In Vivo Platelet Activation in Critically Ill Patients With Primary 2009 Influenza A(H1N1)

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Background: Changes in platelet reactivity during 2009 influenza A(H1N1) (A[H1N1]) have not been characterized.

Methods: We prospectively examined platelet activation and cytokine responses in patients with A(H1N1) (n = 20), matched patients with bacterial pneumonia (n = 15), and nonhospitalized, healthy control subjects (n = 10).

Results: Platelet-monocyte aggregation was higher in patients with A(H1N1) ($21.4\% \pm 4.7\%$) compared with patients with pneumonia ($10.9\% \pm 3.7\%$) and control subjects ($8.1\% \pm 4.5\%$, $P < .05$). Similarly, PAC-1 (antibody that binds to the active conformation of integrin $\alpha_{IIb}\beta_3$) binding to platelets is increased in patients with A(H1N1) ($9.5\% \pm 4.7\%$) compared with patients with pneumonia ($1.0\% \pm 0.7\%$) and healthy subjects ($0.61\% \pm 0.15\%$, $P < .10$). PAC-1 binding was two-fold higher in patients with A(H1N1) with shock vs those without shock. IL-6 levels were elevated in patients with A(H1N1), indicating systemic inflammation consistent with activation of circulating platelets.

Conclusions: These findings, derived from a small but documented cohort of patients, demonstrate that platelet activation responses during A(H1N1) are enhanced—exceeding responses in patients with bacterial pneumonia—and provide new evidence that platelets may contribute to inflammatory responses during A(H1N1).
CHEST 2012; 141(6):1490–1495

Abbreviations: A(H1N1) = 2009 influenza A(H1N1); ALI = acute lung injury; APACHE = Acute Physiology and Chronic Health Evaluation; FITC = fluorescein isothiocyanate; PAC-1 = antibody that binds to the active conformation of integrin $\alpha_{IIb}\beta_3$; PMA = platelet-monocyte aggregate; SSC = side scatter; TRAP = thrombin-receptor activating peptide; TNF- α = tumor necrosis factor- α

The 2009 influenza A(H1N1) (A[H1N1]) is a single-stranded RNA virus that commonly infects the lungs, causing significant morbidity and mortality worldwide. Although the molecular pathogenesis of the influenza virus is not completely understood, influenza A activates primary human cells, including respiratory epithelial cells, neutrophils, and alveolar macrophages.^{1,2} This cellular activation may lead to increased systemic inflammation and the development of acute lung injury (ALI)/ARDS in A(H1N1).³

To date, the roles of platelets in A(H1N1) remain largely uninvestigated. Although thrombocytopenia and thrombosis occur in infected patients,^{4,5} in vivo platelet activation as a mechanism for these complications is unexplored. Mouse models^{6,7} and clinical observations^{3,8} indicate that systemic inflammation

and a prothrombotic state are triggered by influenza infection. There is evidence that human and rodent platelets have a receptor for influenza viruses and that influenza can associate with the platelet surface and be internalized.⁹ Platelets are now known to be effectors of dysregulated inflammatory responses in addition to pathologic hemostasis in systemic infections.^{10–12} For example, platelets interact with and signal circulating monocytes.^{10,13} In addition, however, they have multiple other inflammatory activities in infections and in noninfectious inflammatory syndromes.¹⁴ Thus, platelets are positioned to play central roles in systemic responses to A(H1N1) infections. Given that influenza may interact with platelets and leukocytes, we hypothesized that patients with A(H1N1) and respiratory failure would demonstrate

marked in vivo platelet activation exceeding responses seen in matched patients with bacterial pneumonia.

MATERIALS AND METHODS

Patient Enrollment

The University of Utah and Intermountain Health Care institutional review boards approved this study (protocols 28210 and 1005443), and all subjects provided written, informed consent. This was a prospective study of two groups of ICU patients aged ≥ 21 years with ALI/ARDS enrolled within 24 h of hospital admission. The first group were patients with primary A(H1N1) ($n = 20$). The second group were patients with bacterial pneumonia ($n = 15$). For comparison, a third group of nonhospitalized, healthy, control subjects ($n = 10$) were also prospectively studied. Two investigators (M. T. R. and B. B.) matched the groups on age, sex, and admission APACHE (Acute Physiology and Chronic Health Evaluation) II scores.¹⁵ To minimize bias during the matching process, investigators were blinded to prespecified confounding variables, including comorbidities, hemodynamic and respiratory parameters, vasopressor support, clinical laboratory data, mortality, and length of ICU stay.

Primary A(H1N1) was diagnosed by reverse transcription polymerase chain reaction performed on an appropriate respiratory sample obtained via nasopharyngeal or throat swab (RealTime Ready Influenza A/H1N1 Detection Set; Roche Applied Science). Patients were treated with the antiviral agent oseltamivir (75 mg bid). Patients with A(H1N1) and concurrent secondary bacterial infections were excluded. Pneumonia was diagnosed in patients with typical signs and symptoms of pneumonia and a demonstrable infiltrate by consensus criteria.^{16,17} All patients with pneumonia were treated with antibiotic therapies chosen at the discretion of the primary ICU team. Clinical laboratory variables were determined from blood samples drawn in parallel with blood samples used for platelet activation and cytokine analyses. Patients were followed prospectively for all-cause, in-hospital mortality.

Flow Cytometry

Whole blood, drawn from healthy subjects or from infected patients within 24 h of ICU admission, was collected into sterile acid-citrate-dextrose Vacutainer tubes. Blood was stored in acid-citrate-dextrose until flow cytometry testing was performed. The

average ($\pm$ SEM) time between phlebotomy and preparation for flow cytometry was $32 (\pm 2)$ min and did not differ between groups. The first 3 mL of blood was discarded, and samples with gross hemolysis or clotting were not used. Platelet activation was measured both in unstimulated and thrombin-receptor activating peptide (TRAP)-stimulated conditions ($5 \mu\text{M}$ for 15 min). All antibodies and reagents were obtained from BD Biosciences unless otherwise specified.

To measure PAC-1 (antibody that binds to the active conformation of integrin $\alpha_{\text{IIb}}\beta_3$) binding, whole blood ($20 \mu\text{L}$) was diluted in Hepes-Tyrodes buffer ($180 \mu\text{L}$) and then costained ($10 \mu\text{L}$) with CD41 phycoerythrin, a selective marker of platelets, and the anti-human mouse monoclonal antibody PAC-1 fluorescein isothiocyanate (FITC), which recognizes the active conformation of integrin $\alpha_{\text{IIb}}\beta_3$. Samples were then incubated at 25°C in the dark for 10 min and fixed in fluorescence-activated cell sorter buffer ($250 \mu\text{L}$). Platelets were selected by gating a total of 10,000 CD41⁺ events on a two-parameter dot plot displaying both side scatter (SSC) and forward scatter. The percentage of platelets binding to PAC-1 was adjusted for any nonspecific binding.

For detection of platelet-monocyte aggregate (PMA) formation, whole blood ($800 \mu\text{L}$) was diluted in Hepes-Tyrodes buffer (2.4 mL). Fluorescence-activated cell sorter buffer ($250 \mu\text{L}$) was subsequently added to the blood for 10 min at 25°C in the presence of CD41 phycoerythrin and CD14-FITC, a monocyte marker. Monocytes were selected by gating a total of 1,500 CD41⁺ events on a two-parameter dot plot displaying SSC vs CD14-FITC. Single platelets were excluded using a combination of forward scatter and SSC and positive anti-CD14 fluorescence. The percentage of platelet-marker-positive monocyte conjugates was measured to represent the percentage of monocytes with one or more adherent platelets. The total numbers of PMA were adjusted for any nonspecific binding and displayed as the fraction of monocytes with one or more adherent platelets.

Cytokine and Statistical Analyses

Commercially available sandwich enzyme-linked immunosorbent assays were used to determine IL-6 (manufacturer's limit of detection, 0.7 pg/mL) and tumor necrosis factor α (TNF- α) (manufacturer's limit of detection 5.5 pg/mL) levels from platelet-poor plasma (R&D). Statistical analyses were performed with STATA (version 11.0, StataCorp). Parametric *t* tests were used for continuous variables and the χ^2 test for categorical variables between groups. A one-way analysis of variance was used to compare categorical variables among three-group indices. Logistic linear regression analysis was used to identify correlations between cytokine levels, platelet activation indices, and illness severity. Post hoc analyses were performed using Tukey honestly significant difference pairwise comparisons for unequal groups. Statistical significance was predetermined at $P < .05$.

RESULTS

Hypotension requiring vasopressor support (eg, shock) was common in patients with A(H1N1) and bacterial pneumonia (35% vs 20% , $P = .33$) (Table 1). There were no significant differences in either age or sex between groups (Table 1). As expected given the inclusion criteria of ALI/ARDS, most patients required mechanical ventilation (80% of patients with A[H1N1] and 100% of patients with bacterial pneumonia, $P = .07$). Volume control was the most common mode of mechanical ventilation in the intubated,

Manuscript received November 9, 2011; revision accepted February 4, 2012.

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Funding/Support: This work was supported by the National Institutes of Health [Grants HL091754, HL066277, and K23HL092161] and the National Center for Research Resources public health service [Grant ULI-RR025764].

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DOI: 10.1378/chest.11-2860

Table 1—Demographics of the Study Population and Laboratory Results on ICU Admission

Demographics and Laboratory Results	A(H1N1) (n = 20)	Bacterial Pneumonia (n = 15)	Healthy Control Subjects (n = 10)	P Value
Age, y	41.5 (13.0)	46.8 (14.4)	44.9 (16.5)	.56
Male sex, No. (%)	10 (50)	11 (73)	13 (65)	.35
Comorbidities, No. (%)				
Cancer	2 (10)	1 (6.7)	0 (0)	.73
Diabetes mellitus	2 (10)	4 (2.7)	0 (0)	.20
Chronic liver disease	0 (0)	0 (0)	0 (0)	NA
CVD	0 (0)	0 (0)	0 (0)	NA
Congestive heart failure	0 (0)	0 (0)	0 (0)	NA
Admission data				
APACHE II score	20.5 (7.4)	17.9 (8.7)	...	.35
Shock requiring vasopressors, No. (%)	7 (35)	3 (20)	...	.33
In-hospital mortality, No. (%)	7 (35)	0 (0)	...	.01
WBC count, K/uL	7.6 (4.4)	14.7 (6.2)	...	.0003
Platelet count K/uL	162 (104)	232 (135)	...	.09
Hemoglobin, g/dL	11.6 (1.9)	11.6 (2.6)	...	.93
Fibrinogen, mg/dL	525 (284)	644 (192)	...	.21
Protime, s	15.5 (4.2)	17.0 (3.9)	...	.33
Partial thromboplastin time, s	40.5 (9.8)	46.0 (28.2)	...	.49

Data presented as mean (SD) unless otherwise specified. A(H1N1) = 2009 influenza A(H1N1); APACHE = Acute Physiology and Chronic Health Evaluation; CVD = cardiovascular disease (including stroke, transient ischemic attack, and myocardial infarction); NA = not applicable.

critically ill patients (81% in patients with A[H1N1] vs 87% in patients with pneumonia, $P =$ not significant). The APACHE II scores were similar between groups (Table 1) and, as expected, trended higher in patients with shock requiring vasopressors (22.6 ± 9.5 vs 18.1 ± 7.0 , $P = .13$). All patients with bacterial pneumonia had microbiologic testing performed, including blood and urine cultures, deep sputum culture, BAL, and influenza polymerase chain reaction. Overall, 60% ($n = 9$ of 15) of patients with pneumonia had one or more pathogenic bacteria present in the sputum or BAL cultures. *Staphylococcus aureus* (56%) and *Streptococcus* (44%) were the organisms most commonly identified, whereas *Pseudomonas* was rare (only one culture, 11%). In cultures where *S aureus* was present, pathogens recognized to be common causes of pneumonia (eg, *Streptococcus*) were also frequently present. Only two patients with a positive sputum culture were diagnosed with *S aureus* pneumonia (frequency 13.3%). WBC and platelet counts on ICU admission were lower in patients with A(H1N1) compared with patients with bacterial pneumonia (Table 1). In-hospital, all-cause mortality was significantly higher in subjects with A(H1N1) than patients with bacterial pneumonia (35% vs 0%, $P < .05$).

Although platelets were activated in both critically ill patients with A(H1N1) and patients with bacterial pneumonia, the pattern of platelet activation was distinct. Baseline PMA formation was significantly higher in patients with A(H1N1) compared with healthy control subjects and trended higher compared with patients with bacterial pneumonia (Fig 1A). Similarly, platelets from patients with A(H1N1) dem-

onstrated higher baseline levels of PAC-1 binding compared with patients with bacterial pneumonia and healthy control subjects (Fig 1B). In patients with A(H1N1) with shock ($n = 7$) compared with patients with A(H1N1) without shock ($n = 13$), PAC-1 binding ($15.8\% \pm 10.2\%$ vs $6.1\% \pm 4.7\%$, $P = .17$) and levels of PMA ($25.1\% \pm 11.1\%$ vs $19.4\% \pm 4.5\%$, $P = .29$) trended higher, although the differences did not reach statistical significance.

Upon ex vivo stimulation with TRAP, PAC-1 binding was significantly lower in patients with A(H1N1) or bacterial pneumonia compared with healthy control subjects (Fig 1D). In contrast, levels of PMA with TRAP stimulation did not differ between the three groups (Fig 1C). However, in patients with A(H1N1), levels of TRAP-stimulated PMA formation were lower in patients who died prior to hospital discharge ($n = 7/20$, mortality rate 35%), compared with those who survived ($55.8\% \pm 12.8\%$ vs $72.7\% \pm 5.0\%$, $P = .16$), although the difference did not reach significance.

Because A(H1N1) infections are associated with cytokine release, we also measured plasma levels of IL-6 and TNF- α . Levels of IL-6 were undetectable in healthy control patients (eg, < 0.7 pg/mL) but elevated in patients with A(H1N1) and patients with pneumonia (130.4 ± 22.2 pg/mL vs 172.1 ± 6.0 pg/mL, $P = .43$). Levels of IL-6 trended higher in patients with shock (140 ± 51.0 vs 64 ± 15.8 , $P = .07$). Consistent with these data, IL-6 levels were weakly correlated with APACHE II scores in patients with pneumonia ($r^2 = 0.24$, $P = .07$) but did not correlate in patients with A(H1N1) ($r^2 = 0.0006$, $P = .92$). IL-6 levels did not correlate with levels of PMA or PAC-1 binding in

FIGURE 1. A, Platelet-monocyte aggregate (PMA) formation in baseline whole blood obtained from critically ill patients with 2009 influenza A(H1N1) (A[H1N1]), patients with bacterial pneumonia, and healthy control subjects. B, PAC-1 binding in baseline whole blood obtained from critically ill patients with A(H1N1), patients with bacterial pneumonia, and healthy control subjects. C, PMA formation in TRAP-activated whole blood obtained from critically ill patients with A(H1N1), patients with bacterial pneumonia, and healthy control subjects. D, PAC-1 binding in TRAP-activated whole blood obtained from critically ill patients with A(H1N1), patients with bacterial pneumonia, and healthy control subjects. PMA formation is shown on the y-axis of A and C as the fraction of monocytes with one or more adherent platelets. PAC-1 binding is reported on the y-axis of B and D as the fraction of platelets expressing the active conformation of integrin $\alpha_{IIb}\beta_3$ on their surface as a percentage of the total number of platelets analyzed. H1N1 influenza = 2009 influenza A(H1N1); PAC-1 = antibody that binds to the active conformation of integrin $\alpha_{IIb}\beta_3$; TRAP = thrombin-receptor activating peptide.

either group. Levels of TNF- α were undetectable in patients with A(H1N1) and patients with bacterial pneumonia (eg, < 5.5 pg/mL).

DISCUSSION

A(H1N1), which reached pandemic proportions in 2009, commonly causes pulmonary infections leading to respiratory failure, systemic inflammation, and thrombosis.^{3,4,18} Thrombocytopenia and leukopenia are common during viral respiratory infections, including influenza A,⁴ and we observed similar presentations in our cohort (Table 1). To our knowledge, however, this is the first report characterizing in vivo platelet activation in patients with ALI/ARDS due to primary A(H1N1). Patients with A(H1N1) had evidence of in vivo platelet activation with increased levels of PMA and PAC-1 binding compared with age- and sex-matched healthy control subjects. These data are consistent with published observations of platelet activation in ALI/ARDS from non-A(H1N1) infections¹⁰ and descriptions of soluble CD40L release,

a marker of platelet activation, in influenza-associated encephalopathy.¹⁹

Patients with A(H1N1) also had increased platelet activation compared with patients with bacterial pneumonia of similar age, sex, and APACHE II scores (Fig 1, Table 1), although differences in mechanical ventilation rates cannot be ignored. Furthermore, levels of PAC-1 binding were higher in patients with A(H1N1) with shock requiring vasopressor support, suggesting that the magnitude of platelet activation was greater in more severely ill patients. Although less than one-half of the patients had shock, despite the high admission APACHE II scores, these patients had higher levels of IL-6, consistent with cytokine synthesis inducing a systemic inflammatory response and hypotension.^{8,10}

Although the markers of in vivo platelet activation examined in these patients (eg, PMA and PAC-1) are not used for routine clinical management, their relevance as indices of systemic inflammation and a hypercoagulable state has been demonstrated in similar disease states. Activation of platelets and leukocytes

with formation of heterotypic PMAs in critical illness and infectious syndromes leads to amplified systemic inflammatory responses and cytokine synthesis, contributing to lung injury, microvascular thrombosis, organ failure, and death.^{10,14} Similarly, PAC-1 is a monoclonal antibody that binds to the active conformation of integrin $\alpha_{\text{IIb}}\beta_3$, a platelet surface receptor that binds fibrinogen mediating platelet adhesion and aggregation. The data in the current study support and extend prior reports of thrombotic events in A(H1N1) infections⁵ by demonstrating that platelets—key mediators of thrombosis—are activated during A(H1N1). This in vivo platelet activation may also contribute to leukocyte and endothelial activation, further promoting thrombosis that contributes to organ failure and death.

In our study, patients with A(H1N1) also had a high incidence of in-hospital mortality, similar to other published observations.^{3,18} This high mortality may result from the marked systemic inflammatory responses induced by A(H1N1).

In contrast, none of the patients with bacterial pneumonia died during their hospitalization, a somewhat unexpected finding given their requirements for mechanical ventilation and the average APACHE II score of 17.9. This low mortality rate may be attributable to their younger age (average age, 47 years) and relatively few comorbidities (Table 1), which are associated with a lower risk of mortality.^{16,20,21} As matching was done blinded to all variables except age, sex, and APACHE II scores, the lower mortality of patients with bacterial pneumonia was unlikely to be due to inadvertent bias.

More than one-half of patients with pneumonia had a positive sputum of BAL culture result, thus helping to guide clinical management. *Streptococcus* was the most common cause of pneumonia, consistent with published studies.^{16,17,22} *S aureus* pneumonia occurred in 13.3% of the patients. The prevalence of *S aureus* increases in patients with severe pneumonia requiring ICU-level care^{16,17,23,24} and in patients with health-care associated pneumonia, although we did not capture this information from our cohort.^{16,17,22} Plasma IL-6 levels, but not TNF- α , were elevated in patients with A(H1N1), consistent with published data⁸ and indicating systemic inflammation consistent with our findings of activation of circulating platelets. IL-6 is a potent cytokine released during inflammatory illnesses that has been reported to activate platelets^{25,26} and inhibit TNF- α release²⁷ and may also predict respiratory failure and mortality in critically ill subjects.^{28,29} These data support the key role of IL-6 in host defenses and exaggerated inflammatory responses.³⁰

Our findings of increased PAC-1 binding to unstimulated platelets from patients with A(H1N1) but decreased PAC-1 binding upon TRAP stimulation of platelets from these subjects ex vivo (Fig 1) are intriguing

and require additional investigation. Since ex vivo PMA formation in response to TRAP stimulation was not reduced when cells from infected patients were compared with healthy control subjects, these data suggest that A(H1N1) infection induces altered intracellular signaling pathways to $\alpha_{\text{IIb}}\beta_3$ activation distal to protease-activated receptor-1.^{14,16} The specific mechanism(s) remain to be explored.

The strengths of our study include the prospective study design, inclusion of matched control subjects and patients with bacterial pneumonia, and rigorous assessment of in vivo platelet activation by flow cytometry. The primary limitation is the small sample size, as expected in a pilot study of this nature. In addition, we did not prospectively record specific details regarding the types of antibiotics prescribed or steroid administration, which may influence the inflammatory responses examined in this study. Nevertheless, our analysis identifies key issues of pathophysiologic and clinical relevance for future investigations of larger scale.

ACKNOWLEDGMENTS

Author contributions: Dr Rondina is the guarantor of the final manuscript.

Dr Rondina: contributed to study design and coordination, patient enrollment, data analyses, and manuscript preparation and read and approved the final manuscript.

Ms Brewster: contributed to study design and analysis of flow cytometry and read and approved the final manuscript.

Dr Grissom: contributed to study design and patient enrollment and read and approved the final manuscript.

Dr Zimmerman: contributed to study design and data analyses and interpretation and read and approved the final manuscript.

Ms Kastendieck: contributed to study design, whole blood and plasma analyses, and data interpretation and read and approved the final manuscript.

Dr Harris: contributed to study design and coordination, patient enrollment, and data analyses and read and approved the final manuscript.

Dr Weyrich: contributed to study design and coordination and data analyses and interpretation and read and approved the final manuscript.

Financial/nonfinancial disclosures: The authors have reported to CHEST that no potential conflicts of interest exist with any companies/organizations whose products or services may be discussed in this article.

Role of sponsors: The sponsors had no role in the study design, patient recruitment, data collection and analysis, manuscript preparation, or submission.

Other contributions: We thank Kim Mahoney, MS, Division of General Internal Medicine, for her assistance with figure preparation. From the Program in Molecular Medicine, we thank Harry Men, BS, and Saritha Kalva, MS, for technical assistance.

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