Platelet Activation, P-Selectin, and Eosinophil β1-Integrin Activation in Asthma

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Rationale: Eosinophil β1-integrin activation correlates inversely with FEV_1 and directly with eosinophil-bound P-selectin in subjects with nonsevere allergic asthma.

Objectives: Determine the relationships between β1-integrin activation and pulmonary function or eosinophil-bound P-selectin in subjects with asthma of varying severity and discern the source of eosinophil-bound P-selectin.

Methods: Blood was assayed by flow cytometry for P-selectin and activated β1-integrin on eosinophils and platelets. Plasma was analyzed with ELISA for soluble P-selectin, platelet factor 4, and thrombospondin-1.

Measurements and Main Results: Activated β1-integrin correlated with eosinophil-bound P-selectin among all subjects with asthma even though activated β1-integrin was higher in subjects with nonsevere asthma than severe asthma. Activated β1-integrin correlated inversely with FEV_1 corrected for FVC only in younger subjects with nonsevere asthma. Paradoxically, platelet surface P-selectin, a platelet activation marker, was low in subjects with severe asthma, whereas plasma platelet factor 4, a second platelet activation marker, was high. Correlations indicated that P-selectin–positive platelets complexed to eosinophils are the major source of the eosinophil-bound P-selectin associated with β1-integrin activation. After whole-lung antigen challenge of subjects with nonsevere asthma, a model of asthma exacerbation known to cause platelet activation, circulating eosinophils bearing P-selectin and activated β1-integrin disappeared.

Conclusions: The relationship between eosinophil β1-integrin activation and pulmonary function was replicated only for younger subjects with nonsevere asthma. However, we infer that platelet activation and

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Activated β1-integrins on blood eosinophils of young patients with nonsevere allergic asthma correlate with decreased pulmonary function in two study paradigms, inhaled corticosteroid withdrawal and lung antigen challenge. The relationship of β1-integrin activation to lung function and eosinophil P-selectin in the full range of patients with asthma is unknown.

What This Study Adds to the Field

A correlation between β1-integrin activation and decreased pulmonary function was shown to be restricted to the subpopulation of young subjects with nonsevere disease, whereas a correlation between eosinophil β1-integrin activation and surface-associated P-selectin was shown for the asthmatic population as a whole. Activated P-selectin–bearing platelets associating with eosinophils were the likely source of eosinophil surface-associated P-selectin. Eosinophils with activated α4β1-integrin appear to enter the lungs quickly in severe asthma.

binding of activated platelets to eosinophils followed by P-selectin–mediated eosinophil β1-integrin activation occur in both nonsevere and severe asthma with rapid movement of platelet–eosinophil complexes into the lung in more severe disease.

Keywords: asthma; blood platelets; eosinophils; P-selectin; integrins

Airway inflammation with eosinophilic infiltration of the bronchial mucosa is a characteristic feature of atopic asthma (1–4). Preferential movement of circulating eosinophils to airway is facilitated by induction of vascular cell adhesion molecule-1 (VCAM-1) in bronchial endothelium in response to Th2 immunity mediators (1, 5); VCAM-1 is a ligand for eosinophil α4β1 integrin (6). Integrins exist in different conformations (7), which can be monitored by certain monoclonal antibodies (mAbs). For example, the epitope for activation-sensitive anti-β1 N29 (8) is exposed in the extended active conformations (9).

High N29 epitope expression on circulating eosinophils is associated with a decrease from baseline of FEV_1 in young adult subjects with nonsevere asthma undergoing inhaled corticosteroid (ICS) withdrawal or lung antigen challenge (10, 11). This finding is potentially important inasmuch as eosinophil α4β1 activation is expected to synergize with lung endothelial VCAM-1 expression to direct eosinophils to airway. Recently, it was demonstrated that the amount of P-selectin bound to the surface of circulating eosinophils correlates with β1-integrin activation in subjects with nonsevere asthma and that addition of soluble P-selectin to blood *in vitro* enhances eosinophil β1-integrin activation and adhesion to VCAM-1 (12).

P-selectin is sequestered in Weibel-Palade granules of endothelial cells and α-granules of platelets and translocated to the surface in response to various inflammatory and thrombogenic mediators (13, 14). It is found soluble in plasma at 20 to 40 ng/ml in normal subjects (14–17) with increases described in various disease states (15), including in asthma after whole-lung antigen challenge or exercise (18, 19). Thus, eosinophil-bound P-selectin in asthma may be derived directly from interactions with activated platelets or endothelial cells or indirectly from platelets or endothelial cells via plasma.

The goals of the present study were to determine whether the correlations between eosinophil β_1-integrin activation and eosinophil-bound P-selectin (12) or pulmonary function (10, 11) are true for subjects with severe and nonsevere asthma and to learn the source of eosinophil-bound P-selectin, the likely cause of β_1-integrin activation (12). Blood was assayed by flow cytometry for P-selectin, activated β_1-integrin, the abundant platelet-surface protein α_{IIb} integrin subunit (CD41) as platelet marker (12, 20), and the P-selectin counter-receptor P-selectin glycoprotein ligand-1 (PSGL-1, CD162) (21) on eosinophils and for P-selectin on platelets, and by ELISA for soluble P-selectin and platelet α-granule proteins, platelet factor 4 (PF4) (18, 22, 23), and thrombospondin-1 (TSP-1) (24–26). Subjects were those studied locally as part of the Severe Asthma Research Program (SARP) (27–29) or undergoing whole-lung antigen challenge. The results indicate that platelet activation and binding of activated platelets bearing surface P-selectin to eosinophils followed by P-selectin–mediated eosinophil β_1-integrin activation occur in both nonsevere and severe asthma and are in accord with previous studies linking platelets to airway inflammation and hyperreactivity in humans and animal models (18, 30–41). Some of the results have been reported previously in the form of an abstract (42).

METHODS

Subjects and Assessments

Subjects with severe or nonsevere asthma judged by American Thoracic Society criteria (43) were screened and enrolled in SARP (27, 29) at the University of Wisconsin (28) and were analyzed by flow cytometry and ELISA; subjects with no asthma enrolled in SARP were also studied. Data on SARP subjects, grouped by severity, are shown in Table 1 and based on methods described in the online supplement. Non-SARP subjects with allergic nonsevere asthma were studied in the setting of whole-lung antigen challenge as described in the online supplement. These subjects had the following values before antigen challenge: mean FEV_1 of 96 % predicted ($\pm$ SD of 18% predicted), median provocative concentration of methacholine producing a 20% fall in FEV_1 of 0.9 mg/ml (with 25th and 75th percentiles of 0.4 and 4.4 mg/ml, respectively), mean of 180 ($\pm$ 100) blood eosinophils/μl, mean of 220 ($\pm$ 60) $\times$ 10^3 platelets/μl, median sputum eosinophils of 0.3 % (0.0, 2.2%), and median fraction of exhaled nitric oxide of 43 ppb (26, 60 ppb). None of the SARP or antigen challenge subjects participated in our earlier studies (10–12). Both studies were approved by the University of Wisconsin-Madison Health Sciences Institutional Review Board. Written consent was obtained from each subject.

Antibodies, ELISA, and Flow Cytometry

Antibodies, ELISA, and flow cytometry are described in the online supplement.

Statistics and Time-to-Time and Subject-to-Subject Variability of Flow Cytometry and ELISA Measurements

The Mann-Whitney or χ^2 tests were used to compare ordinal or nominal data, respectively, between two groups; the Wilcoxon test or the Friedman two-way analysis of variance to compare data from different visits of single subjects or single visits of different subjects. The Spearman rank test was used to analyze correlations. A *P* value $\leqslant$ 0.05 was considered significant. Analyses were performed using Prism (GraphPad; La Jolla, CA) or as described (44). Group data are reported as mean $\pm$ SD if the variable was normally distributed (passed Prism's normality test) and as median with 25th and 75th percentiles if the variable was non-normally distributed.

Individual-to-individual versus visit-to-visit variability was analyzed in three SARP subjects who had blood analyzed at three different visits. Coefficients of variation (SD/mean) for N29 reactivity and plasma P-selectin measured at different visits in the same subject were smaller than the coefficients of variation of the means of the subjects such that there were significant differences among subjects but not among visits.

RESULTS

Description of SARP Subjects

To learn the relationships among β_1-integrin activation, eosinophil-bound P-selectin, and pulmonary function in subjects with asthma of variable severity, we studied 37 SARP subjects, 10 with severe asthma, 21 with nonsevere asthma, and 6 normal control subjects (Table 1). Criteria for severe and nonsevere asthma were as described (27, 28, 43). Power analyses of sample sizes required to detect possible correlations were performed based on previously observed correlations in nonsevere asthma between N29 reactivity and FEV_1 (Spearman rank correlation coefficients [r_s] = −0.50 to −0.56) (10) or eosinophil-associated P-selectin (r_s < 0.8) (12). The analyses estimated that a sample size of 8 or 22 subjects was needed to detect with 80% power a true correlation with $r = \pm 0.8$ or 0.5, respectively, with the alternative hypothesis $r = 0$. Thus, the target was to study at least eight subjects in each asthma status group. Subject status was blinded until after flow cytometry was performed. Accrual was discontinued when 10 subjects with severe asthma and 22 with nonsevere asthma had been enrolled. One of these subjects with nonsevere asthma was later removed from analysis due to the interim diagnosis of possible sarcoidosis.

The mean age was higher in subjects with severe asthma than nonsevere asthma and lowest in the normal subjects. Subjects with severe asthma had lower FVC than subjects with nonsevere asthma, as described in larger studies (27, 28). The concentrations of serum IgE were higher in both asthma groups than in normal subjects. Mean platelet count was lower in nonsevere asthma than normal with a trend to a lower count also in severe asthma. Only one subject had a count below the lower end of the normal range, at 1.1×10^6/ml. All the subjects with severe and a majority of those with nonsevere asthma were receiving inhaled corticosteroids (ICS). Some subjects with severe asthma also received oral corticosteroids. The daily ICS dose in the severe asthma population was higher than that of the nonsevere asthma group. All the subjects with asthma except two (one with severe asthma and one with nonsevere asthma) had at least one positive skin test. The two skin test–negative subjects had experienced allergic symptoms. Thus, no subjects were nonallergic. Plasma from additional 54 SARP subjects (25 subjects with nonsevere asthma, 18 with severe asthma, and 11 normal subjects) was studied by ELISA for P-selectin and PF4. The demographics and screening results were similar to the 37 SARP subjects described in Table 1.

Eosinophil β_1-Integrin Activation Correlates with Eosinophil-Bound P-Selectin in Subjects with Asthma as a Whole but with FEV_1/FVC Only in Young Subjects with Nonsevere Asthma

For the 31 subjects with asthma grouped together or the 21 subjects with nonsevere asthma, mean eosinophil β_1-integrin

TABLE 1. SEVERE ASTHMA RESEARCH PROGRAM SUBJECT CHARACTERISTICS

Variable	Group		
	Severe Asthma	Nonsevere Asthma	No Asthma
N	10	21	6
Sex, female, male	6, 4	12, 9	3, 3
Age, yr	34 ± 13	29 ± 10	25 ± 8
FEV_1, % predicted	74 ± 25	92 ± 13	94 ± 6
FEV_1/FVC, % predicted	88 ± 20	91 ± 8	94 ± 5
FVC, % predicted	82 ± 16[*,†]	100 ± 12	102 ± 6
PC_{20}, mg/ml	1.2 (0.7, 4.9)[‡]	1.5 (1.0, 6.8)[‡]	50 (50, 50)
IgE, IU/ml	160 (74, 270)[‡]	100 (36, 300)[‡]	9 (2, 56)
Positive skin tests, No.	6 ± 3[†]	6 ± 3[‡]	0 ± 0
Blood eosinophils, per μl	210 ± 70[†]	240 ± 170[†]	110 ± 60
Blood neutrophils, per μl	$3,500 \pm 1,100$	$3,500 \pm 980$	$3,500 \pm 1,360$
Platelets, thousands per μl	250 ± 30	240 ± 40[†]	290 ± 40
Sputum eosinophils, % of WBC	0.7 (0.0, 7.2)	0.4 (0.1, 1.2)	0.1 (0.0, 0.2)
Sputum neutrophils, % of WBC	43 ± 21	35 ± 17	53 ± 23
FeNO, ppb	30 (22, 84)[†]	24 (17, 47)	13 (11, 21)
No. (%) on ICS	10 (100)[§,‖]	14 (67)[‡]	0 (0)
No. (%) on oral CS	4 (40)[*]	0 (0)	0 (0)
ICS dose, μg/d	1,000 (940, 1,000)[‡,¶]	250 (0, 500)	0 (0, 0)

Definition of abbreviations: CS = corticosteroid; FeNO = fraction of exhaled nitric oxide; ICS = inhaled CS; PC_{20} = provocative concentration of methacholine producing a 20% fall in FEV_1; SARP = Severe Asthma Research Program; WBC = white blood cells.

Data are presented as mean $\pm$ SD or median (25th, 75th percentiles) unless otherwise noted. The allergen extracts used for skin testing were Eastern seven-tree mix, grass mix, short ragweed, common weed mix, dog, cat, *Alternaria*, *Cladosporium*, *Aspergillus* mix, *Dermatophagoides farinae*, *Dermatophagoides pteronyssinus*, and American cockroach, with histamine as positive control and diluting fluid as negative control, and were from Greer Laboratories (Lenoir, NC). ICS dose is expressed as fluticasone equivalent, based on the equivalence of 1,200 μg budesonide or 1,260 μg beclomethasone with 880 μg fluticasone (43). Twenty-one subjects were on fluticasone, two on budesonide, and one on beclomethasone. Of the 28 subjects with asthma with reliable sputum data (*see* the online supplement for exclusion criterion), 20 subjects (71%) had a paucigranulocytic asthma inflammatory phenotype, 6 (21%) were eosinophilic, 2 (7%) neutrophilic, and 0 (0%) mixed granulocytic, using the cutoffs 2% eosinophils and 61% neutrophils as described by Gibson and colleagues (60). Alternatively, using the cutoffs 2% eosinophils and 40% neutrophils as done in a larger SARP cohort (59), 13 subjects (46%) were paucigranulocytic, 2 (7%) eosinophilic, 9 (32%) neutrophilic, and 4 (14%) mixed.

[*] $P \leqslant 0.01$ versus nonsevere asthma.
[†] $P \leqslant 0.05$ versus no asthma.
[‡] $P \leqslant 0.01$ versus no asthma.
[§] $P \leqslant 0.05$ versus nonsevere asthma.
[‖] $P \leqslant 0.001$ versus no asthma.
[¶] $P \leqslant 0.001$ versus nonsevere asthma.

activation on circulating eosinophils was significantly increased by 1.9-fold or 2.2-fold, respectively, when compared with normal subjects (Table 2). Eosinophil surface P-selectin was not significantly different in subjects with asthma as compared with normal subjects, but there was a trend to a lower level in subjects with severe asthma (Table 2). Results were similar for eosinophil surface-associated α_{IIb} integrin, which is an abundant platelet surface protein (20) and which we used as a platelet marker as before (12), with a trend to lower signal in severe asthma (Table 2). We also measured eosinophil expression of PSGL-1, the eosinophil surface counter-receptor for P-selectin (21). PSGL-1 expression was high in all groups and not significantly different among them (Table 2).

A plot of β_1-integrin activation versus eosinophil-surface P-selectin demonstrated considerable scatter, with more variability for subjects with nonsevere asthma (Figure 1A). Nevertheless, β_1-integrin activation correlated directly with eosinophil surface P-selectin in subjects with asthma ($r_s = 0.42$, $P = 0.02$) (Table 3). The correlation was less in subjects with nonsevere asthma ($r_s = 0.23$, $P = 0.32$) than in severe asthma ($r_s = 0.81$, $P = 0.006$).

In earlier studies correlating eosinophil β_1 activation with pulmonary function (10, 11), subjects had median ages of 21 years, with only one of 27 subjects older than 30 years. The measure of function was change in FEV_1 from baseline before

steroid withdrawal or lung antigen challenge (10, 11). No intervention was taken in the present study to warrant a baseline, and therefore the measure of pulmonary function here was FEV_1 divided by FVC, both expressed as percent of the predicted value. As the subjects in the previous studies all had nonsevere asthma and most were less than 30 years of age, a question was whether the correlation between eosinophil β_1 activation and lung function would hold up across all subjects with asthma or would be restricted to such a subgroup. To address this issue, we analyzed both the whole population with asthma and subgroups. For all subjects with asthma, r_s for N29 reactivity versus FEV_1/FVC was -0.26, which was not significant (Table 3). For subjects with nonsevere asthma, r_s was -0.38, still nonsignificant but a greater absolute value than the correlation of r_s of -0.18 for subjects with severe asthma. When we analyzed the 14 subjects with nonsevere asthma and aged 30 years or younger, activated β_1 correlated inversely with FEV_1/FVC with an r_s of -0.54 ($P = 0.05$).

The results were initially puzzling. On the one hand, we found that the previously described relationship between activated eosinophil β_1-integrin and airway physiology (10, 11) is specific for younger subjects with nonsevere asthma. On the other hand, we found that the correlation between activated eosinophil β_1 integrin and eosinophil-bound P-selectin holds for all subjects with asthma as a group.

TABLE 2. EOSINOPHIL β_1-INTEGRIN ACTIVATION, P-SELECTIN, PLATELET FACTOR 4, AND THROMBOSPONDIN-1 IN SEVERE ASTHMA RESEARCH PROGRAM SUBJECTS WITH ASTHMA, NONSEVERE ASTHMA, SEVERE ASTHMA, OR NO ASTHMA

Measurement	All Asthma	Nonsevere Asthma	Severe Asthma	No Asthma
Eosinophil N29, specific gMCF	210 ± 110* (31)	240 ± 100* (21)	150 ± 110 (10)	110 ± 100 (6)
Eosinophil P-selectin, specific gMCF	250 ± 180 (31)	260 ± 200 (21)	210 ± 120 (10)	250 ± 150 (6)
Eosinophil α_{IIb} integrin, specific gMCF	640 ± 350 (31)	670 ± 390 (21)	560 ± 260 (10)	640 ± 420 (6)
Eosinophil PSGL-1, specific gMCF	1,250 ± 230 (31)	1,230 ± 260 (21)	1,280 ± 150 (10)	1,180 ± 250 (6)
Platelet P-selectin, specific gMCF	110 (60, 590)* (31)	150 (60, 780) (21)	90 (0, 460)* (10)	500 (240, 1,220) (6)
Plasma P-selectin, ng/ml	35 ± 14 (31)	36 ± 11 (21)	33 ± 19 (10)	40 ± 8 (6)
	25 ± 13 (74)	25 ± 12 (46)	26 ± 14 (28)	23 ± 12 (17)
Platelet factor 4 (PF4), ng/ml	9.7 (5.5, 36) (31)	7.9 (5.2, 16) (21)	31 (12, 62)† (10)	16 (9.4, 130) (6)
	7.2 (4.0, 17) (74)	6.3 (3.7, 13)* (46)	13 (5.2, 22)‡ (28)	12 (5.8, 21) (17)
Thrombospondin-1 (TSP-1), ng/ml	250 ± 200 (30)	230 ± 160 (20)	290 ± 270 (10)	300 ± 200 (5)

Definition of abbreviations: gMCF = geometric mean channel fluorescence; mAb = monoclonal antibody; PF4 = platelet factor 4; PSGL-1, P-selectin glycoprotein ligand-1; SARP = Severe Asthma Research Program; TSP-1 = thrombospondin-1.

Data are presented as mean ± SD (n) or median (25th, 75th percentiles) (n). The reactivity of blood eosinophils with activation-sensitive anti-β_1 integrin mAb N29, the level of blood eosinophil surface-associated P-selectin and α_{IIb} integrin subunit, blood eosinophil expression of PSGL-1, and platelet surface expression of P-selectin were measured by flow cytometry; and the concentrations of soluble plasma P-selectin, PF4, and TSP-1 were measured by ELISAs as described in the online supplement. Expression level values for platelet P-selectin are not comparable to those for eosinophil N29, P-selectin, α_{IIb}, and PSGL-1 due to different settings of detector sensitivity for platelets and leukocytes, as described in the online supplement. Data are from the SARP subjects described in Table 1, except that the data in the bottom entry for plasma P-selectin and PF4 also include the additional 54 SARP subjects mentioned in the text under Subject Accrual and Characteristics.

* $P \leqslant 0.05$ versus no asthma.
† $P \leqslant 0.01$, versus nonsevere asthma.
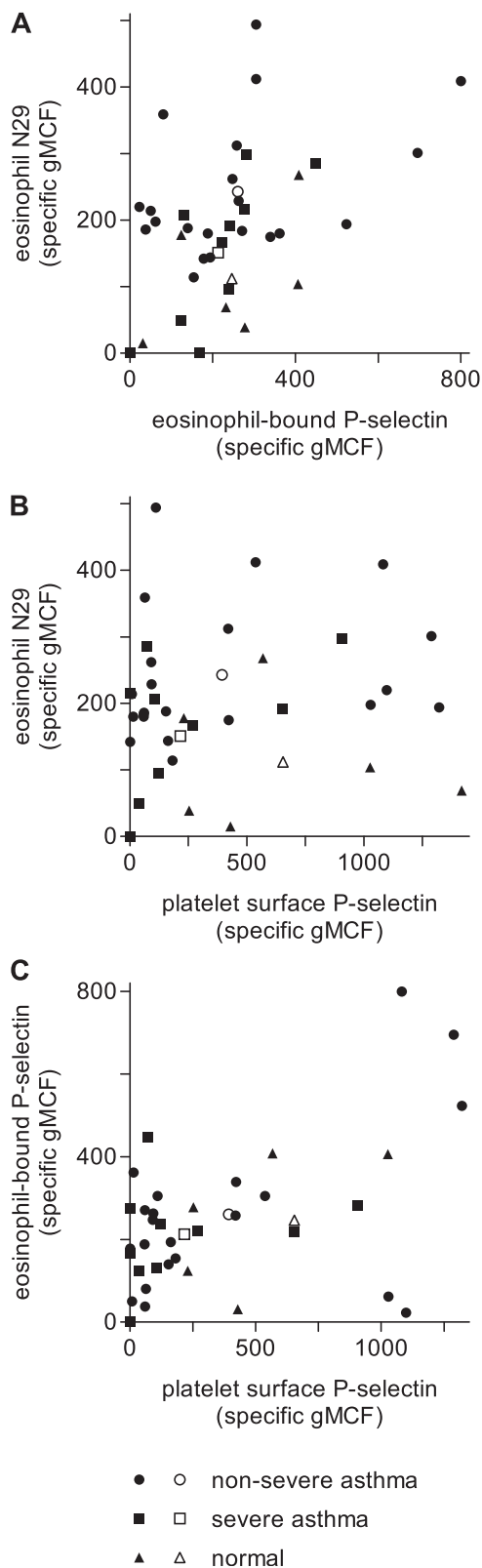‡ $P \leqslant 0.05$ versus nonsevere asthma.

The Primary Source of the Eosinophil-Bound P-Selectin Associated with Eosinophil β_1-Integrin Activation in Asthma Is Likely the Activated Platelet

Activated platelets bind to leukocytes, including eosinophils, via P-selectin (45) binding its counter-receptor PSGL-1 (21). Studies of platelet-depleted mice reconstituted with activated wild-type or P-selectin–deficient platelets implicate platelet P-selectin as a cause of airway eosinophilic inflammation in the ovalbumin-sensitization model (38, 40). An *in vitro* study observed enhanced complex formation between activated P-selectin–bearing platelets and eosinophils from subjects with allergic asthma and indicated that platelet association contributes to the enhanced tethering of such eosinophils to activated endothelium in a P-selectin–dependent manner (46). Previous studies have demonstrated variable increases in P-selectin in plasma in subjects with asthma after antigen challenge or exercise (18, 19) and on the platelet surface in aspirin-sensitive subjects or associated with acute disease (33, 34). In immunofluorescence microscopic studies of P-selectin associated with circulating eosinophils from subjects with nonsevere asthma, P-selectin was found in a patchy distribution, with some but not all of the patches colocalizing with platelet markers (12). A possible explanation for the platelet-free P-selectin–positive sites is the transient nature of the selectin-dependent association of platelets to eosinophils; activated platelets may "deliver" P-selectin to the eosinophil surface and then dissociate. To learn the possible source(s) of the eosinophil-bound P-selectin, we measured the concentration of soluble P-selectin in plasma by ELISA and P-selectin on the surface of activated platelets by flow cytometry. Samples were processed to minimize artifactual platelet activation due to blood collection and handling, and results were correlated with activated β_1-integrin and P-selectin on the surface of eosinophils.

ELISA estimates of plasma P-selectin concentration were no different from normal subjects for the subjects with asthma as a whole or for the groups with nonsevere or severe asthma (Table 2). The failure to demonstrate differences in plasma P-selectin persisted when additional individuals were studied (Table 2). The variability in measurements of platelet-surface P-selectin by flow cytometry

was considerable (Table 2, Figures 1B and 1C), as has been found before (33). Nevertheless, flow cytometry revealed a significant decrease in platelet surface P-selectin in subjects with severe asthma (Table 2). Platelet surface P-selectin, but not plasma P-selectin concentration, correlated directly with β_1-integrin activation ($r_s = 0.42$, $P = 0.02$) and eosinophil-bound P-selectin ($r_s = 0.35$, $P = 0.05$) in subjects with asthma as a whole (Table 3, Figures 1B and 1C). The correlations had similar r_s values for both nonsevere and severe subjects (activated β_1-integrin versus platelet surface P-selectin: $r_s = 0.34$, $P = 0.13$ for nonsevere and $r_s = 0.50$, $P = 0.14$ for severe; eosinophil P-selectin versus platelet surface P-selectin: $r_s = 0.33$, $P = 0.15$ for nonsevere and $r_s = 0.42$, $P = 0.22$ for severe). The results suggest that the major source of eosinophil-bound P-selectin associated with eosinophil β_1-integrin activation in asthma is activated platelets that associate with eosinophils. In addition, platelet association with eosinophils assessed by eosinophil-bound α_{IIb} integrin correlated with $r_s = 0.61$, $P = 0.0003$ with eosinophil P-selectin in subjects with asthma (Table 3), further strengthening the conclusion that platelets associating with eosinophils are the source of eosinophil-bound P-selectin.

The observations that subjects with severe asthma have low platelet surface P-selectin and their eosinophils have trends to low eosinophil-bound P-selectin and α_{IIb} and do not have increased β_1-integrin activation (Table 2) raise the possibility that platelet and eosinophil β_1-integrin activation, and platelet-eosinophil complex formation, are ongoing in severe asthma but not detected by flow cytometry of venous blood because of decreased entry into the circulation or increased clearance. P-selectin is a type I membrane protein that is sequestered in platelet α-granules and mobilized to the platelet surface upon platelet activation (13, 14). We therefore quantified plasma PF4 (18, 22, 23) and TSP-1 (24–26), which also originate in α-granules and are released into the circulation upon platelet activation. Plasma PF4 has previously been demonstrated to be higher in patients with asthma when symptomatic (32, 39), after exercise (30), or after whole-lung antigen challenge (18, 32). Plasma PF4 was significantly higher and TSP-1 trended to a higher level in subjects with severe asthma than with nonsevere

Eosinophil β_1-Integrin Activation and Eosinophil-Bound P-Selectin after Whole-Lung Antigen Challenge

The results described above on subjects with stable asthma of variable severity suggest that platelet activation, association of activated platelets with eosinophils, and binding of P-selectin to eosinophils lead to activation of eosinophil β_1-integrin and are linked to increased vascular clearance of eosinophils with surface P-selectin and activated β_1-integrin. To examine trafficking of such eosinophils in a second experimental paradigm, we studied 16 subjects with mild allergic asthma undergoing whole-lung inhaled antigen challenge. Such challenge has been shown to lead to platelet activation, a decrease in the number of eosinophils in the circulation at 0.5 to 6 hours followed by a recovery at 24 hours, and a decrease in the platelet count at 0.5 hours (and 6 h in subjects with a dual-response phenotype) followed by recovery at 6 hours (at 24 h in dual responders) (18), and an increase in soluble VCAM-1 and eosinophils in the airway at 4 to 48 hours (47–49).

There was a significant decrease in β_1-integrin activation at 8 hours after challenge followed by a significant recovery at 48 hours (Figures 2A and 2E). Eosinophil-bound P-selectin was also significantly decreased at 8 hours, followed by a trend to a recovery at 48 hours (Figures 2B and 2F). The two measurements tracked together ($r_s = 0.42$, $P = 0.0005$ assuming that the 64 samples are independent of one another). Eosinophil-bound α_{IIb} similarly showed trends to a greatest decrease at 8 hours followed by recovery at 48 hours (Figures 2C and 2G). Eosinophil PSGL-1 expression had a minor but significant decrease at 2 hours, a somewhat greater decrease at 8 hours, and a significant recovery at 48 hours (Figures 2D and 2H). Mean blood eosinophil concentration, measured by a Unopette method, was decreased at 2 hours (150 per μl vs. 180 per μl at 0 h, $P = 0.31$; then recovered to 180 and 220 per μl at 8 and 48 h, respectively; $P = 0.03$ for 48 h versus 2 h). In contrast, the percentage of eosinophils in sputum increased from a median of 0.3% (25th and 75th percentiles, 0.0 and 2.2%, respectively) at 0 hours to 7.0% (4.0, 8.8%) at 48 hours ($P = 0.002$), and the number of eosinophils per weight sputum increased from a median of 1,100 (0, 19,000) per g at 0 hours to 38,000 (14,000, 62,000) per g at 48 hours ($P = 0.02$). Platelet counts are available from 14 of these 16 subjects; mean platelet concentration was $2.2 \times 10^6/\mu$l at 0 hours and $2.4 \times 10^6/\mu$l at 2, 8, and 48 hours. Thus, whereas Kowal and colleagues recorded a transient decrease in platelet count in all their subjects at 0.5 hours (and in dual responders at 6 h) (18), we did not observe a decrease in platelet numbers. Perhaps a decrease in platelets occurred also in our subjects at 0.5 hours, which we did not record. The disappearance of circulating eosinophils bearing surface P-selectin, high PSGL-1 expression, and activated β_1-integrin; the trend for disappearance of eosinophils bearing α_{IIb}; and appearance of more eosinophils in sputum suggest that those eosinophils with the highest PSGL-1 level that have associated with activated P-selectin–bearing platelets triggering β_1-integrin activation preferentially traffic to the airway.

asthma; the difference in PF4 persisted when a larger number of subjects was tested (Table 2). Remarkably, there was an inverse correlation of r_s of -0.38 or -0.47 between plasma PF4 or TSP-1 and activated eosinophil β_1-integrin for the asthmatic group as a whole and trends to inverse correlations between plasma PF4 or TSP-1 and eosinophil or platelet surface P-selectin (Table 3).

TABLE 3. CORRELATIONS AMONG P-SELECTIN, PLATELET FACTOR 4, THROMBOSPONDIN-1, EOSINOPHIL β_1-INTEGRIN ACTIVATION, AND MEASUREMENTS OF PULMONARY FUNCTION IN SEVERE ASTHMA RESEARCH PROGRAM SUBJECTS WITH ASTHMA

Protein/mAb	PF4 r_s/P Value	TSP1 r_s/P Value	Eos N29 r_s/P Value	Eos PS r_s/P Value	Eos α_{IIb} r_s/P Value	Platelet PS r_s/P Value	FEV$_1$ r_s/P Value	FEV$_1$/FVC r_s/P Value
Plasma PS	0.02/0.93	0.01/0.97	0.23/0.22	0.14/0.46	0.13/0.48	0.09/0.62	−0.03/0.80	0.02/0.88
PF4		0.44/0.02	−0.38/0.04	−0.32/0.08	−0.20/0.28	−0.26/0.16	0.03/0.83	0.08/0.51
TSP-1			−0.47/0.01	−0.19/0.33	−0.08/0.68	−0.20/0.31	0.05/0.80	0.02/0.91
Eos N29				0.42/0.02	0.17/0.37	0.42/0.02	−0.10/0.60	−0.26/0.15
Eos PS					0.61/0.0003	0.35/0.05	0.07/0.72	−0.01/0.96
Eos α_{IIb}						0.32/0.08	0.36/0.05	0.28/0.13
Platelet PS							0.05/0.78	−0.01/0.95
FEV$_1$								0.71/<0.0001

Definition of abbreviations: Eos = eosinophil; mAb = monoclonal antibody; PF4 = platelet factor 4; PS = P-selectin; r_s = Spearman rank correlation coefficient; TSP-1 = thrombospondin-1.

For measurements, *see* Table 2.

DISCUSSION

Asthma is a heterogeneous disease based on symptoms, allergen sensitization, airflow limitation, response to medication, airway inflammation, and outcomes; and establishing subgroups and relating subgroups to pathophysiology are considered important steps in determining optimal treatments (2, 3, 29, 50–52). Working from studies demonstrating associations between blood eosinophil β₁-integrin activation and lung function (10, 11) and effects of P-selectin binding to eosinophils on β₁-integrin activation (12) in young subjects with nonsevere asthma, we undertook the present study of subjects with allergic asthma of variable severity. We reproduced the association between eosinophil β₁-integrin activation and pulmonary function only in subjects with nonsevere asthma similar to those studied previously. Nevertheless, the association between β₁-integrin activation and eosinophil-bound P-selectin was true for the asthmatic population as a whole, suggesting that similar pathophysiology underlies eosinophil β₁-integrin activation in both nonsevere and severe allergic asthma. Platelet surface P-selectin correlated with eosinophil β₁-integrin activation and eosinophil-bound P-selectin, whereas soluble P-selectin did not. Furthermore, degree of platelet association with eosinophils, assessed by α$_{IIb}$ integrin subunit, correlated strongly with eosinophil-bound P-selectin. Thus, the major source of eosinophil-bound P-selectin that likely triggers β₁-integrin activation in allergic asthma appears to be activated platelets that associate with eosinophils.

Expression of P-selectin on the surface of free platelets was significantly lower than normal in subjects with severe asthma but not in subjects with nonsevere asthma. One possible explanation for this phenomenon is that in severe asthma there is rapid shedding of P-selectin from the surface of free circulating activated platelets that have not associated with circulating leukocytes or endothelium. Rapid P-selectin shedding from circulating activated platelets have been described in animal models (53–55). Plasma PF4 was higher in severe than in nonsevere asthma, indicating that, despite low P-selectin on surfaces of platelets and eosinophils, there is more platelet activation in severe asthma.

Figure 3 depicts a model that addresses this paradox and is buttressed by the findings that transfusion of platelet-depleted mice with activated wild-type but not P-selectin–deficient platelets enhances eosinophil recruitment to the airway after lung antigen challenge (38, 40) and that *in vitro* there is enhanced complex formation between activated P-selectin–bearing platelets and eosinophils from subjects with allergic asthma contributing to enhanced P-selectin–dependent tethering of such eosinophils to activated endothelium (46). Circulating eosinophils bearing activated β₁-integrins and P-selectin are shown as becoming increasingly less common in more severe asthma even as platelet activation, as assessed by systemic PF4 concentration, increases. This discrepancy occurs because complexes of activated platelets and eosinophils are removed efficiently from the circulation. Efficient removal may be due to greater lung endothelial VCAM-1 expression, as has been observed in bronchial biopsies of subjects with severe compared with nonsevere asthma (56). Increased density of VCAM-1 would create a "sink" for arrest and transmigration of eosinophils with activated α₄β₁. The model is consistent with the trends toward lower numbers of blood eosinophils in SARP subjects with severe compared with nonsevere asthma (27) and higher sputum eosinophil numbers in severe asthma (Table 1). The model is also consistent with the decreased eosinophil β₁-integrin activation, surface P-selectin, and expression of the P-selectin counter-receptor PSGL-1 found after whole-lung inhaled antigen challenge, accompanied by decreased circulating eosinophils and increased sputum eosinophils. The model predicts that increased eosinophil β₁-integrin activation correlates with obstructive airway physiology only in patients with the mildest form of asthma (Figure 3). Presumably, β₁ activation on circulating eosinophils does not correlate with physiology in more severe asthma due to enhanced extravasation of activated eosinophils. Furthermore, the correlation did not hold up in older subjects, including older subjects with nonsevere asthma. Perhaps pulmonary function becomes less associated with eosinophil activation and recruitment as the disease evolves with time and increased age. Finally, the model is consistent with the lower or trends to lower platelet counts in nonsevere and severe asthma (Table 1). The lower numbers of free platelets may be due to platelets associating with eosinophils and other leukocytes or extravasating along with the complexes or possibly also due to migration of isolated platelets, as has been described in mice (57). In this context, it can be noted that atopic asthma has been reported to be accompanied by shorter platelet survival time (41, 58).

As mentioned, there was a trend to higher sputum eosinophil numbers in severe asthma. Still, these sputum eosinophil levels were, although variable among subjects, relatively low. Using criteria for the definition of inflammatory phenotypes in asthma (59, 60), a plurality of our subjects can be considered to have paucigranulocytic asthma with smaller groups having eosinophilic or neutrophilic asthma dependent on cutoffs used (*see* legend of Table 1). Perhaps the relatively low level of sputum eosinophils in both severe and nonsevere asthma is an effect of steroid treatment and maybe more subjects would be considered "eosinophilic" if their steroid dose were reduced; sputum eosinophil percentage tends to increase if ICS are withdrawn in
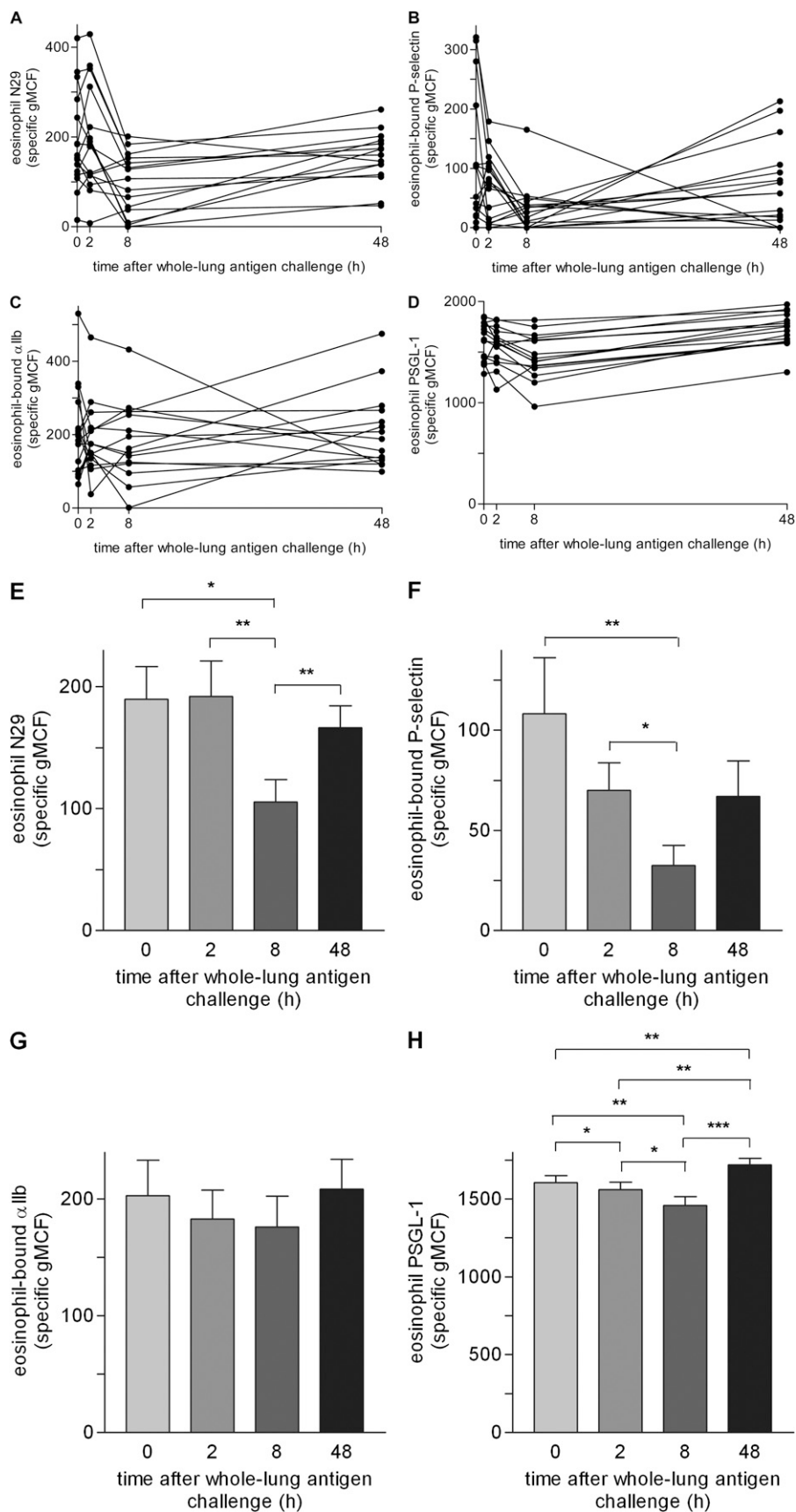
Figure 2. Time courses of eosinophil β_1-integrin activation, assessed by reactivity with activation-sensitive anti-β_1 integrin monoclonal antibody (mAb) N29, levels of eosinophil-bound P-selectin and the platelet marker α_{IIb} integrin, and P-selectin glycoprotein ligand-1 (PSGL-1) expression after whole-lung inhaled antigen challenge in non–Severe Asthma Research Program subjects with mild allergic asthma. (*A*) Individual time courses of eosinophil N29 reactivity. (*B*) Individual time courses of eosinophil-bound P-selectin. (*C*) Individual time courses of eosinophil-bound α_{IIb}; (*D*) Individual time courses of eosinophil PSGL-1. (*E–H*) Means of values (± SEM) of (*E*) eosinophil N29 reactivity, (*F*) eosinophil-bound P-selectin, (*G*) eosinophil-bound α_{IIb}, and (*H*) eosinophil PSGL-1 at indicated times. *$P \leqslant 0.05$, **$P \leqslant 0.01$, ***$P \leqslant 0.001$. gMCF = geometric mean channel fluorescence.
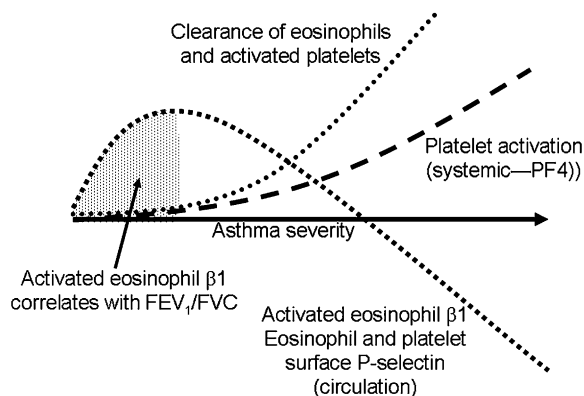
Figure 3. Model of platelet activation, eosinophil β₁-integrin activation, and airway physiology in asthma of increasing severity. Platelet activation and appearance of platelet factor 4 (PF4) in circulation increase with increasing disease severity. P-selectin appears on the surface of platelets upon activation, activated platelets bind to eosinophils via P-selectin, and P-selectin triggers activation of eosinophil β₁-integrins. Eosinophils with activated β₁ integrins persist in the circulation in stable nonsevere asthma but are lost in more severe asthma or during asthma exacerbation. Loss is likely due to interaction of activated α₄β₁ integrin with endothelial cell vascular cell adhesion molecule-1 followed by extravasation in the asthmatic lung. Increased eosinophil β₁-integrin activation is therefore inversely associated with decreased pulmonary function only in the subpopulation with stable nonsevere asthma and minimal eosinophil clearance.

nonsevere asthma (10). An additional possibility may be that these subjects may have significant numbers of lung eosinophils that are retained in the airway wall or tissue and not recovered from induced sputum.

The time courses of eosinophil β₁-integrin activation and P-selectin after whole-lung antigen challenge can be compared with that of eosinophil reactivity with mAb A17 after antigen challenge. A17 recognizes an activated form of Fcγ receptor II (CD32) (61). A17 reactivity increased 6 hours after whole-lung antigen challenge and then decreased at 24 hours (62), indicating that this receptor was first activated followed by extravasation of eosinophils with activated CD32. In addition, circulating eosinophils from subjects with allergic asthma have greater responsiveness to chemoattractants, which is more pronounced after allergen challenge (63). Such priming of eosinophils to greater responsiveness is achieved by IL-5 and related cytokines (64). However, we do not believe that IL-5 family cytokines are involved in the platelet P-selectin–β₁-integrin activation axis described here. Although P-selectin activates eosinophil β₁-integrins *in vitro*, IL-5 does not (12). On the other hand, IL-5 activates eosinophil β₂-integrins, but P-selectin does not (12). Thus, the present *in vivo* and the previous *in vitro* data indicate that eosinophil β₁ activation reports and is a result of preactivation by P-selectin and not priming by IL-5. Still, it remains possible that activity of eosinophil β₂-integrins are also involved in eosinophil arrest and extravasation *in vivo* to some degree, since α$_M$β₂ together with α₄β₁ contributed to arrest of purified eosinophils to VCAM-1 under physiological flow conditions *in vitro* (65), and both α₄ (presumably α₄β₁) and β₂ (presumably α$_M$β₂) are required for eosinophil trafficking to the lung and airway lumen in acute and chronic mouse antigen challenge models (66, 67).

The present study has several limitations. First is the trend to an age discrepancy among the severe asthma, nonsevere asthma, and normal groups. Thus, some of the group differences in Table 1 may be due to age or duration of asthma. Future studies are

needed to address this issue. Second, the SARP study was observational and a snapshot of disease in only a short time window. Visit-to-visit variability was low and there was concordance of values over the three blood draw visits. However, it would be useful to perform the same analyses over a longer time span (e.g., one to several years) and relate measurements to the evolution of the disease over time as well as comparing periods of stability to asthma exacerbations.

Third, differences in measures of platelet activation have the potential to be misleading due to activation resulting from uneven quality of blood collection and sample preparation. We used a protocol designed to minimize platelet activation and measured three markers of activation. The findings that platelet surface P-selectin and plasma PF4 or TSP-1 trended toward inverse correlations, whereas PF4 and TSP-1 correlated tightly and directly with one another indicate that, despite considerable variability in values, the flow cytometry and ELISA data can be interpreted without worry about systematic changes in postphlebotomy platelet activation. Thus, we believe that none of the limitations invalidate a model in which platelet activation is an important determinant of the severity of allergic asthma.

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