for pH and blood gas measurements was collected after 60-min ventilation and decapitation in the newborn (the total blood volume of the animal was 0.5 ml, and only by decapitation was enough collected for these measurements). The pH was 7.42 ± 0.04 and 7.35 ± 0.03, and the PCO2 was 43 ± 4 and 47 ± 3 Torr for the 10 (n = 3) and 30 ml/kg (n = 6) ventilated animals, respectively.

Adult rat preparation. The adult rats (n = 23) were cannulated and ventilated on air. Nonventilated, briefly anesthetized animals served as controls (n = 18). The mechanical ventilation settings for adult animals are shown in Table 1, and the arterial blood gases after 60-min mechanical ventilation were pH 7.34 ± 0.03, PCO2 38 ± 4 Torr, and PaO2 108 ± 7 Torr (n = 4).

Lung lavage. The newborn animals underwent a modified whole lung lavage previously described for adult rats (28) and adapted for newborn rats (29). Briefly, the lungs were infused with 1 ml of sterile saline, followed by withdrawal and reinfusion two more times. This procedure was repeated a total of three times, and the combined volume of the total lavage was recorded. The total lavage material obtained from each animal (0.5 ml average) was centrifuged at 150 g for 10 min, and the supernatant was collected and immediately frozen for future studies. There were no differences in the total volume of saline infused or recovered after the lavage procedure between the experimental and control groups. Previous studies have shown that this lavage procedure removes >90% of the alveolar surfactant in both normal animals and those with acute lung injury (42).

Blood collection. Blood was obtained from the newborn animals, immediately after removal from the ventilator (experimental) or after short-term (5 min) anesthesia (control), by decapitation and collected in a tube containing 3.2% sodium citrate (volume citrate: blood = 1:9). The blood was centrifuged at 2,000 g for 15 min, and the supernatant plasma was stored at −80°C.

Clot time measurement. Plasma and lung lavage samples were tested for their ability to accelerate recalcified plasma thrombin generation using modifications of an assay previously described by our laboratory (8). Experiments were conducted in 6-mm inner diameter × 50-mm-long borosilicate glass tubes containing a 3- to 4-mm iron ball. For rat plasma samples, 5 μl of Cryo Check normal adult human plasma (Precision Biologic, Dartmouth, Nova Scotia, Canada; added as a reagent to normalize for variation in rat plasma’s coagulation factor levels) were mixed with 25 μl of test sample in a tube heated in a 37°C H2O bath. Thirty microliters of prewarmed 0.04 M CaCl2, 0.016 M sodium acetate, 0.036 M sodium diethyl barbiturate, and 0.145 M NaCl, pH 7.40, were added as a clock was started, and the tube was tilted back and forth to allow movement of the iron ball in the mixture. The clot time was recorded when the ball stopped moving. In the case of lung lavage samples, the same procedure was used as that for plasma, except that 15 instead of 5 μl of normal adult human plasma (added as a source of clotting factors) and 15 μl of lavage material in place of 25 μl of test plasma were used. Decreases in clotting time, compared with that of controls, were assumed to be due to procoagulant material present in the various samples. In some experiments, factor VII (FVII)-depleted human plasma (Affinity Biologicals, Ancaster, Ontario, Canada) was used in place of normal adult human plasma to determine whether decreased clot times were associated with TF-like activity.

TF content. TF in plasma was determined by SDS-PAGE, and Western blot analysis of FVII/FVIIa-TF was isolated by immunoblot. Twenty-microliter aliquots of plasma samples were incubated with protein G-Agarose beads (Sigma, Mississauga, Ontario, Canada) containing bound sheep anti-human FVII IgG that cross-reacts with rat FVII/FVIIa (Affinity Biologicals). After incubation, the beads were copiously washed with buffer, and the FVII/FVIIa-TF was eluted according to the manufacturer. SDS-PAGE of eluted material was carried out according to the method of Laemmli (24). FVII/FVIIa-TF samples run on SDS-PAGE were further subjected to Western blotting. This methodology was utilized because all active TF was bound to the circulating excess FVII/FVIIa, and thus it was imperative to ensure complete TF removal. For this purpose, in the immunoblotting procedure, protein M beads containing vast excess anti-FVII antibody was used to ensure that all uncomplexed FVII and FVIIa-TF complexes were captured to achieve quantitative recovery. Blots were probed with goat anti-human TF polyclonal antibody (American Diagnostica) that has been shown to partially cross-react with rat TF (45). Densitometric analysis was used to determine the relative intensity of TF bands from different samples on the blot. To determine the method specificity, initial control experiments were run in which nonimmune IgG was used in place of the anti-factor VII IgG and did not result in TF bands.

Factor Xa and thrombin content. Concentrations of activated factor X (Xa) and thrombin were measured in plasma and lung lavage samples from ventilated and control rats to assess the effect of age and ventilation on activation of the coagulation cascade. For experiments analyzing plasma samples, 10 μl of plasma plus 80 μl of 0.02 M Tris·HCl, 0.15 M NaCl, and 0.6% (mass/vol) PEG 8000, pH 7.4, plus 10 μl of stock substrate were mixed in 96-well plates, and absorbance readings were taken every 10 s at 405 and 450 nm for 3 h at room temperature. Curves of absorbance at 450 nm, due to background, were subtracted from curves of absorbance at 405 nm, due to substrate cleavage, to give net substrate-cleavage maximum velocity (Vmax).

Table 1. Mechanical ventilation setting for newborn and adult animals

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<th>Newborn</th>
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<td>Tidal volume, ml/kg</td>
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<td>Rate, breaths/min</td>
<td>60</td>
<td>30</td>
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<td>Minute ventilation, ml/kg·min⁻¹</td>
<td>600</td>
<td>900</td>
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<td>Initial peak inspiratory pressure, cmH2O</td>
<td>20</td>
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<td>Positive end-expiratory pressure, cmH2O</td>
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values. The average V\textsubscript{max} values in milli-absorbance U/min were calculated and taken to represent activity of enzyme against either Xa-selective substrate (4.21 mM stock S-2222, diaPharma, West Chester, OH) or thrombin-selective substrate (5 mM stock S-2238, diaPharma). Data from experiments done previously with purified Xa or thrombin (Enzyme Research Laboratories, South Bend, IN), reacted against the same substrates under similar conditions, were used to determine the theoretical concentration of enzyme in the plasma. Similar experiments were carried out with lavage fluid except that 40 \mu l of lavage fluid were reacted with 50 \mu l of stock substrate for 2 h. Negative control experiments were carried out with 100 \mu l of TSP alone in the well or 90 \mu l of TSP plus 10 \mu l of substrate alone in the well.

**Fibrinogen/fibrin degradation.** Aliquots of plasma samples were run on SDS-PAGE under reducing conditions and then transferring to polyvinylidene difluoride membranes by Western blotting. The blot was probed with horseradish peroxidase linked to sheep anti-rat fibrinogen IgG (Affinity Biologicals). Bands from fibrinogen, fibrin, or fibrin(ogen) degradation products were visualized using ECL technology (Perkin Elmer, Boston, MA) according to the manufacturer’s instructions.

**Plasma AT concentration.** Aliquots of 1 \mu l of plasma samples were run on SDS-PAGE, followed by Western blotting as described for analysis of fibrin(ogen) degradation. Blots were probed with polyclonal goat anti-human AT IgG that cross-reacts with rat AT (Affinity Biologicals). AT bands were detected by probing with donkey anti-sheep IgG conjugated to alkaline phosphatase (Sigma), followed by reaction with nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate substrate (Bio-Rad). Relative intensity of the bands was determined by densitometry.

**Statistical analysis.** Results were reported as means \pm SE unless otherwise indicated. Experimental and control data were compared by unpaired t-test (only when 2 groups were involved), one-way or two-way ANOVA, as noted. Multiple comparisons were done by Tukey’s test utilizing the Number Cruncher Statistical Software (Kaysville, UT). Significance was accepted at P < 0.05.

**RESULTS**

Influence of age and ventilation duration on plasma clot time. Plasma clot time in normal nonventilated rats was proportional to age and significantly lower (P < 0.01) in the newborn (44 ± 1 s) compared with the adult (88 ± 4 s). Ventilation induced a significant decrease (P < 0.01) in the newborn plasma clot time in the animals subjected to high, but not low, tidal volume. In contrast, high tidal volume ventilation did not alter the plasma clot time of adult rats (Fig. 1). As little as 15 min of high tidal volume ventilation was sufficient to significantly reduce the time for blood clot formation in the newborn, whereas in the adult a similar injurious ventilatory strategy for up to 90 min showed no change in this parameter (Fig. 1).

Influence of age and ventilation on plasma TF concentration. Plasma TF content was also age dependent and significantly increased (P < 0.01) in the newborn compared with the nonventilated adult (Fig. 2). High tidal volume ventilation more than doubled the plasma TF content in newborn rats, whereas no significant change in the plasma TF level was observed in ventilated adult animals (Fig. 2).

Influence of age and ventilation on activated coagulation factors and coagulation inhibitors. Comparing the nonventilated newborn and adult rats, we noted no age-related statistically significant difference for the Xa plasma level (Fig. 3A), whereas the thrombin content was significantly higher (P < 0.05) in the newborn (Fig. 3B). High tidal volume ventilation significantly increased the newborn plasma Xa (P < 0.01) and thrombin content (P < 0.05), whereas (in spite of a trend) no significant changes (P = 0.17) were observed in the experimental adult animals (Fig. 3, A and B).

For further evidence that mechanical ventilation in the newborn activated the coagulation cascade, we evaluated plasma fibrin(ogen) degradation products and AT concentrations. Low-molecular-weight degradation products were present only in the newborns (Fig. 4). The AT plasma content was significantly reduced in the ventilated, compared with the nonventilated, newborn samples (P < 0.01). No ventilation-induced change in AT content was observed in the adult animals (Fig. 3C).

Bronchoalveolar lavage material. Last, we evaluated the newborn animals’ bronchoalveolar lavage material. As shown
in Table 2, the ventilated animals’ lavage material significantly reduced the in vitro clot time measurement and showed significantly higher Xa and thrombin concentrations (P < 0.01), suggestive of TF release into the alveolar compartment.

DISCUSSION

In the present study we evaluated and compared the newborn and adult rat coagulation parameters using standard techniques. We have found that the factors whose measurements can be compared with values quoted in the literature are in keeping with the previously reported age differences. This is the case for relative plasma levels of AT that, in this study, were found to be twofold higher in adults compared with newborns and similar to the findings in humans (3, 4). The fact that the newborn plasma TF protein and activity concentrations were increased relative to adults is in agreement with what has been reported for human blood immediately after birth, especially in the case of premature neonates (37).

Of note, however, is the fact that due to the increased level of endogenous TF measured in the newborn rat plasma, clot times were shorter than the corresponding values for adult plasma. Detection of these TF-related differences has been overlooked in clinical studies where massive amounts of external activators are added during the assays. When the usual clinical coagulation screening tests were utilized, clot times measured in newborn plasma were actually longer than those of adults due to the decreased plasma concentrations of coagulation factors found in newborns (4).

Lung inflammatory disease is commonly associated with activation of the clotting cascade. This is the case for conditions such as acute lung injury and adult respiratory distress syndrome where intra-alveolar and intravascular fibrin deposition (17, 18), enhanced lung procoagulant activity (15, 35), and TF levels in bronchoalveolar lavage have been demonstrated (1). It has been suggested that the adult lung possesses a cell-specific alveolar coagulation and fibrinolysis system, independent of the vascular coagulation cascade that can rapidly respond to stimuli such as endotoxin exposure (47).

Present evidence supports the existence in newborns of a similar association between lung disease and activation of the clotting system, which is best illustrated by the observation of
hyaline membrane formation in the lungs of infants with respiratory distress syndrome. Such hyaline material results from disease-induced activation of the clotting system in the lung leading to thrombin formation and fibrin deposition in the alveoli (6, 7). That such thrombin release is mediated by the lung’s disease-associated inflammatory processes is supported by the fact that, in premature sheep, granulocyte depletion prevents alveolar hyaline membrane formation in these immature lungs (22).

Whether the inflammation and/or clotting present in experimental newborn animal models of respiratory syndrome (19, 20) is related to the surfactant deficiency, the mechanical ventilation, or a combination of both has not been previously addressed. To our knowledge, the role of mechanical ventilation alone in the extrinsic coagulation pathway activation, independent of any primary lung disease, has not been previously investigated in the neonate.

We and others have previously demonstrated that high tidal volumes have adverse effects in animal models of adult ventilator-associated lung injury (14, 21, 30). In adult acute respiratory distress syndrome, increased tidal volume ventilation is associated with worsened mortality (2, 39), suggesting that ventilation compounds the lung injury in this disease. In the adult rat, high-pressure amplitude mechanical ventilation suppresses alveolar fibrinolytic activity due to local production of plasminogen activator inhibitor (12). We have recently shown that high tidal volume ventilation induces cytokine expression in the rat lung (10), and significant differences in the pattern of upregulation of these proinflammatory factors are observed when comparing newborn and adult animals (11). In addition, the degree of lung injury after high tidal ventilation is significantly greater in the adult compared with the infant rat lung (23).

Lung infection, mechanical ventilation, and activation of the clotting system are commonly clinically associated. In adults with respiratory failure requiring prolonged mechanical ventilation and who ultimately develop pneumonia, thrombin, TF, and FVIIa concentrations in the bronchoalveolar lavage are increased (34). As the main trigger of the extrinsic coagulation pathway, TF plays an important role by being stimulated in life-threatening conditions such as septicemia (27). Under these conditions, endotoxin is known to stimulate TF release (25) and induce cytokine procoagulant activity (1). Specific cytokines known to upregulate TF expression include TNF-α and IL-1β (26). Clotting factors can also act as inflammatory mediators (33), thus challenging the current mechanistic paradigm that disease-induced inflammation is the trigger for activation of the clotting cascade.

Under physiological conditions, TF is produced by cells in the adventitia of normal vessels and only minute amounts are present in the circulation (46). Yet, it appears that TF expression is developmentally regulated. In humans, plasma TF content during fetal life is higher compared with the postnatal period (38), and newborns have a greater vascular potential for TF formation (41). Our data indicate that the rat plasma TF content in the newborn was significantly higher compared with the adult.

In the present study, we further documented that as little as 15 min of high tidal volume ventilation shortens the clot time, suggesting activation of the extrinsic coagulation pathway. That volutrauma is the initiator of such processes is further supported by the increase in lung alveolar fluid factor Xa and thrombin content as well as shortening of lavage material clot time in ventilated newborn animals. Thus our finding that high tidal volume ventilation is associated with activation of the TF-dependent extrinsic pathway provides further support for a causal relationship between ventilation-induced cytokine release in the lung and procoagulant activity in the blood.

The mechanism by which high tidal volume ventilation activates TF release is unclear but is likely related to the magnitude of stretch of lung parenchymal tissue. To this effect,
it is interesting to note that a stretch stimulus comparable to physiological breathing downregulates secretion of TF procoagulant activity by fetal lung epithelial cells (8). Yet a higher degree of stretch, as induced by the high tidal volume ventilation protocol utilized in this study, upregulates TF secretion. Of potential clinical significance is the fact that even a relatively short duration of high tidal volume ventilation is capable of activating the clotting cascade, because there are times such as the initial newborn resuscitation and hand ventilation where tidal volume delivery is higher than desired since it is not routinely monitored.

AT is a plasma glycoprotein, produced by the liver that inactivates thrombin and inhibits several other coagulation factors such as IXa, Xa, XIa, XIIa, plasmin, and kallikrein. In this study, we demonstrated that the nonventilated newborn had decreased AT plasma content compared with the adult. Similar age-related differences are present in humans whereby plasma AT is known to be deficient in sick newborns (31). Although speculative, the data from this study suggest that the developmental differences in TF and AT plasma content may account for the volutrauma-dependent activation of coagulation in the newborn but not adult rat.

Figure 5 illustrates the proposed mechanism by which high tidal volume activates the clotting cascade. The cells responsible for TF release (vascular or lung parenchymal) and the magnitude of stretch required to produce this effect are the objects of current investigation in our laboratory. Yet, it is clear from the data presented in this study that TF’s release results in thrombin deposition in the alveoli and activation of the extrinsic coagulation cascade, likely via cytokine release.

Whether the high tidal volume ventilation-induced lung injuries of the newborn and adult, as employed in this study, are comparable merits further comment. In a recently published study, our laboratory explored the effects of age and lung development on susceptibility to such injury. In that study, we observed that comparable ventilator settings are significantly more injurious in the adult than infant rat lung (23).

In addition we have shown previously that, whereas the newborn can withstand ventilation with a tidal volume of 40 ml/kg, the adult rat succumbs in 20 min under these conditions (11). Utilizing a tidal volume of 30 ml/kg in the present study, we were able to ventilate the adult rat for up to 90 min, beyond which significant mortality was observed. Thus the high tidal volume ventilation-induced changes in clot time of the newborn, but not adult, rat are likely unrelated to the degree of lung injury, given that less damage is expected in the former when the strategy employed in this study is used.

In summary, this is the first study to document that mechanical ventilation in the newborn can promote the release of lung-associated TF and activation of the extrinsic coagulation cascade, resulting in decreased time for clot formation. This age-dependent phenomenon may predispose mechanical ventilation-treated neonates to thrombus formation and fibrin deposition in the alveoli.

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