Reproduction of Transfusion-Related Acute Lung Injury in an Ex Vivo Lung Model

By Werner Seeger, Udo Schneider, Bettina Kreusler, Esther v. Witzleben, Dieter Walmrath, Friedrich Grimminger, and Jürgen Neppert

Leukoagglutinins are implicated in transfusion-related acute lung injury (TRALI). In the present study, severe lung vascular leakage was reproduced by application of a leukoagglutinating antibody of anti-5b specificity in an ex vivo lung model. The antibody originated from a multiparous donor-plasma, observed to cause noncardiogenic edema during transfusion therapy. Heated full plasma (anti-5b-titer 1/128) or purified immunoglobulin G fraction was used for the studies. Ex vivo isolated rabbit lungs were perfused with albumin buffer, and human granulocytes (PMN) were admixed to the recirculating perfusate. In presence of anti-5b antibody plus 5b-positive PMN plus rabbit plasma as complement-source, severe lung edema occurred after a latent period of 3 to 6 hours. Pulmonary artery pressure was only transiently and moderately increased, and the leakage reaction could be traced back to a several-fold increase in lung vascular permeability. In contrast, no vascular leakage was noted in lungs perfused in the absence of anti-5b antibody, PMN, or rabbit plasma. Moreover, no permeability increase occurred on use of 5b-negative PMN. This reproduction of TRALI in an ex vivo lung model corroborates the role of leukoagglutinating antibodies in initiating PMN-dependent respiratory distress and suggests a contribution of concomitant complement activation.

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NONCARDIOGENIC pulmonary edema after transfusion therapy is an infrequent but hazardous complication. The occurrence of this entity, characterized as transfusion-related acute lung injury (TRALI), is linked to the presence of circulating leukoagglutinins. Clinical features include chills, fever, tachycardia, cough, and various degrees of respiratory distress. Transient leukopenia may be noted. The chest x-ray demonstrates bilateral pulmonary infiltrates in the absence of cardiac enlargement and pulmonary vascular engorgement. Normal pulmonary capillary wedge pressure on right heart catheterization verifies the "noncardiogenic" origin of the pulmonary edema formation. The onset of respiratory distress ranges between a few minutes and 40 hours after transfusion, with a maximum at 4 to 8 hours. In most cases, the symptoms subside within 1 or 2 days with full recovery.

The underlying mechanisms of TRALI are not fully understood. Initiation is attributed to passive transfer of donor antibodies against leukocytes or, more rarely, presence of leukoagglutinins in the recipient serum. Leukocyte antibodies of different specificity have been implicated. These include HLA-related antibodies and granulocyte-specific antibodies (ie, anti-NA); however, a positive cause and effect relationship could not conclusively be established in all cases. In addition, antibodies against the diallelic (5a/5b) group five antigen system, assumed to be present on granulocytes, lymphocytes, and platelets, have been associated with onset of nonhemolytic febrile transfusion reactions. Plasma containing agglutinating immunoglobulin G (IgG) antibody of anti-5b specificity was recently described to induce acute respiratory distress after transfusion. This is noteworthy, as more than 80% of the western population express the 5b-epitope on their polymorphonuclear leukocytes (PMN), and particularly multiparous 5b-negative women were noted to develop significant anti-5b antibody titers. In accordance with this notion, the cause of a recent transfusion-related lung edema in the Blood Center of the University of Giessen (FRG) could be traced back to a high anti-5b titer in the donor plasma, originating from a multiparous woman. In the present study, performed in an established model of blood-free perfused rabbit lungs, we reproduced acute lung injury by intravascular administration of this antibody, 5b-positive PMN, and rabbit plasma as complement source. This mimicry of TRALI in an ex vivo model will allow further elucidation of underlying mechanisms resulting in the development of severe lung vascular leakage in this syndrome.

MATERIALS AND METHODS

Ex vivo lung preparation. The model of isolated rabbit lungs has been previously described (Fig 1). Briefly, rabbits of either sex (body weight 2.3 to 2.8 kg) were deeply anesthetized with pentobarbital (60 to 90 mg/kg) and anticoagulated with 1,000 U/kg heparin. Tracheotomy and thoracotomy were performed and lungs were excised while being ventilated and perfused. A gas mixture of 4% CO2, 17% O2, and 79% N2 was used. The lungs were perfused via canulas in the pulmonary artery and the left ventricle with Krebs-Henseleit albumin (1% wt/vol) buffer (KHAB) with a constant pulsatile flow of 100 mL/min (total recirculating volume 250 mL). The use of two different perfusion circuits allowed repeated exchange of perfusion fluid by fresh buffer medium. The lungs were placed in a 38°C equilibrated chamber, freely suspended from a force transducer. Pulmonary arterial pressure (PAP), pulmonary venous pressure (PVP), ventilation pressure (VP), and the weight of the isolated organ were continuously registered. A left atrial pressure of 2 mm Hg was measured at zone III conditions at endexpiration. The capillary filtration coefficient (Kf) and the total vascular compliance (COM) were repeatedly determined from the slope of weight gain versus time. The sudden venous pressure elevation (hydrostatic challenge maneuver) of 10 cm H2O for 8 minutes (gravimetric technique). Zero time extrapolation of the slope of weight gain by
semilogarithmic plot was performed as described.\textsuperscript{26,27} The increase in net lung weight evoked by the hydrostatic challenge maneuver is given as $\Delta W$, read between onset of the venous pressure elevation and 3 minutes after termination of maneuver. Lungs included in the study were those that (1) showed no signs of hemostasis or edema, (2) had pulmonary artery and ventilation pressures in the normal range, and (3) were isogravimetric during an initial steady-state period of at least 45 minutes. Random light microscopical examinations of these lungs showed virtually no erythrocytes or platelets and only few resting (marginated) leukocytes in the intravascular space, as previously described.\textsuperscript{29} Nearly quantitative sequestration of the PMN in the pulmonary vascular bed, as previously described,\textsuperscript{30}

Preparation of human granulocytes and rabbit plasma. Heparinized human donor blood was centrifuged in a discontinuous Percoll gradient\textsuperscript{30,32} to yield a PMN fraction of approximately 97% purity. The granulocytes were kept in RPMI 1640 medium with 20% fetal calf serum for 90 to 120 minutes. Immediately before experimental use the cells were washed twice and resuspended in KHB. Donor PMN were tested for 5b-antigen by leukoagglutinating technique.\textsuperscript{27} Rabbit plasma was obtained from anesthetized animals by femoral artery catheterization after anticoagulation with heparin. Blood was centrifugated for 10 minutes at 3,000 g and was stored at $-70^\circ$C until experimental use.

Preparation of anti-5b-containing human plasma and its IgG fraction. The antibody specificity as anti-5b IgG was determined with the generous support of donors and reagents by Dr F.H.J. Claas (Leiden, The Netherlands). The titer of the anti-5b antibody was 1/128. The plasma of the donor with high anti-5b titer (1/128) was obtained by plasmapheresis and heated for 30 minutes at $57^\circ$C to inactivate complement factors. IgG was fractionated purified by ionic exchange chromatography, using a DEAE 32 column. Plasma, 89 mL, yielded an IgG-eluante of 29 mL in 0.9% saline with a protein concentration of 1.66 g/100 mL and a leukoagglutinating titer of 1/64.\textsuperscript{30}

Analytical procedures. Thromboxane A\textsubscript{2} (TxA\textsubscript{2}) and prostacyclin (PGI\textsubscript{2}) were assayed serologically from the recirculating buffer fluid as their stable hydrolysis products TxB\textsubscript{2} and 6-keto PGF\textsubscript{1\alpha}. The method has been previously described.\textsuperscript{31} Leukotriene (LT) B\textsubscript{4}, omega-oxidation products of LT\textsubscript{B}x, 5-hydroxyeicosatetraenoic acid (5-HETE), and nonenzymatic hydrolysis products of LTA\textsubscript{x} in PMN stimulated in vitro were assayed as described.\textsuperscript{32}

Experimental protocol. After an isogravimetric steady-state period of 45 minutes, perfusate was exchanged, time was set zero, and the first hydrostatic challenge was immediately performed in all lung experiments (Kfc 0 minutes). In the standard protocol, the perfusate medium was next exchanged by KHB with 15% (vol/vol) rabbit plasma and 4% (vol/vol) anti-5b containing human plasma. In a subgroup of the standard protocol, the antibody-containing human plasma was replaced by the purified antibody-containing IgG fraction to give a corresponding anti-5b-titer in the recirculating perfusate. A second hydrostatic challenge was performed (Kfc 15'), and $2 \times 10^8$ PMN were injected directly into the pulmonary artery in a total volume of 2-mL buffer fluid. Three and fifteen minutes after PMN application, the circulating leukocytes were $30 \pm 4$ and $25 \pm 5/\mu$L in the lung effluent, documenting a nearly quantitative sequestration of the PMN in the pulmonary vascular bed, as previously described.\textsuperscript{33}

Subsequent hydrostatic challenges were performed at 30, 45, 60, 120, 180, and 360 minutes. Control experiments included those with (1) use of 5b-negative PMN (AB + PMN\textsuperscript{-} + PL in Table 1); (2) absence of PMN (AB + PL in Table 1); (3) replacement of anti-5b-containing plasma by heated human plasma without significant antibody titer (PL + PMN\textsuperscript{5b} in Table 1); (4) omission of rabbit plasma (AB + PMN\textsuperscript{5b}). Exchange of perfusate and hydrostatic challenges in these experiments were performed according to the standard protocol. Per fusate samples, of 2 mL for detection of prostanoids (TxA\textsubscript{2} and PGI\textsubscript{2}) were taken according to the time schedule in Table 2.

In additional in vitro experiments, $4 \times 10^8$ PMN (5b-negative or -positive) were incubated at $37^\circ$C in a total volume of 5-mL buffer fluid, in the absence or presence of anti-5b containing plasma and/or rabbit plasma. Cells were centrifuged after 30 or 90 minutes, and the supernatant was analyzed for leukotrienes and 5-HETE.

Materials. Bovine albumin (92% purity, reduced in free fatty acids to <5 mg/g) was purchased from Paesel GmbH (Frankfurt, FRG). TxB\textsubscript{2} were graciously supplied by Ono Pharmaceutical (Osaka, Japan). 6-Keto PGF\textsubscript{1\alpha} was obtained from Sigma GmbH (Munich, FRG), and rabbit anti-6-keto PGF\textsubscript{1\alpha} as well as anti-TxB\textsubscript{2} were purchased from Paesel GmbH. Tritiated 6-keto PGF\textsubscript{1\alpha} (120 to 180 Ci/mol) and TxB\textsubscript{2} (100 to 150 Ci/mol) were obtained from New England Nuclear GmbH (Dreieich, FRG). RPMI 1640 medium and fetal calf serum were from Boehringer Mannheim GmbH (Mannheim, FRG), and Percoll from Pharmacia Fine Chemicals (Uppsala, Sweden). All others biochemicals were obtained from Merck (Munich, FRG).

Statistical methods. Values are given as mean ± standard deviation (SD). Data were analyzed by two-way analysis of variance.
Table 1. PAP and COM in the Absence or Presence of Anti-5b Antibody Rabbit Plasma, and 5b-Positive or 5b-Negative Granulocytes

<table>
<thead>
<tr>
<th>Variables</th>
<th>Groups</th>
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<tr>
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<td>n = 5</td>
<td>8.60</td>
<td>11.90</td>
<td>11.60</td>
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<td>± 2.58</td>
<td>± 5.48</td>
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<td>± 4.75</td>
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<td>± 4.76</td>
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<tr>
<td>AB + PMNSb + PL†</td>
<td>n = 5</td>
<td>0.50</td>
<td>0.45</td>
<td>0.49</td>
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<td>± 0.08</td>
<td>± 0.09</td>
<td>± 0.09</td>
<td>± 0.14</td>
<td>± 0.14</td>
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<td>± 0.09</td>
<td>± 0.08</td>
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<td>± 0.12</td>
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<td>± 0.09</td>
<td>± 0.19</td>
<td>± 0.14</td>
<td>± 0.15</td>
<td>± 0.08</td>
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</table>

Abbreviations: AB, anti-5b antibody; PL, rabbit plasma; PMNSb, 5b-positive granulocytes; PMN−, 5b-negative granulocytes.

*The pulmonary artery pressure was read immediately before onset of each hydrostatic challenge (mm Hg).
†This group includes n = 3 experiments with anti-5b antibody containing human plasma and n = 2 experiments with purified IgG fraction.
‡This group includes n = 2 experiments with autologous PMN taken from the antibody donor and n = 2 experiments with 5b-negative PMN obtained from a different donor.
§The vascular compliance (COM) gives the change in vascular volume per change in microvascular pressure (mL/cm H2O).

RESULTS

Under baseline conditions, pulmonary artery pressure, ventilation pressure, Kf, data, and lung weight of all lungs ranged at normal values, corresponding to previous isolated lung studies. Application of anti-5b antibody, 5b-positive human PMN, and rabbit plasma as complement source in the standard protocol caused a transient, moderate increase in pulmonary artery pressure (Table 1). The total pressure rise averaged ~5 mm Hg, with a maximum at about 45 to 120 minutes. At the end of experiment (360 minutes), baseline pulmonary artery pressure was reached again. However, the outstanding biophysical alteration in this group was a delayed, severe increase in lung vascular permeability (Fig 2, Table 3). The Kf values determined after 180 minutes ranged at a slightly increased level (~2 mL per cm H2O per gram wet lung weight per second × 10^-4), and the hydrostatic challenge-induced weight gain was moderately augmented (~2.5 g). In the subsequent 180-minute perfusion

Table 2. Perfusate 6-keto-PGF1α, Concentrations Detected in the Absence or Presence of Anti-5b Antibody, Rabbit Plasma, and 5b-Positive or 5b-Negative Granulocytes

<table>
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<tr>
<td>AB + PMNSb + PL†</td>
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<td>394.6</td>
<td>386.9</td>
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<td>± 396.0</td>
<td>± 460.9</td>
<td>± 607.0</td>
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See Table 1 for abbreviations. 6-keto-PGF1α levels in the perfusate samples taken at various times are given for the different groups in picograms per milliliter. Respective baseline values in differently composed perfusion fluids, analyzed before recirculation in the lungs, have been subtracted.

*This group includes n = 3 experiments with anti-5b antibody containing human plasma and n = 2 experiments with purified IgG fraction.
†P < 0.01 compared with all control groups.
‡This group includes n = 2 experiments with autologous PMN taken from the antibody donor and n = 2 experiments with 5b-negative PMN obtained from a different donor.
Fig 2. Repetitive hydrostatic challenge maneuvers induced in a lung undergoing standard protocol (A) as well as two control experiments (B and C). The original registrations of weight gain ($\Delta W$) evoked by 10 cm H$_2$O sudden venous pressure elevations are given for selected time points (time scale interrupted). The controls include an experiment with use of 5b-negative PMN (B) and a study with omission of rabbit plasma (C). The protracted increase in lung weight and the several-fold increased steepness of fluid filtration induced by the 360-minute hydrostatic challenge in the standard protocol (A) are evident. The dotted line indicates baseline reset to allow continued registration of weight in this experiment.

Table 3. Kfc and Hydrostatic Challenge-Induced Weight Gain ($\Delta W$) in the Absence or Presence of Anti-5b Antibody, Rabbit Plasma, and 5b-Positive or 5b-Negative Granulocytes

<table>
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<td>1.85</td>
<td>2.04</td>
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<tr>
<td>$\Delta W$</td>
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<tr>
<td>n = 5</td>
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<td>1.28</td>
<td>1.89</td>
<td>2.46</td>
<td>&gt;10$^*$</td>
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See Table 1 for abbreviations.

$^*$The capillary filtration coefficient is expressed in cm$^3$ per second per cm H$_2$O per g wet lung weight $\times 10^{-4}$.

$^\dagger$This group includes $n = 3$ experiments with anti-5b antibody containing human plasma and $n = 2$ experiments with purified IgG fraction.

$^\ddagger$This group includes $n = 2$ experiments with autologous PMN taken from the antibody donor and $n = 2$ experiments with 5b-negative PMN obtained from a different donor.

$\Delta W$ gives the total weight gain per hydrostatic challenge in grams.
was applied and lungs with administration of purified IgG fraction. The ventilation pressure was not altered until the 360 minute hydrostatic challenge; only in parallel with the severe edema formation during this challenge a moderate increase occurred.

In all control experiments in which PMN were administered, a transient, moderate increase in pulmonary artery pressure was noted, corresponding to that observed in the standard protocol (Table 1). In contrast, no permeability increase was observed on omission of anti-5b antibody, rabbit plasma, or PMN (Table 3). The same was true for the use of 5b-negative PMN, whether taken from the anti-5b antibody donor or obtained from a different donor with granulocytes not expressing this epitope. The Kfc values remained virtually unchanged throughout the 6-hour experimental procedure. Ventilation pressure was unchanged in these lungs.

In all isolated lung experiments, a progressive accumulation of 6-keto-PGF1α in the recirculating buffer medium was noted (Table 2). This increase was significantly more pronounced in the standard experiments than in the control studies (Table 2). TxB2 ranged below 100 pg/mL in the perfusate samples of all isolated lung experiments.

Incubation of PMN in vitro resulted in marked generation of LTB4, omega-oxidation products of LTB4, nonenzymatic hydrolysis products of LTA4, and 5-HETE in the presence of anti-5b and rabbit plasma as complement source, but not in appropriate controls (Table 4).

**DISCUSSION**

In the present study, the key events of transfusion-related acute lung injury were reproduced in an ex vivo isolated lung model. The reaction was dependent on both 5b-positive PMN and antibody with anti-5b specificity, as well as rabbit plasma as complement source.

The outstanding biophysical alteration noted in the present experiments was an increase in lung vascular permeability, which exhibited delayed onset (approximately 3 hours), but progressed to severe vascular leakage toward the end of the 6-hour observation period. This reaction was dependent on both the 5b-anti-5b system and the presence of rabbit plasma as a complement source. The reaction was dependent on both 5b-positive PMN and antibody with anti-5b specificity, as well as rabbit plasma as complement source.

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system (data not given in detail). Thus, several aspects of neutrophil activation, all implicated in the triggering of microvascular disturbances, may underlie the mimicry of TRALI in the presently used isolated lung preparation and need further elucidation. Moreover, complement activation occurring at sites of PMN antigen-antibody reaction in direct vicinity of microvascular endothelial cells (conditions of vascular sticking) might attack endothelial cell membranes. The significantly increased PGI$_2$ generation in the anti-5b–PMN$_{5b}$ system might correspond to the induction of PGI$_2$ generation noted after membrane insertion of the terminal complement complex in cultured lung endothelial cells. This speculative view is further supported by the recent finding that in situ complement activation in the lung microvasculature causes severe vascular leakage with similarly delayed kinetic as presently noted after administration of anti-5b and PMN$_{5b}$. Initiation, extent, and location of concomitant complement activation, whether causing activation of neutrophils, of endothelial cells, or both, thus might represent an important variable of leukoagglutinin-induced acute lung injury.~

The onset of vascular leakage was preceded by a transient lung vasoconstrictor response. However, this was comparable in all lungs with application of human PMN and thus is not specifically related to the anti-5b–PMN$_{5b}$ system. The absence of any significant thromboxane levels in the perfusate strongly argues against a contribution of lung thromboxane generation in this pressor response. This notion, as a large variety of soluble and particulate stimuli are known to cause pulmonary artery pressure increase via induction of this vasoconstrictive prostanoid. Taken together, lung vasoconstrictor responses apparently do not contribute to the leukoagglutinin-induced acute lung injury to a major extent. This is in accordance with the clinical observation that pulmonary artery pressure is only infrequently elevated in patients with TRALI.

The present experimental study corroborates repeated clinical observations that leukoagglutinating antibodies are capable of evoking severe pulmonary vascular leakage. Initiation of concomitant complement activation may contribute to the development of this injury. The subsequently formed protein-rich lung edema is known to be associated with serious disturbances of gas exchange. The predominance of lung fluid balance alteration, not accompanied by major alterations in pulmonary hemodynamics, may explain the fact that transfusion-related lung edema is often misdiagnosed as circulatory overload. The establishment of an ex vivo model of TRALI can be expected to allow further elucidation of pathogenetic events underlying the development of this severe transfusion-related incident.

**ACKNOWLEDGMENT**

We are grateful to Dr. S. Baudner and H. Haupt, Behringwerke Marburg, FRG, for help with the purification of the IgG fraction. We thank Ch. Ernst and H. Michnass for excellent technical assistance, and P. Müller for skillful graphical illustration.

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The present experimental study corroborates repeated clinical observations that leukoagglutinating antibodies are capable of evoking severe pulmonary vascular leakage. Initiation of concomitant complement activation may contribute to the development of this injury. The subsequently formed protein-rich lung edema is known to be associated with serious disturbances of gas exchange. The predominance of lung fluid balance alteration, not accompanied by major alterations in pulmonary hemodynamics, may explain the fact that transfusion-related lung edema is often misdiagnosed as circulatory overload. The establishment of an ex vivo model of TRALI can be expected to allow further elucidation of pathogenetic events underlying the development of this severe transfusion-related incident.

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Reproduction of transfusion-related acute lung injury in an ex vivo lung model [see comments]

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