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Identification of lipids that accumulate during the routine storage of prestorage leukoreduced red blood cells and cause acute lung injury

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Abstract

BACKGROUND—Lipids accumulate during the storage of red blood cells (RBCs), prime neutrophils (PMNs), and have been implicated in transfusion-related acute lung injury (TRALI). These lipids are composed of two classes: nonpolar lipids and lysophosphatidylcholines based on their retention time on separation by high-pressure liquid chromatography. Prestorage leukoreduction significantly decreases white blood cell and platelet contamination of RBCs; therefore, it is hypothesized that prestorage leukoreduction changes the classes of lipids that accumulate during storage, and these lipids prime PMNs and induce acute lung injury (ALI) as the second event in a two-event in vivo model.

STUDY DESIGN AND METHODS—RBC units were divided: 50% was leukoreduced (LR-RBCs), stored, and sampled on Day 1 and at the end of storage, Day 42. Priming activity was evaluated on isolated PMNs, and the purified lipids from Day 1 or Day 42 were used as the second event in the in vivo model.

RESULTS—The plasma and lipids from RBCs and LR-RBCs primed PMNs, and the LR-RBC activity decreased with longer storage. Unlike RBCs, nonpolar lipids comprised the PMN-priming activity from stored LR-RBCs. Mass spectroscopy identified these lipids as arachidonic acid and 5-, 12-, and 15-hydroxyeicosatetraenoic acid. At concentrations from Day 42, but not Day 1, three of four of these lipids individually, and the mixture, primed PMNs. The mixture also caused ALI as the second event in a two-event model of TRALI.

CONCLUSION—We conclude that the nonpolar lipids that accumulate during LR-RBC storage may represent the agents responsible for antibody-negative TRALI.

Transfusion-related acute lung injury (TRALI) remains the most common cause of transfusion-related mortality.^{1,2} TRALI has been linked to a number of mediators including antibodies against white blood cell (WBC) antigens, biologically active lipids that accumulate during routine storage of cellular blood components, and soluble CD40 ligand.^{3–6} During routine storage of red blood cells (RBCs), lipids accumulate in the plasma fraction, supernatant.⁷ These lipids consist of nonpolar lipids and a mixture of lysophosphatidylcholines (lyso-PCs), as defined by their retention times on normal-phase high-pressure liquid chromatography (HPLC).⁷ Lyso-PCs at concentrations that accumulate during RBC and platelet (PLT) concentrate storage precipitate polymorphonuclear

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neutrophil (PMN)-mediated endothelial damage and acute lung injury (ALI) in a two-event in vivo model of TRALI.⁸

Universal prestorage leukoreduction has been instituted in a number of countries, and despite decreasing the numbers of febrile transfusion reactions, outside of a single institution in the United States, there has been no mention of its effects on the rates of TRALI.^{9,10} In addition, prestorage leukoreduction by filtration is known to decrease WBC contamination by greater than 3 logs and decrease PLT contamination by 4 to 5 logs, as evidenced by eradication of soluble CD40 ligand accumulation during routine storage because it is PLT derived.³ It is hypothesized, therefore, that prestorage leukoreduction changes the lipids that accumulate during routine storage but does not affect their ability to rapidly prime the PMN oxidase or to serve as the second event to precipitate ALI in an in vivo model.

MATERIALS AND METHODS

Materials

All chemicals were purchased from Sigma Chemical Co. (St Louis, MO) unless otherwise stated. Solutions were made from sterile water for injection, USP (Baxter Healthcare Corp., Deerfield, IL). All buffers were made from stock USP solutions as previously described and sterile-filtered with disposable sterilization filter units (Nalgene, MF75 series, Fisher Scientific Corp., Pittsburgh, PA).⁷

RBC collection

Whole blood (450 mL) was collected from 10 healthy volunteer donors after obtaining informed consent under a protocol approved by the Colorado Multiple Internal Review Board at the University of Colorado Denver.⁸ Each unit was equally divided (weight) with 50% being leukoreduced (LR-RBCs) via filtration (Pall BPF4 filter, Pall Corp., Westbury, NY) and stored at 4°C per industry standards.¹¹ Sterile couplers were employed to obtain samples on Day 1 and Day 42, and the supernatant was isolated and stored at -80°C.¹¹

HPLC separation of lipids and analysis by mass spectrometry

Plasma lipids were solubilized and separated by normal-phase HPLC and these lipid fractions were solubilized in albumin for use in PMN-priming assays and for the identification of the active lipids.⁷ To identify the nonpolar lipids in the supernatant ice cold methanol was added (50% vol/vol) to the acellular supernatant, the proteins were precipitated, a stable internal standard was added (2 ng ²H₈-5- hydroxyeicosatetraenoic acid [HETE]), and the nonpolar lipids were extracted and analyzed as reported using an HPLC system directly interfaced into the electrospray source of a triple quadrupole mass spectrometer (liquid chromatography coupled to electrospray ionization mass spectrometry [LC/MS/MS]).^{12,13} An estimation of lipid concentration was completed using ratios to an internal standard (1.0 = 2ng of lipid).¹²⁻¹⁴

PMN isolation and priming of the oxidase

Heparinized whole blood was drawn from healthy human donors after obtaining informed consent employing a protocol approved by Colorado Multiple Internal Review Board, and the PMNs were isolated by standard techniques.⁷ PMNs (3.75×10^5) were then incubated with albumin (vehicle) or the purified lipids, both individually and as a mixture from Day 1 or Day 42 for 5 minutes at 37°C. The PMNs were then activated with 1 μ mol/L formyl-methionyl-leucyl-phenylalanine (fMLP), and the maximal rate of superoxide production was measured.⁷ Priming activity was measured as the augmentation of the maximal rate of O₂⁻ in response to fMLP.

Two-event in vivo model

Male Sprague-Dawley rats (Harlan, Indianapolis, IN) underwent a treatment protocol approved by the Animal Care and Use Committee, University of Colorado Denver.⁸ Briefly, rats were injected intraperitoneally with 2 mg/kg lipopolysaccharide (LPS; *Salmonella enteritidis*) incubated for 2 hours and anesthetized with 60 mg/kg pentobarbital, and the femoral vessels were cannulated.⁸ Blood was removed (10 min) equal to 5% of the total blood volume¹⁵ followed by infusion of an identical volume of the neutral lipids at concentrations from Day 1 or Day 42 at 4 mL/hr followed by 30 mg/kg of Evans blue dye (EBD). Six hours later the rats were reanesthetized, blood (3 mL) was drawn, and the rats were euthanized with an overdose of pentobarbital followed by a bronchoalveolar lavage.⁸ Lung leak was measured as the percentage of EBD that leaked from the plasma into the bronchoalveolar lavage fluid.⁸

Statistical analysis

All data are presented as the mean $\pm$ the standard error of the mean. Statistical differences among groups were measured by independent analyses of variance followed by either a Bonferroni's or a Newman-Keuls post hoc test for multiple comparisons depending on the equality of variance with significance determined at the p value level of less than 0.05.

RESULTS

The accumulation of PMN-priming activity during routine storage

PMN-priming activity accumulated during the routine storage of both RBCs and LR-RBCs and reached a relative maximum on Day 42 (Fig. 1A). Compared to unmodified RBCs, the priming activity was attenuated by prestorage leukoreduction but was still significantly increased beginning on Day 14 of storage, reached a relative maximum on Day 28, and only modestly increased by Day 42, the last day the unit may be transfused, whereas the priming activity in the control units continued to increase (Fig. 1A). The lipids extracted from the plasma fraction of Day 42 RBCs primed the PMN oxidase compared to the lipids from the extractions of the identical RBCs on Day 1 (data not shown). Lipids were then separated by normal-phase HPLC and solubilized in 1.25% albumin, and the fractions were assessed for priming activity. Unmodified RBCs contained two peaks of lipid-priming activity: the first at the retention time of nonpolar lipids, which were not retained on the column, and the second at the retention time of lyso-PCs, which is a broad peak of activity (Fig. 1B).⁷ In contrast, the LR-RBCs contained lipid-priming activity only at the retention time of nonpolar lipids (Fig. 1B).

Gas chromatography/MS identification of the neutral lipids from RBCs

The nonpolar lipids were separated by reverse-phase HPLC and analyzed by LC/MS/MS with an estimation of concentration employing a stable isotopic internal standard.^{7,12,13} These lipids were identified as arachidonic acid, 5-HETE, 12-HETE, and 15-HETE and were present in the Day 42 RBCs (data not shown) and LR-RBCs (Table 1). In addition, the nonpolar lipids from the identical units from Day 1 RBCs and LR-RBCs were also analyzed by LC/MS/MS. All four lipid moieties were present on Day 1 and significantly increased by Day 42 in both RBCs (data not shown) and LR-RBCs, as demonstrated by the analyte ratios and the estimation of concentration (Table 1).

Biologic activity of nonpolar lipids in vitro

The ability of the lipids, both separately and as a mixture, to prime the fMLP-activated PMN oxidase was assessed. Neither the individual lipids nor the mixture from Day 1 LR-RBCs primed the fMLP activation of the oxidase versus the controls (Fig. 2). In contrast, when the

concentrations of the lipids from Day 42 were used, three of the four individual compounds (arachidonic acid, 5-HETE, and 12-HETE) and the mixture of the nonpolar lipids primed the PMN oxidase except for the 15-HETE (Fig. 2). Moreover, the mixture of nonpolar lipids elicited increased priming activity compared to the constituents alone (Fig. 2).

The lipids from stored LR-RBCs cause ALI in an in vivo model

To demonstrate if these lipids could cause ALI as the second event in a two-event model of in vivo ALI, rats were injected intraperitoneally with saline or LPS (first event) and then infused with the lipids at concentrations present on Day 1 or Day 42 of LR-RBC storage. The lipids from Day 1 LR-RBCs did not elicit ALI in either normal saline (NS)- or LPS-treated rat (Fig. 3). However, the lipids at concentrations from Day 42 induced ALI as the second event in this two-event in vivo model (Fig. 3).

DISCUSSION

These data have identified the nonpolar lipids that accumulate in the supernatant during routine storage of RBCs, both prestorage LR or unmodified. These lipids reach a relative maximum concentration on Day 42, the last day the unit may be transfused, and three of the four individual lipid compounds, arachidonic acid, 5-HETE, and 12-HETE, and the mixture primed the PMN oxidase at concentrations reached at the end (Day 42) of storage compared to Day 1. Furthermore, the mixture of lipids from stored, but not fresh LR-RBCs, causes ALI as the second event in a two-event in vivo model of TRALI. The ability of these nonpolar lipids to induce ALI in vivo may explain why 1) the rates of antibody-negative TRALI have been largely unaffected by prestorage leukoreduction and 2) the observation that the plasma from stored LR-RBCs may cause ALI in a two-event in vivo model of ALI.^{8,9}

The reported data also provide some insight into the sources of the lipids that accumulate during the routine storage of blood products. PLT concentrates, those either derived from whole blood or isolated via apheresis, contain large amounts of lyso-PCs, which prime PMNs through activation of a specific G-protein-linked receptor, cause PMN-mediated cytotoxicity of human pulmonary endothelial cells, and can serve as the second event in two-event in vivo models of TRALI.^{8,16–20} Furthermore, PLT concentrates, both whole blood-PLTs and apheresis-PLTs, do not generate nonpolar lipids during routine storage; however, unlike whole blood-PLTs, apheresis-PLTs are LR by definition and both types of concentrates still produce large amounts of lyso-PCs.^{17,21} Thus, one may infer that lyso-PCs are PLT derived. Unmodified RBCs contain large amounts of PLT and WBC contamination, and during storage both nonpolar lipids and lyso-PCs accumulate.⁷ If prestorage LR, the identical RBC units generated only nonpolar lipids; thus, the nonpolar lipids appear to be derived from RBC membranes.

It is likely that these lipophilic compounds that accumulate during RBC storage were produced by peroxidation and not enzymatic conversion from arachidonate.^{22,23} Moreover, the concentrations of arachidonate and the HETEs were much higher than previously reported in human plasma; however, one should not compare the concentrations in the supernatant of a unit of RBCs to the circulating plasma from an intact human.^{24,25} Importantly, although antibody-negative TRALI is responsible for the minority of TRALI deaths, recent prospective data demonstrated that its incidence is not decreasing unlike antibody-mediated TRALI, which has declined with the use of male-only transfusion regimens (M.R. Looney, personal communication, data presented at a platform session, AABB National Meeting, Baltimore, MD, 2010).^{26–28} More detailed research into the proteome of blood components may help delineate the cellular and subcellular interactions that occur during routine storage which cause the accumulation of proinflammatory

substances that may induce ALI or other effects in the transfused host. Delineation of such subcellular processes may reveal possible points of intervention to decrease the production of such proinflammatory compounds to make transfusions safer.

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ABBREVIATIONS

ALI	acute lung injury
EBD	Evans blue dye
fMLP	formyl-methionyl-leucyl-phenylalanine
HETE	hydroxyeicosatetranoic acid
LC/MS/MS	liquid chromatography coupled to a triple quadrupole mass spectrometer
LPS	lipopolysaccharide
LR	leukoreduced
lyso-PC(s)	lysophosphatidylcholine(s)
NS	normal saline

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Fig. 1.

The plasma- and lipid-priming activity of stored RBCs. (A) The plasma-priming activity of stored RBCs. Plasma-priming activity (nmol of O_2^- /min) is depicted as a function of storage time (weeks) of both prestorage LR-RBCs or unmodified RBCs. Compared to the buffer-treated controls ($\square$) and Day 1 plasma from both LR-RBCs and RBC-priming activity was augmented on Day 14 for both unmodified RBCs ($\square$) and LR-RBCs ($\blacksquare$) and steadily increased in the plasma from unmodified RBCs reaching a relative maximum on Day 42. In contrast the plasma-priming activity from LR-RBCs appeared to plateau on Day 28 of storage and was significantly decreased compared to the plasma-priming activity from unmodified RBCs on Days 35 and Day 42 of storage. * $p < 0.05$ compared to buffer or Day 7 plasma primed PMNs; # $p < 0.05$ compared to the nonmodified plasma from Day 35 and Day 42. (B) Separation of the lipids from RBCs that cause priming activity in PMNs. The lipid peaks that cause priming activity in PMNs (multiple of albumin-treated controls) are shown as a function of the retention time of lipid separation by normal-phase HPLC. The unmodified RBC plasma ($\circ$) demonstrated two peaks of PMN-priming activity, the first at the retention time of nonpolar lipids and the second a broad peak of activity known to contain lyso-PCs. In contrast separation of the plasma lipid peaks from Day 42 LR-RBCs ($\blacktriangle$) yielded only a single lipid peak of involved in PMN-priming activity at the retention time of nonpolar lipids. The data are expressed as fold concentration of the mean from three separate experiments using disparate RBCs from different donors. D = day; NL = nonpolar lipids; PE = phosphatidylethanolamine; PI = phosphatidylinositol; PC = phosphatidylcholine; L-PC = lyso-PC.

Fig. 2.

PMN-priming activity and ALI induced by purified lipids from fresh (Day 1) versus stored (Day 42) LR-RBCs. The priming activity, the maximal rate of O_2^- production (nmol O_2^- /min), is depicted as a function of treatment group. The controls consist of albumin- (■) or buffer-primed (□) PMNs, and the priming activity from both the lipid mixtures and the individual lipid moieties from Day 1 (■) or Day 42 (▨) of LR-RBC storage are shown. * $p < 0.05$ versus the controls, both buffer and albumin treated, and the concentrations of the individual lipid moieties or mixture on Day 1 of storage. Each bar represents a sample size of seven. AA = arachidonic acid.

Fig. 3.

The lipids from stored LR-RBCs cause ALI in a two-event in vivo model. ALI is shown as the percentage of EBD leak from the plasma into the bronchoalveolar lavage (BAL) as a function of treatment group. * $p < 0.05$ versus the NS/lipids from Day 1 LR-RBCs, LPS/lipids from Day 1 LR-RBCs, and NS/lipids from Day 42 LR-RBCs. Each bar represents five different rats that underwent identical treatment protocols with differing first and second insults. (■) NS; (■) LPS.

TABLE 1

Gas chromatography/MS/MS identification and quantification of the nonpolar lipids from the plasma from Day 1 and Day 42 of LR-RBC storage*

Nonpolar lipid	Day 1 LR-RBCs		Day 42 LR-RBCs	
	Analyte ratio	Concentration (nmol/L)	Analyte ratio	Concentration (nmol/L)
Arachidonic acid	4.81 ± 0.13	63.15 ± 1.73	105.83 ± 9.80 [†]	1390 ± 129 [†]
5-HETE	0.07 ± 0.03	0.90 ± 0.33	2.33 ± 0.35 [†]	29.05 ± 4.34 [†]
12-HETE	0.56 ± 0.21	6.95 ± 2.59	7.1 ± 2.17 [†]	88.60 ± 27.12 [†]
15-HETE	0.03 ± 0.003	0.45 ± 0.05	0.68 ± 0.08	8.45 ± 1.05 [†]

* The numbers are the mean ± the SEM in ng/mL. The concentrations are estimated from an internal standard, ²H₈-5-HETE with a mass of 2 ng such that a ratio of 1.0 = 2 ng.

[†] p < 0.05 compared to the Day 1 concentrations of the individual lipid compounds.