Time Course of Hemosiderin Production by Alveolar Macrophages in a Murine Model*

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Study objectives: The diagnosis of alveolar hemorrhage is assisted by the presence of hemosiderin-laden macrophages (HLMs) in the BAL fluid or lung tissue. Despite the importance of this diagnostic method in clinical settings, limited information is available on the formation and clearance of HLMs as a function of time. The objectives of this study are to determine the time course of HLMs within the BAL and lung tissue, and to evaluate the effect of a single blood aspiration on the recruitment of inflammatory cells within the BAL.

Design: Under light anesthesia, Balb/c mice received a single intranasal instillation of species-specific blood (50 μL). Control animals received heparinized sterile saline solution in a similar manner. At several time points after blood aspiration, BAL was recovered for cell differentials and determination of HLMs. The time course for HLMs was also established in the lung tissue.

Results: Hemosiderin staining within alveolar macrophages was first detected in the BAL and lung tissue at day 3, peaked at day 7, and persisted through 2 months. The analysis of the BAL revealed an increased number of total cells, with an acute inflammatory reaction that resolved within 2 weeks.

Conclusions: Our findings demonstrate the validity of this model for the study of HLM production after blood aspiration. Additional work using animal models of lung hemorrhage is needed to further characterize the cellular events leading to clearance of erythrocytes within the lung.

Key words: hemosiderin; macrophages; mouse; pulmonary hemorrhage

Abbreviations: HLM = hemosiderin-laden macrophage; IPH = idiopathic pulmonary hemosiderosis

Pulmonary hemosiderosis is an uncommon disorder in both children and adults characterized by recurrent alveolar hemorrhage and associated with significant morbidity and mortality.1–5 Classifications6 of alveolar hemorrhage syndromes categorize the various disorders into those with and without pulmonary capillaritis. Disorders without capillaritis include idiopathic pulmonary hemosiderosis (IPH), mitral stenosis, primary pulmonary hypertension, pulmonary vascular malformations, hypersensitivity to cow’s milk (Heiner’s syndrome), and celiac disease. Furthermore, an association between exposure to fungal agents and acute pulmonary hemorrhage in infants has been suggested.7–10

The diagnosis of alveolar hemorrhage is assisted by the presence of hemosiderin-laden macrophages (HLMs) in the BAL or lung tissue. Despite the importance of this diagnostic method in clinical settings, limited information is available on the production of hemosiderin by alveolar macrophages as a function of time. Previous studies11–13 have provided little information on the appearance of HLMs and did not clearly define the time course for clearance of the hemosiderin. In addition, Sherman et al12 reported that HLMs were not present in the BAL 2 to 4 weeks after an acute episode of pulmonary hemorrhage in two infants.

In this study, we investigated the time course of HLMs within the BAL and lung tissue in a murine model. In addition, we evaluated the effect of a single blood aspiration on the recruitment of inflammatory cells within the BAL.

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**Materials and Methods**

**Experimental Animals and Aspiration**

Eight-week-old female Balb/c mice (Harlan Sprague Dawley; Indianapolis, IN) were used for all studies. All of the procedures were approved by the Animal Care and Use Committee of the University of Texas Health Science Center and conformed to National Institutes of Health guidelines.

Mouse blood mixed with heparin, to prevent clotting, was used as the aspiration media. After the mice were anesthetized with an intraperitoneal injection of ketamine, 200 mg/kg, and xylazine, 10 mg/kg, 50 μL of blood was placed in the nose of quietly breathing mice while the mouth was closed. This technique is a modification of methods previously described by our laboratory that allows instillations of exogenous substances into the mouse lung. Control animals received heparinized sterile saline solution (50 μL) in a similar manner. Mice were studied at nine time points (2 to 60 days) after blood aspiration (n = 3 to 6 at each time point).

**Lung Lavage and Cell Differentials**

After anesthesia with ketamine and xylazine, a polyethylene catheter (3.0 cm in length and 0.81 mm in diameter) was inserted into the trachea through a cervical incision. Lung lavage was performed with three successive aliquots of 0.6 mL of phosphate-buffered saline solution. Cell counts were determined using a Coulter counter. Following cell isolation, 50 × 10^6 cells were placed in sterile cytofunnels and centrifuged at 1,000 revolutions per minute for 5 min onto positively charged glass slides (Superfrost/plus; Fisher Scientific; Pittsburgh, PA) using a centrifuge (Cytospin 2; Shandon; Pittsburgh, PA). The cells were stained with Leukostat (Fisher Scientific) and evaluated by light microscopy (Olympus Bx60; Olympus; Tokyo, Japan). Two hundred random cells were counted; characterized as macrophages, lymphocytes, neutrophils, eosinophils, or other; and divided by two. Results were expressed in terms of total number of cells (mean ± SE).

**Determination of HLMs**

Slides of centrifuged cells were prepared as described above. Immediately following centrifugation, slides were fixed with 95% ethanol for 15 min. Staining for hemosiderin (a complex of ferric iron, lipid, protein, and carbohydrate) was then performed using the Prussian blue reaction. The Prussian Blue (Perls) reaction stains for iron when it exists in its ferric form. This material reacts with potassium ferrocyanide to form a blue colored compound, ferriferoxyanide, thus a positive test. The cells were examined for presence of granules within intact alveolar macrophages using a microscope and scored by a previously described method. Two hundred random cells were counted by two investigators and divided by two. Results were expressed in terms of the percentage of positive cells (mean ± SE).

**Lung Histology**

After the mice were anesthetized, a catheter was inserted into the trachea through a cervical incision. The lungs were inflated with 1 mL of 10% formalin, excised from the chest, and fixed in 10% formalin. Lung sections were stained with hematoxylin–eosin for light microscopy. Staining for hemosiderin was performed using the Prussian blue reaction. The airways and lungs were examined for evidence of inflammation and injury. The time course of HLMs within the lung tissue was also determined.

Statistical Analysis

All data were expressed as mean ± SE. Comparisons were done by paired and unpaired Student’s t test or one-way analysis of variance where appropriate. A p value < 0.05 was considered significant.

**Results**

**Analysis of BAL**

The complete time course of hemosiderin staining within alveolar macrophages in the BAL is shown in Figure 1. HLMs were first detected in the BAL at day 3, with 2.8 ± 1.2% of the cells staining positive for iron. Peak staining for hemosiderin in the BAL, 60.0 ± 4.2% of the alveolar macrophages, was detected at 7 days and persisted through 10 days. By 60 days, the number of positive cells had decreased to 10.3 ± 1.4%. Control animals were examined at 2 days and 7 days after heparinized sterile saline solution aspiration and did not demonstrate positive staining for hemosiderin. Figure 2 illustrates iron staining of alveolar macrophages in the BAL from control and study animals (top left, A, control; middle left, B, 7 days after aspiration; bottom left, C, 60 days after aspiration).

The results of the total BAL cell count at various time points are shown in Figure 3. The cell counts were significantly higher from 2 through 14 days after aspiration of blood as compared to control values (p < 0.05). Figures 4–6 show the effect of blood aspiration on macrophages, neutrophils, and lymphocytes in the BAL. The number of macrophages was significantly higher than controls from 2 through 14 days (Fig 4, p < 0.05). After 2 days, the number of neutrophils (Fig 5) and lymphocytes (Fig 6) was significantly increased in the BAL when compared to control values: 4.8 ± 0.8 × 10^4/mL and 1.9 ± 0.3 × 10^4/mL, respectively (p = 0.01). By day 5, the number of neutrophils and lymphocytes decreased to 0.5 ± 0.2 × 10^4/mL and 1.0 ± 0.1 × 10^4/mL, respectively. Although BAL neutrophils returned to control values by day 5, the number of lymphocytes remained elevated through day 10 (Fig 6).

**Histopathologic Studies**

Whole lung sections demonstrated evidence of an acute inflammatory reaction 2 days after blood aspiration. There was a mild peribronchiolar infiltration of a mixed inflammatory cell population, consisting of both polymorphonuclear and lymphocytic cells. In addition, patchy areas of a mixed inflammatory infiltrate were also demonstrated in the parenchyma. These histologic changes resolved by 5 days after aspiration of blood.
Figure 2 illustrates representative sections of iron staining within the lung tissue in control animals (Fig 2, top right, D) and study animals (Fig 2, middle right, E, and bottom right, F). A few HLMs were first visible at day 3 (not shown). From days 4 through 14, a large number of HLMs were detected. After 60 days, iron staining within lung macrophages substantially decreased. As shown in Figure 2 (middle right, E), numerous HLMs were detected in clusters, mostly in the alveolar spaces with a few in the interstitium. Although the amount of clusters decreased, this pattern persisted through 2 months (Fig 2, bottom right, F). Of interest, the iron staining in lung macrophages paralleled the findings obtained in the BAL.

**DISCUSSION**

IPH is an uncommon disorder characterized by widespread hemorrhage from the microvasculature of the lung into the airspaces. Virchow first described this condition as “brown lung induration” in 1864. IPH presents primarily in infancy and childhood and causes a significant amount of morbidity and mortality. Patients often require long-term therapy, with potentially serious side effects. One study showed a 5-year survival rate of 86%, with massive pulmonary hemorrhage most often the cause of death. A subsequent study confirmed a better prognosis for IPH than previously documented, with a mean duration of survival of 17.2 years after diagnosis. Suggested mechanisms for the development of recurrent bleeding include a structural defect in the alveolar capillary bed, an immunologic defect that interferes with the integrity of the alveolar capillaries, and genetic or environmental factors. However, the precise etiology of IPH remains unknown.

The diagnosis of pulmonary hemosiderosis is suggested by hemoptysis, iron-deficiency anemia, and widespread, patchy intra-alveolar infiltrates on chest radiographs. The diagnosis is usually established or confirmed by the detection of HLMs in gastric aspirate, BAL, or lung biopsy specimen. The detection of HLMs in BAL has been shown to reliably diagnose alveolar hemorrhage in adults, but it does not help to identify the cause of bleeding. In a pediatric study of IPH, HLMs were detected in the BAL in all patients who underwent this procedure. In addition, the presence of HLMs may be a poor prognostic factor for patients requiring bone marrow transplantation.

Although an important diagnostic method in clinical settings, limited information is available on the formation and clearance of HLMs. In a rabbit model, aspirated blood alone did not lead to the formation of hemosiderin by alveolar macrophages. However, the addition of a hypertonic
solution of dextrose to the blood resulted in lung injury and the production of hemosiderin by alveolar macrophages. Although this study showed that HLMs were first visible 24 h after blood/dextrose aspiration and persisted through 14 days, the clearance of the hemosiderin from the alveolar macrophages was not determined. Another study utilizing human alveolar macrophages incubated with sheep erythrocytes in vitro demonstrated hemosiderin formation at 3 days with more intense staining shown at 5 days. This study also did not evaluate the time course for clearance of the hemosiderin. A recent study in a rat model designed to evaluate iron disequilibrium after instillation of blood into the trachea showed that hemosiderin within the lung tissue cleared after 2 weeks. While this work revealed new insights into potential mechanisms involved in the pathogenesis of lung injury in response to blood aspiration, a detailed description of the changes in iron staining within the lung was not a primary end point of this study. In this respect, the minimal iron staining...
found in rat lung differs from the clinical experience in which significant amounts of HLMs develop in patients after a pulmonary hemorrhage. The formation and clearance of HLMs in humans have not been well studied. After reviewing data obtained from two infants with pulmonary hemorrhage, Sherman et al.\textsuperscript{12} suggested that the clearance of hemosiderin from alveolar macrophages occurred within 2 to 4 weeks. However, a high hemosiderin score was found up to 14 weeks

Figure 3. Effect of blood aspiration on BAL total cell count. The number of cells within the BAL was significantly higher at 2 to 14 days after blood aspiration when compared to control animals (*p < 0.05).

Figure 4. Effect of aspiration of blood on BAL macrophages. The total number of macrophages was significantly higher at 2 to 14 days after aspiration of blood when compared to control animals (*p < 0.05).
in a selected group of patients after heart transplantation for severe congestive heart failure.\textsuperscript{23} 

In this study, we investigated the time course for HLMs within the lungs in a murine model. Our findings demonstrate that hemosiderin staining within alveolar macrophages is first detected in the BAL at 3 days, thus supporting previously published data.\textsuperscript{11,12} The iron staining of alveolar macrophages peaks at 7 days and persists through 2 months. In addition, iron staining in the lung tissue parallels the time course of the BAL.

In light of our observations, it appears that alveolar...
macrophages require a prolonged period of time to clear hemosiderin after a single episode of blood aspiration. Although this information cannot be directly extrapolated to humans, our data suggest that it may take months for hemosiderin to clear after an episode of pulmonary hemorrhage. Therefore, it may be difficult to differentiate between an acute bleed into the lung and chronic recurrent bleeding. As a result, it may be challenging to determine the timing of a bleed if HLMs are found in a BAL sample.

The explanation for the persistence of hemosiderin 2 months after one episode of blood aspiration is unclear. Several hypotheses, alone or in combination, could potentially explain this phenomenon. Human alveolar macrophages are shown to have limited ability to metabolize hemoglobin as compared to macrophages obtained by culture of blood monocytes.\(^{27}\) If the alveolar macrophage is unable to completely degrade the iron, it may release the iron into the alveolar space to be phagocytosed by another macrophage. It is also possible that an intact macrophage may engulf adjacent cell(s) that have already phagocytosed iron. Furthermore, the clearance mechanisms for macrophages may be overwhelmed and thus take a prolonged period of time to clear the hemosiderin.

In this study, we also described the cellular alterations that occurred after blood aspiration. The analysis of the BAL revealed an increased number of total cells with an acute inflammatory reaction characterized by a mixed infiltrate of mononuclear cells. In addition, the total number of macrophages remained significantly elevated from 2 through 14 days after aspiration of blood. While direct evidence for macrophage activation is not apparent in our work, macrophage activation has been demonstrated in a rat model of Goodpasture’s syndrome with pulmonary hemorrhage.\(^{28}\) Alveolar macrophage activation commonly occurs after phagocytosis.\(^{29}\) Activation of macrophages and subsequent damage to the normal lung structure may result from repeated ingestion of a variety of noxious stimuli or the inability to clear these agents from the alveolar macrophage.\(^{30}\) In addition, the ferritin within alveolar macrophages has a predominance of L subunits, which are less effective than the H type for detoxifying iron and inhibiting peroxidation, a potential source for tissue damage.\(^{30}\) The presence of neutrophils and lymphocytes in the BAL is not surprising due to the well-established pattern of cellular responses after intratracheal administration of noxious agents.\(^{31}\) Of interest, an increased number of neutrophils were also described in a rat model of blood instillation.\(^{13}\)

Although our model did not reveal diffuse histologic changes after a single aspiration of blood, the rat model of experimental Goodpasture’s syndrome with pulmonary hemorrhage demonstrated widespread fibrinogen deposition, interstitial infiltration of mononuclear cells, and edema within the lung.\(^{25}\)

Extensive histologic abnormalities seen in the latter model are likely due to the lung injury caused by the antibody deposition and not the resulting hemorrhage. While our study was not designed to define histologic changes, widespread tissue abnormalities would not be expected after a single aspiration event. It is feasible that a model of repeated blood aspiration would provide more information on structural changes within the lung.

Our findings demonstrate the validity of this model for the study of HLM production. Since it is difficult to perform this type of study in human subjects, our animal model may be useful to explain the mechanisms leading to persistence of hemosiderin within alveolar macrophages. It is important to note that the mechanism for bleeding in IPH is likely different than our murine model. Additional experiments using models of pulmonary hemorrhage are needed to further characterize the cellular events leading to clearance of erythrocytes within the lung.

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