

Platelets are essential for leukocyte recruitment in allergic inflammation

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Background: The role of platelets in inflammation is recognized but poorly characterized, and little is known of their interaction with leukocytes. However, platelet-leukocyte interactions have been demonstrated in cardiovascular disease, culminating in enhanced leukocyte recruitment.

Objectives: This study was undertaken to assess the possibility and potential role of similar phenomena occurring in asthmatic patients, a murine model of allergic inflammation, and in vitro adhesion studies.

Methods: Asthmatic patients had blood taken at various time points to document the degree of leukocyte activation and the presence of platelet-leukocyte aggregates through FACS analysis before and after allergen exposure. Similar studies were carried out in mice exposed to allergen after previous sensitization, with some groups being selectively depleted of platelets through both an immunologic (antiplatelet antiserum) and nonimmunologic (busulfan) method. Additionally, lavage fluid and airway tissue were analyzed to assess the degree of pulmonary leukocyte recruitment. The importance of platelets on leukocyte adhesion to the endothelium was then assessed with in vitro incubation of radiolabeled leukocytes in the presence of activated platelets on cultured human vascular endothelial cells.

Results: We have observed circulating platelet-leukocyte aggregates in the blood of allergic asthmatic patients during the allergen-induced late asthmatic response and in sensitized mice after allergen exposure. In platelet-depleted mice infiltration of leukocytes into airways after allergen challenge was significantly reduced and could be restored by means of infusion of platelets from allergic animals, indicating an essential role for platelets in leukocyte recruitment. CD11b expression on leukocytes involved in aggregates with platelets, although not on free leukocytes, was upregulated. Furthermore, the presence of autologous platelets augmented the adhesion of human polymorphonuclear leukocytes to cultured vascular endothelial cells, an effect that was found to be endothelial cell dependent and to involve platelet activation.

Conclusion: These results suggest that platelet participation in cell recruitment occurs at the level of the circulation and

might involve the priming of leukocytes for subsequent adhesion and transmigration into tissues. (*J Allergy Clin Immunol* 2003;112:109-18.)

Key words: Platelet, leukocyte, platelet-leukocyte interaction, asthma, allergy, eosinophil, lymphocyte, vascular endothelium, CD11b

Cell-cell interactions play a critical role in the pathogenesis of inflammatory diseases. It is well recognized that circulating leukocytes adhere to endothelial cells in response to inflammatory stimuli, a process directed by interactions between vascular and leukocyte adhesion molecules, before transmigration into tissues.^{1,2} Platelets have been suggested to be involved in inflammation, as well as in disorders of the cardiovascular system, and are known to be able to interact with both leukocytes and endothelial cells, suggesting a potential role for platelets in leukocyte adhesion and hence trafficking into tissues.^{3,4} Indeed, various studies have shown that platelet aggregates accumulate in inflammatory lesions concomitantly with leukocytes.^{5,6} Furthermore, a pivotal role for platelets in the inflammatory response has been strongly indicated by the results of in vivo studies, which show that platelet depletion by means of lytic antiplatelet antiserum (APAS) can reduce leukocyte accumulation in tissues in response to specific allergen exposure^{7,8} and ischemia and reperfusion.⁹ However, the mechanisms by which platelets contribute to leukocyte adhesion and recruitment are poorly understood.

In recent years, bronchial asthma has been recognized as a chronic inflammatory disease associated with bronchial eosinophilic infiltration and airway epithelial damage. It is now clear that eosinophil, mast cell, and T-lymphocyte activation occurs in allergic asthma,¹⁰ and data showing the activation of circulating white blood cells of patients undergoing allergen challenge have been taken as a proof of the systemic involvement of leukocytes in the inflammatory reaction typical of allergic asthma. In particular, the activation of peripheral lymphocytes was shown by the enhanced expression of CD25 and HLA-DR antigen¹¹ and by the increase of circulating CD4 helper T cells and CD23⁺ cells,¹² whereas for neutrophils, both an increased number and increased Fcγ receptor-mediated chemiluminescence were observed in asthmatic patients after allergen challenge.¹³ Furthermore, the presence of intravascular platelet aggregates within bronchial biopsy specimens in asthmatic

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Abbreviations used

ACD:	Acid citrate dextrose
APAS:	Antiplatelet antiserum
BAL:	Bronchoalveolar lavage
CS:	Control serum
fMLP:	Formyl-methionyl-leucyl-phenylalanine
HUVEC:	Human umbilical vein endothelial cell
LAR:	Late asthmatic response
MFI:	Mean fluorescence intensity
MPO:	Myeloperoxidase
NPRP:	Naive platelet-rich plasma
OVA:	Ovalbumin
PE:	Phycoerythrin
PGI ₂ :	Prostaglandin I ₂
PPP:	Platelet-poor plasma
PRP:	Platelet-rich plasma

patients have also been reported,⁵ extending earlier observations that have visualized platelets at sites of inflammation in other chronic inflammatory conditions, such as rheumatoid arthritis.¹⁴ Interestingly, platelets have been observed extravascularly in bronchial tissue and in the intra-alveolar space in patients with asthma.¹⁵ Several studies have also shown signs of intravascular platelet activation accompanying allergen-induced bronchoconstriction in asthmatic patients,¹⁶⁻²¹ suggesting a participation of these blood elements in the pathophysiology of asthma.

In the present study we investigated interactions between platelets and leukocytes in allergic patients with mild asthma undergoing allergen challenge. Such aggregates have been found to be increased in other inflammatory diseases, such as acute myocardial infarction,²² unstable angina,²³ coronary heart disease,²⁴ and chronic venous insufficiency.²⁵ In addition, we have evaluated the effect of platelet depletion on leukocyte recruitment in a mouse model of allergic inflammation of the airways and have assessed the effect of platelets on the adhesion of leukocytes to vascular endothelial cells.

METHODS**Studies in allergic asthmatic patients**

Eight patients with mild asthma (7 men; age range, 24-32 years) who were free of medication other than pro re nata use of inhaled β_2 -agonists and with demonstrable early and late airways obstruction after airway allergen challenge were enrolled in the study in accordance with the principles of the Declaration of Helsinki, approved by King's College Hospital ethics committee.

Lung function was assessed²⁶ at intervals of 10 minutes to 1 hour for 10 hours after challenge. Venous blood samples were obtained before inhalational allergen challenge²⁷ and 8 and 24 hours after challenge. On a second visit at least 1 week later, subjects received inhalational challenge with saline solution, and blood samples were taken 20 hours later.

Platelet-leukocyte aggregates were measured in 50- μ L samples of citrated blood by means of the addition of 5 μ L of anti-CD42b FITC-labeled (Pharmingen) and 5 μ L of anti-CD11b phycoerythrin (PE)-labeled (Pharmingen) or isotype control antibodies within 10 minutes of sampling. After 10 minutes' incubation at room temper-

ature, 200 μ L of FACSlyse solution (Beckton Dickinson) was added for a further 10 minutes before the addition of 500 μ L of paraformaldehyde (1% in PBS).^{28,29} Samples were kept at 4°C and analyzed within 24 hours on a FACScan flow cytometer (Beckton Dickinson) by the same operator, who was unaware of the identity of the samples, using a previously published method.^{30,31} Live gating on leukocyte-sized events was performed to exclude single platelets by using either forward and side scatter alone or in combination with a specific leukocyte fluorescence marker. Events staining positive for both CD11b and CD42 were considered to represent platelet-leukocyte aggregates and were distinguishable from events staining for CD11b alone.

Studies in the allergic mouse model

Male C57BL/6 mice (20-25 g, Charles River) were immunized with chicken egg albumin (grade V, ovalbumin [OVA], Sigma) at a dose of 10 μ g per mouse (administered intraperitoneally) on days 0 and 7 and dissolved in 0.4 mL of 0.1 mol/L aluminum hydroxide (Al₂(OH)₃) in saline solution. Sham-immunized animals received Al₂(OH)₃ in saline solution alone. Mice were subsequently exposed to a nebulizer solution of OVA (10 mg/mL saline solution) for 20-minute periods 3 times daily at hourly intervals on days 15, 16, and 17 (de Villbis Ultraneb 90 nebuliser). Twenty-four hours after the first allergen challenge on day 17, mice were used in the studies outlined below. All studies were carried out according to The Animals (Scientific Procedures) Act (1986) and local ethical approval from King's College London.

Protocol for APAS and immune-mediated platelet depletion. APAS was prepared as previously described by others.³² Blood was collected from normal mice and centrifuged at 150g for 10 minutes. The platelet-rich plasma (PRP) obtained was washed by using the method of Mustard et al.³³ Pure murine platelets were then homogenized in Freund complete adjuvant and injected subcutaneously in rabbits. A second immunization was performed 10 days later by means of intramuscular injection of pure murine platelets in Freund incomplete adjuvant. Thirty days after the second immunization, the rabbits were bled, and the antiserum was inactivated at 56°C for 60 minutes and stored at -70°C. Nonimmune serum was obtained from nonimmunized rabbits (control serum [CS]). The APAS was administered undiluted to allergic mice (0.1 mL administered intravenously) 30 minutes before the first allergen challenge on days 15 and 16. This protocol for administration was found to be the most appropriate because a time course of the recovery of platelet numbers after treatment with this APAS demonstrated a gradual increase in platelet numbers 48 hours after administration. Control rabbit serum was administered in control experiments.

Busulfan-induced platelet depletion. Busulfan, a bone marrow precursor cell-specific toxin was used to deplete platelets, as previously described.³⁴ Busulfan (Sigma-Aldrich, UK) was prepared in polyethylene glycol 400 (25 mg/mL) at room temperature for 1 hour and then treated at 65°C to 70°C for a further hour before dilution (1:8) in warm saline solution for injection on days -4, -2, 1, 8, and 14 of the immunization protocol (25 mg/kg, 0.2 mL administered intraperitoneally).

Restoration of the platelet population. PRP from mice exposed to the above immunization protocol and PRP from naive mice (NPRP) were injected intravenously into immunized and naive mice rendered thrombocytopenic by means of busulfan administration 30 minutes before the start of the first allergen challenge. PRP was prepared by taking blood from anesthetized animals by means of cardiac puncture, diluting 1:9 with acid citrate dextrose (ACD), and centrifuging at 200g for 10 minutes. The top layer was aspirated gently and added to 0.2 μ mol/L prostaglandin I₂ (PGI₂). After 30 minutes' incubation at room temperature to allow for the decay of PGI₂ activity, platelets were counted, and then animals were injected intravenously with 0.2 mL of PRP on days 15 and 16, with each

animal receiving between 2.5 and 4.0×10^8 platelets per animal. The cellular content of PRP was greater than 99% platelets. Platelet reconstitution typically restored the circulating platelet population to approximately 40% of OVA-challenged mice. Platelet-poor plasma (PPP) was produced by centrifuging blood from OVA-challenged animals at 2000g for 10 minutes.

Bronchoalveolar lavage. Twenty-four hours after the first aerosol challenge on day 17, mice were anesthetized with 25% urethane (2 g/kg) i.p. The trachea was then exposed and cannulated. Three 0.5-mL aliquots of warm sterile saline solution were injected into the lung, and the resulting fluid was drawn out with a 1-mL syringe and put on ice. Total cell counts were obtained by adding 50 μ L of bronchoalveolar lavage (BAL) fluid to 50 μ L of hemolysis solution (0.1% methylene blue in 1% acetic acid). Cells were counted on an improved Neubauer haemocytometer with a 20 $\times$ objective. Differential cell counts were obtained from cytospin preparations stained with the DiffQuick system (Gamidor Ltd).

Blood sampling. Blood (50 μ L) was collected before challenge through the retro-orbital route by using glass pipettes dipped in ACD for platelet and leukocyte enumeration. After BAL, blood samples (up to 0.5 mL) were obtained by means of cardiac puncture and smeared onto microscope slides for differential leukocyte counts with the DiffQuik method. Blood (10 μ L) was taken and mixed with hemolysis solution (90 μ L) for total leukocyte counts.

Platelets were enumerated in accordance with the method of Brecher and Cronkite.³⁵ Briefly, blood was mixed with ACD in a 9:1 ratio and diluted 1:20 with 1% acetic acid in saline solution. Platelets were counted under a hemocytometer by using a thin coverslip (thickness 1) with a phase microscope, dark contrast, 40 $\times$ objective after allowing the platelets to settle for 20 minutes.

Serum IgE profiles. Blood was taken from mice at the time of BAL fluid collection (24 hours after the start of the challenge on day 17) and analyzed for total IgE levels in serum by using an IgE detection ELISA kit (Pharmingen).

Lung morphometry. At the end of the experiment, after achievement of terminal anesthesia, lungs were recovered from mice, inflated with a tracheal cannula with 10% formalin, and then immersed in 10% formalin for at least 1 week before tissue processing. The lungs were then processed into paraffin-embedded blocks. Lungs were cut along the lateral axis into 4 segments, placed in cartridges and blocked in paraffin wax, sectioned (5 μ m) with a microtome, and stained with hematoxylin and eosin for detection of cellular infiltrate.

Quantitative image analysis was then carried out on distal airways (small <0.75-mm perimeter) by using the computer-assisted Leica KS300 program (Leica). Briefly, the number of eosinophils was counted to a depth of 50 μ m below the epithelial basement membrane and then divided by the total area in which the eosinophils were counted to determine the number of eosinophils per square millimeter of submucosal tissue.

Detection of platelet-leukocyte aggregates. Blood was obtained by means of cardiac puncture 24 hours after the last day of aerosol challenge, diluted 1:10 in PBS, and analyzed for the presence of platelet-leukocyte aggregates by means of a modification of the method used for human blood samples. Specific antibodies used in saturating concentrations were FITC-labeled anti-CD41 (gpIIb-IIIa), anti-CD45-PerCP (common leukocyte antigen), and PE-conjugated anti-CD11b-PE (all from Pharmingen).

Leukocyte-endothelial adhesion studies

Preparation of PMNs and platelets. Peripheral venous blood was collected from healthy volunteers who were free from medication into tubes, diluted 1:10 in ACD. PRP, obtained by means of centrifugation of the citrated blood at 200g for 10 minutes at room temperature, was again centrifuged at 1200g for 15 minutes on a 50%

BSA cushion. After centrifugation, the platelet fraction separated from red blood cells on top of the albumin cushion was collected and diluted to a concentration of 5×10^8 platelets/mL in PBS supplemented with 36 mmol/L citric acid, 5 mmol/L glucose, 2 mmol/L CaCl_2 , 1 mmol/L MgCl_2 , 0.35% BSA, 0.1 U/mL apyrase, and 0.2 μ mol/L PGI_2 .³⁶ The platelet suspension was further washed by means of gel filtration performed with the use of Sepharose 2B in the same buffer, except for the concentration of PGI_2 (0.02 μ mol/L). The gel-filtered platelets were centrifuged at 1000g for 10 minutes, resuspended to the desired final concentration in HBSS (phenol red free) supplemented with 5 mmol/L HEPES (pH 7.3) and 0.35% BSA, and used immediately in adhesion assays. Platelets isolated in this manner had a discoid appearance, and populations used in these studies contained less than 0.1% contaminating leukocytes. In some experiments, before gel filtration, platelets were incubated with wortmannin at a concentration of 100 nmol/L (a gift from Dr S. Nakanishi and Dr Y. Matsuda, Kyowa Hakkō Kogyō Co, Ltd) or vehicle at 37°C for 30 minutes. The blood fraction remaining after removal of PRP was mixed with an equal volume of a 6% dextran solution (Hespan) and left at room temperature for 40 minutes to sediment erythrocytes. PMNs were then isolated from the white cell-rich supernatant by means of density-dependent centrifugation on Percoll gradients (55% on 70% on 81%), with the PMNs being collected from the 70%-81% interface. Contaminating erythrocytes were removed by means of hypotonic lysis, and cell pellets were washed 3 times with modified HBSS (Ca^{2+} and Mg^{2+} free). Cells were then radiolabeled with chromium 51 by means of incubating cell pellets for 1 hour at 37°C in aqueous sodium chromate (37 kBq per 10^6 cells, Amersham Lifesciences Ltd) and then washing 3 times with modified HBSS. Cells were resuspended at a concentration of 2×10^6 cells/mL in HBSS (phenol red free with Ca^{2+} and Mg^{2+}) supplemented with 5 mmol/L HEPES (pH 7.3) and 0.35% BSA and was ready for use in the adhesion assays. PMN populations isolated in this manner were found to be more than 98% viable (as measured by means of trypan blue exclusion), consisted of not less than 95% neutrophils, and contained no more than 1 platelet per 100 PMNs. The process of radiolabeling did not affect cell viability.

Adhesion assay. The adhesion assay used is essentially based on a previously described method.³⁷ Briefly, cryopreserved human umbilical vein endothelial cells (HUVECs; BioWhittaker UK Ltd) were cultured in EGM-2 medium (BioWhittaker) in 96-well plates. Monolayers of HUVECs were washed with warmed HBSS before the addition per well of 100 μ L of radiolabeled PMN suspension (2×10^5 cells per well) and 100 μ L of platelet suspension (2×10^5 to 5.6×10^8 cells per well) or medium control. In some experiments various concentrations of N-formyl-methionyl-leucyl-phenylalanine (fMLP; 22 μ L per well) were added as a PMN stimulus. Ninety-six-well plates were then incubated for 30 minutes at 37°C. After incubation, nonadherent cells were removed by means of gentle aspiration and washing with warmed HBSS, adherent cells in each well were lysed with 1% Triton X-100, and samples were placed in scintillation vials and γ counted. Counts per minute in samples are expressed as the percentage of total counts added to the well (percentage of input). Before lysing, cells remaining in the well were studied microscopically to confirm the appearance and integrity of HUVEC monolayers.

Myeloperoxidase release. PMNs and platelets were incubated on HUVEC monolayers under the same conditions as those in the adhesion assay. Seventy-five microliters of medium was collected into new 96-well plates centrifuged at 700g for 3 minutes, and 50 μ L of supernatant was collected to measure myeloperoxidase (MPO) activity in the assay medium. MPO activity in supernatants was measured by using a spectrophotometric assay on the basis of MPO-catalyzed oxidation of 3,3',5,5'-tetramethylbenzidine by H_2O_2 with the 3,3',5,5'-tetramethylbenzidine substrate system (Sigma-Aldrich

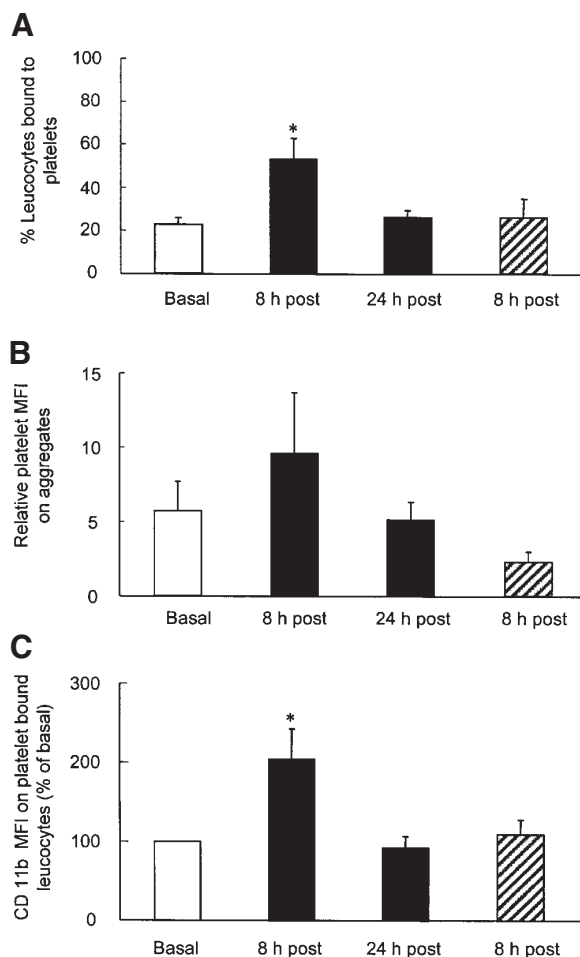


FIG 1. Platelet-leukocyte aggregates in the blood of asthmatic patients. **A**, The percentage of circulating leukocytes positive for platelet markers was measured in whole blood drawn from allergic asthmatic patients before (open bar) and after (filled bar) allergen challenge or after challenge with saline solution (hatched bar). **B**, The relative index of the number of platelets per leukocyte was assessed again before and after challenge with allergen or saline solution. **C**, CD11b MFI was measured on leukocytes involved in aggregates with platelets in circulating blood and is expressed as a percentage of the level observed before challenge. All data are expressed as mean $\pm$ SE values ($n = 8$). * $P < .05$.

Ltd). MPO activity in supernatants is expressed as the percentage of MPO activity remaining in the well after incubation, as assessed by lysing the contents of identical wells at the end of the assay (1% Triton-X) and quantifying colorimetrically, as before.

Statistical analysis

Data from all studies are expressed as means $\pm$ SEM. Data from studies investigating platelet-leukocyte interactions in allergic asthmatic patients were then analyzed by means of repeated-measures ANOVA, followed by the Tukey multiple comparison test. A P value of less than .05 was considered significant.

Data from in vivo studies incorporating BAL fluid leukocyte numbers, blood leukocyte and platelet numbers, serum IgE levels, and FACScan data were analyzed by means of ANOVA, followed by the Bonferroni multiple comparisons test. A P value of less than .05 was considered significant.

Data from in vitro studies were taken from 4 to 6 separate donor experiments, each carried out in triplicate where appropriate, and concentration dependency was assessed by means of ANOVA, followed by the Tukey multiple comparison test. Other data were analyzed by means of ANOVA, followed by the Dunnett test. A P value of less than .05 was considered significant.

RESULTS

Allergen challenge studies in allergic asthmatic patients

All subjects had an early asthmatic reaction after allergen challenge (mean decrease in FEV_1 of $40.4\% \pm 2.7\%$, $n = 8$), as well as a late asthmatic reaction (mean decrease in FEV_1 of $42\% \pm 4.8\%$). On the contrary, no reduction in lung function was observed after saline challenge in any patient (maximum average decrease in FEV_1 of $3\% \pm 0.8\%$).

The percentage of leukocytes positive for the platelet marker GP1b in circulating blood was significantly greater during the late asthmatic response (LAR; 8 hours after challenge; $53\% \pm 9.8\%$, $P < .05$) than the values measured in circulating blood before challenge ($22.7\% \pm 3.0\%$) or 24 hours after challenge ($26\% \pm 3.5\%$). In contrast, 8 hours after challenge with saline solution, no significant increase in platelet-leukocyte aggregates was observed ($26\% \pm 9\%$; Fig 1, A). When the value was expressed as the relative platelet fluorescence index, similar changes were observed, although the increase did not attain the level of statistical significance (Fig 1, B). Representative dot plots demonstrating these changes in GP1b fluorescence are shown in Fig 2.

CD11b expression on circulating free leukocytes did not significantly change after allergen challenge. However, there was a significant increase in CD11b expression on leukocytes involved in platelet-leukocyte aggregates during the LAR ($204\% \pm 39.6\%$, $P < .05$) compared with prechallenge and 24-hour postchallenge values ($92\% \pm 15\%$). No significant change in the expression of CD11b on leukocytes involved in platelet-leukocyte aggregates was seen 8 hours after saline challenge ($109\% \pm 19\%$). Furthermore, CD11b expression on platelet-leukocyte aggregates was significantly greater during the LAR than expression on free leukocytes (Fig 1, C). Representative dot plots demonstrating these changes in CD11b fluorescence on circulating leukocytes are shown in Fig 3.

There was no significant relationship between the number of platelet-leukocyte aggregates and the decrease in FEV_1 observed after allergen challenge.

During the LAR and after allergen challenge, no differences were observed in the number of circulating platelets, neutrophils, or lymphocytes compared with values observed after saline challenge.

Studies in the allergic mouse model

Allergen sensitization and challenge resulted in significant recruitment of eosinophils ($2.9 \pm 0.6 \times 10^5$ cells/mL from 0.0 ± 0.0 cells/mL) and lymphocytes ($0.4 \pm 0.4 \times 10^3$ cells/mL to $4.3 \pm 1.8 \times 10^3$ cells/mL) to the lung. Reg-

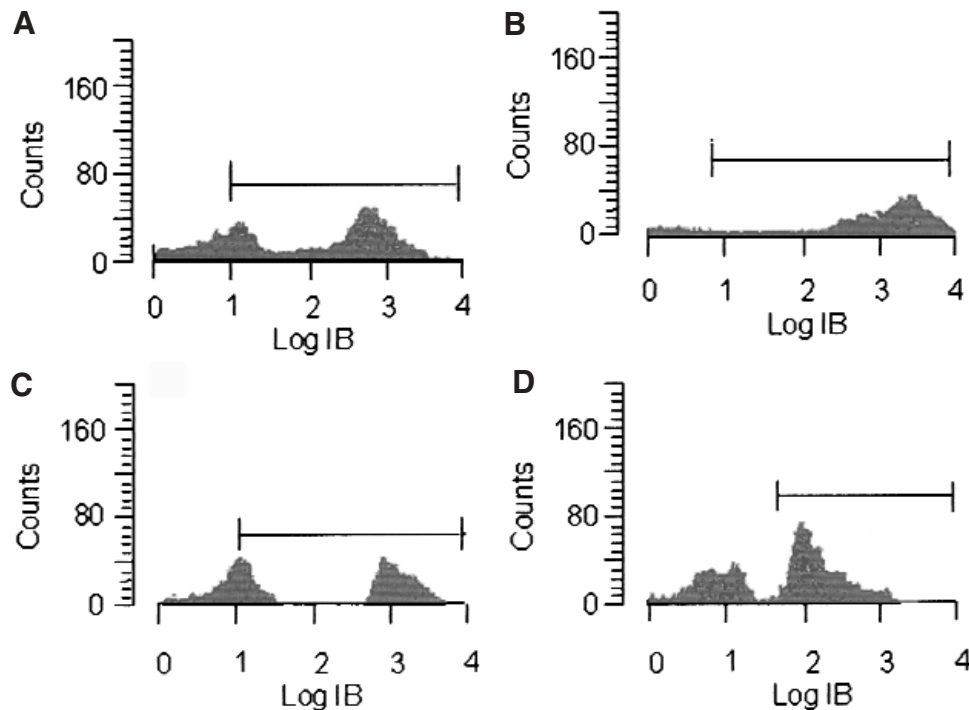


FIG 2. Representative dot plots of GP1b fluorescence on circulating leukocytes in asthmatic blood before allergen challenge (A), 8 hours after allergen challenge (B), 24 hours after allergen challenge (C), and 8 hours after saline challenge (D).

ular treatment with busulfan resulted in sustained platelet depletion throughout the period of challenge, from $5.60 \pm 0.33 \times 10^8$ platelets/mL to $0.7 \pm 0.09 \times 10^8$ platelets/mL before the first challenge, between the OVA- and busulfan-treated groups. Animals remained thrombocytopenic 24 hours after the last day of challenge ($4.40 \pm 0.4 \times 10^8$ platelets/mL compared with $0.46 \pm 0.09 \times 10^8$ platelets/mL in the OVA- and busulfan-treated groups, respectively; $P < .05$). Depletion of platelets with busulfan was found to significantly reduce the recruitment of eosinophils and lymphocytes into the lung after allergen challenge (Fig 4), whereas levels of circulating leukocytes were similar across all treatment groups before challenge with aerosolized OVA. Importantly, the long-term thrombocytopenia induced by busulfan administration was found to be cell specific because peripheral blood leukocytes remained unaffected between the OVA- and busulfan-treated groups after allergen challenge ($7.6 \pm 0.6 \times 10^6$ cells/mL and $5.4 \pm 0.9 \times 10^6$ cells/mL, respectively).

Busulfan, a bone marrow cell-specific toxin, induces a gradual, long-term, nonimmune thrombocytopenia, allowing the immediate restoration of the platelet population through transfusion of PRP. Sensitized animals previously made thrombocytopenic with busulfan and subsequently treated with PRP from sensitized donors had restored levels of eosinophil ($3.5 \pm 0.9 \times 10^5$ cells/mL from $0.3 \pm 0.09 \times 10^5$ cells/mL, $P < .01$) and lymphocyte ($2.8 \pm 1.0 \times 10^3$ cells/mL from $0.3 \pm 0.2 \times 10^3$ cells/mL, $P < .05$) infiltration to BAL fluid in response to inhaled allergen. This restoration in leukocyte recruitment was not mimicked by the administration

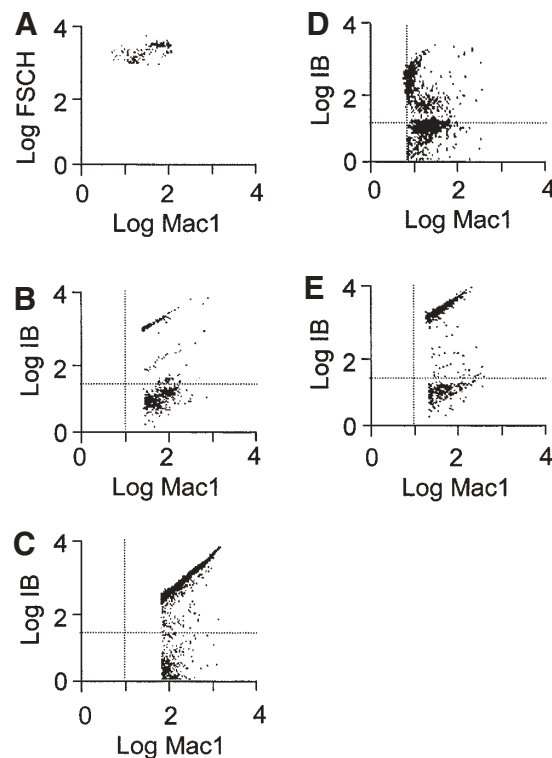


FIG 3. Representative dot plots of CD11b fluorescence on circulating leukocytes in asthmatic blood: gating strategy to identify leukocytes expressing CD11b (A) and CD11b expression on leukocytes before allergen challenge (B), 8 hours after allergen challenge (C), 24 hours after allergen challenge (D), and 8 hours after saline challenge (E).

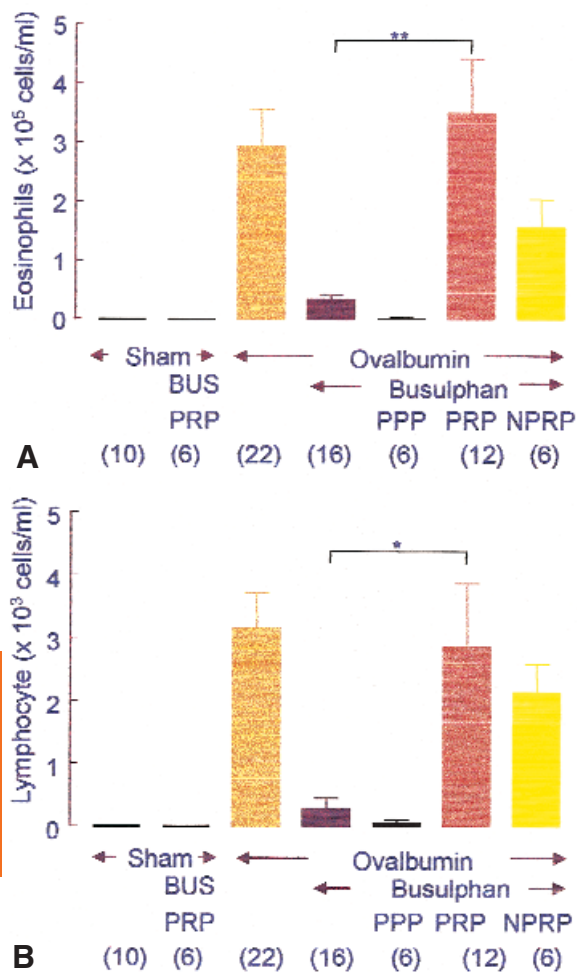


FIG 4. Effect of platelet depletion and restoration on pulmonary leukocyte recruitment in allergic mice. C57BL/6 mice, sham sensitized or sensitized to OVA, were subsequently exposed to aerosolized antigen. BAL was carried out 24 hours after the start of the last day of challenge, and BAL fluid was analyzed for eosinophil (A) and lymphocyte (B) numbers. In some experiments animals were rendered thrombocytopenic by means of busulfan (BUS) administration before sensitization. Groups of thrombocytopenic animals were injected intravenously with either PPP or PRP obtained from sensitized donor animals before inhaled antigen challenge. A further group of thrombocytopenic animals was administered NPRP. Numbers of animals per group are indicated in parentheses. Data are expressed as means $\pm$ SE. * $P < .05$, ** $P < .01$.

of PPP from OVA-challenged animals to busulfan-treated animals. Sensitized and thrombocytopenic mice transfused with NPRP from sham-immunized mice showed only partial restoration of leukocyte recruitment to the lungs compared with sensitized thrombocytopenic mice that received PRP from sensitized mice ($1.6 \pm 0.5 \times 10^5$ eosinophils/mL and $3.5 \pm 0.9 \times 10^5$ eosinophils/mL, respectively, and $2.1 \pm 0.5 \times 10^3$ lymphocytes/mL and $2.8 \pm 1.0 \times 10^3$ lymphocytes/mL, respectively). Finally, PRP obtained from sensitized mice transfused to sham-immunized thrombocytopenic mice was unable to induce leukocyte recruitment to the lungs (Fig 4).

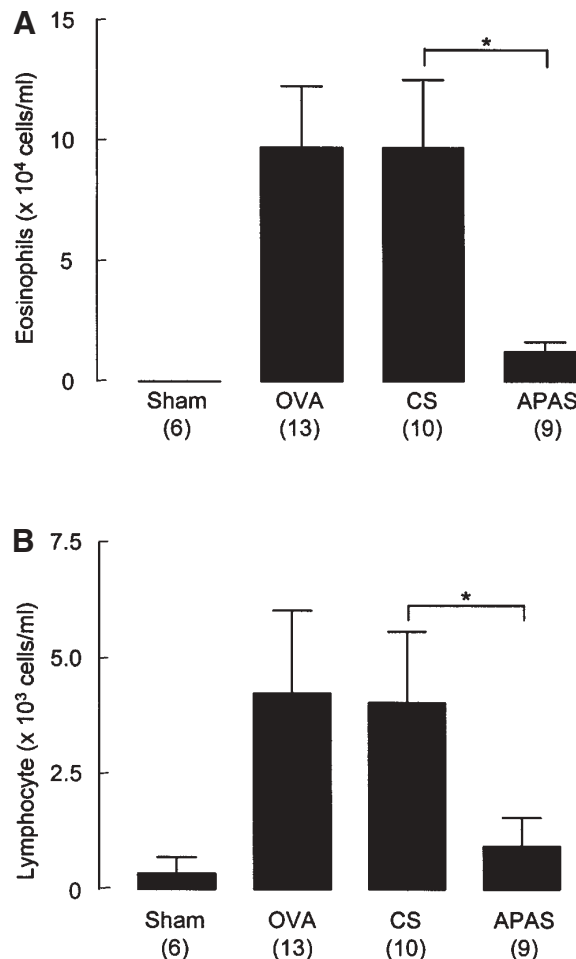


FIG 5. Effect of immune-mediated platelet depletion on pulmonary leukocyte recruitment in allergic mice. C57BL/6 mice, sham sensitized or sensitized to OVA, were subsequently exposed to aerosolized antigen. BAL was carried out 24 hours after the start of the last day of challenge, and BAL fluid was analyzed for eosinophil (A) and lymphocyte (B) numbers. Some animals received intravenous APAS or CS 30 minutes before allergen challenge on the first 2 days of exposure. Numbers of animals per group are indicated in parentheses. Data are expressed as means $\pm$ SE. * $P < .05$.

Moreover, in control experiments mice were rendered thrombocytopenic by over 95% (OVA plus CS: $3.4 \pm 0.7 \times 10^8$ cells/mL vs OVA plus APAS: $0.1 \pm 0.03 \times 10^8$ cells/mL, $P < .001$) through APAS administration, showing similar reductions in eosinophil and lymphocyte levels in the BAL fluid after allergen challenge (Fig 5). In this alternative protocol short-term thrombocytopenia was induced after the sensitization process, further suggesting that the effects of busulfan on cell recruitment were due to effects on circulating platelet numbers rather than interference with the development of allergy in treated animals. Again, of importance, the thrombocytopenia induced by APAS administration was found to be cell specific because peripheral blood leukocyte levels remained unaffected between OVA plus CS- and OVA plus

APAS-treated groups after allergen challenge ($5.3 \pm 0.7 \times 10^6$ cells/mL and $3.8 \pm 0.5 \times 10^6$ cells/mL, respectively).

Furthermore, morphometric analysis of lungs revealed a significant increase in eosinophilia within the submucosa in OVA-sensitized mice compared with that in sham-sensitized control animals ($1.1 \pm 0.3 \times 10^3$ cells/mm² and $0.02 \pm 0.01 \times 10^3$ cells/mm², respectively; $n = 8$; $P < .001$). There was an absence of tissue eosinophilia in sections from mice rendered thrombocytopenic by busulfan, and this was significantly different compared with that seen in OVA-sensitized mice ($0.03 \pm 0.01 \times 10^3$ cells/mm², $n = 7$, $P < .001$).

Analysis of total IgE titers from serum taken 24 hours after the start of the last OVA challenge showed a significant increase in levels between sham- and OVA-sensitized mice (busulfan studies: 73.7 ± 21.0 ng/mL for sham-sensitized mice vs 294.3 ± 47.3 ng/mL for OVA-sensitized mice, $n = 9$, $P < .001$; APAS studies: 17.5 ± 9.1 ng/mL for sham-sensitized mice vs 422.2 ± 131.4 ng/mL for OVA plus CS-sensitized mice, $n = 7$ to 9 , $P < .001$). The levels of total serum IgE were not reduced in animals rendered thrombocytopenic with busulfan (843.3 ± 194.9 ng/mL, $n = 10$) or APAS (360.1 ± 69.0 ng/mL, $n = 11$).

Analysis of blood obtained through cardiac puncture 24 hours after the last allergen challenge revealed a significant increase in the number of platelets bound to leukocytes because the mean platelet fluorescence as a percentage of sham control animals (anti-CD41-FITC) for each leukocyte-positive event increased from $100.5\% \pm 3.5\%$ mean platelet fluorescence in the sham-sensitized groups to $147.3\% \pm 10.1\%$ mean platelet fluorescence in the OVA-sensitized groups ($P < .001$; Fig 6, A). However, the percentage of leukocytes attached to at least one platelet remained constant ($25.4\% \pm 3.1\%$ for sham-sensitized mice and $26.3\% \pm 3.1\%$ for OVA-sensitized mice). In addition, levels of CD11b expressed on leukocytes involved in interactions with platelets were again found to be increased in sensitized animals ($117.0\% \pm 4.8\%$ mean fluorescence intensity [MFI]) compared with those seen in sham-sensitized animals ($100.6\% \pm 3.6\%$ MFI, $P < .001$; Fig 6, B). No difference, however, was found between the sham and OVA treatment groups with respect to CD11b expression found on circulating leukocytes, irrespective of platelet attachment (Fig 6, C), although the level of CD11b expression was significantly reduced in mice depleted of platelets ($106.6\% \pm 2.1\%$ MFI for the OVA group and $86.6\% \pm 8.1\%$ MFI for the APAS group, $P < .001$; Fig 6, C). Representative dot plots of changes in both CD41 and CD11b expression are also shown in Fig 6.

Leukocyte-endothelial adhesion studies

First, PMN activity and adhesion on HUVEC monolayers were examined in the presence of various densities of platelets. The adhesion of PMNs was $5.4\% \pm 0.5\%$ of input (total cells added to well) in the absence of platelets and increased in the presence of platelets (Fig 7, A). Although no statistically significant concentration-dependent relationship was observed, adhesion was found to be

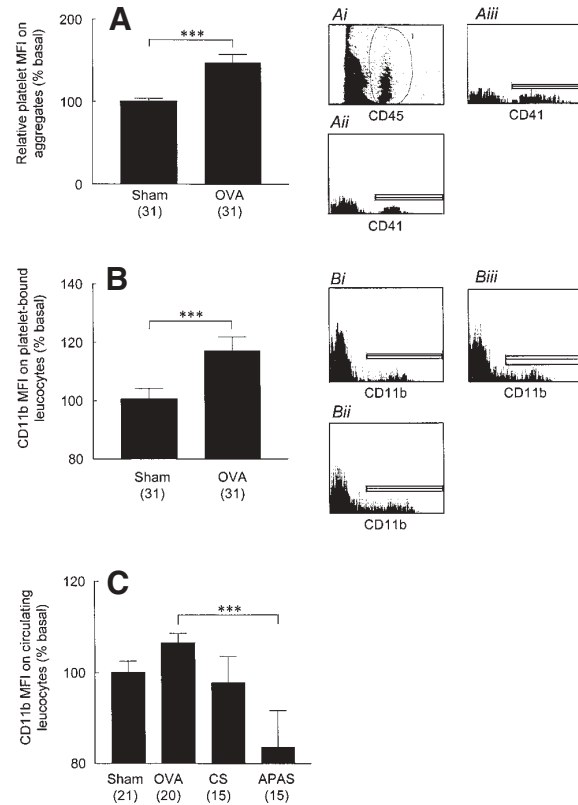


FIG 6. Platelet-leukocyte aggregates in the blood of allergic mice. C57BL/6 mice, sham sensitized or sensitized to OVA, were subsequently exposed to aerosolized antigen. Blood sampling was carried out 24 hours after the start of the last day of challenge and analyzed for the presence of platelets attached to leukocytes (A), the intensity of CD11b expression on leukocytes attached to platelets (B), and the intensity of CD11b expression on circulating leukocytes irrespective of platelet attachment (C). Some animals received intravenous APAS or CS 30 minutes before allergen challenge on the first 2 days of exposure. Numbers of animals per group are indicated in parentheses. Data are expressed as means $\pm$ SE (*** $P < .001$). Also shown are representative dot plots of CD41 and CD11b fluorescence on circulating leukocytes in blood from allergic mice: gating strategy to identify leukocytes (1) from other blood cells (Ai); circulating platelet-leukocyte aggregates on sham-sensitized (Aii) and OVA-sensitized (Aiii) mice; and CD11b expression on circulating leukocytes of sham-sensitized (Bi), OVA-sensitized (Bii), and OVA plus APAS-sensitized (Biii) mice.

enhanced by a physiologic ratio of platelets to PMNs (2.25×10^7 platelets per well: 2×10^5 PMNs per well), an effect that increased significantly (up to $38.9\% \pm 2.9\%$ of input) when platelet concentrations exceeded this ratio. When fMLP was introduced to the assay in the absence and presence of the various concentrations of platelets, further increases in PMN adhesion were observed (Fig 7, B). The combined effects of platelets and fMLP on PMN adhesion were additive rather than synergistic. In the absence of an endothelial monolayer, platelets did not affect the adhesion of PMNs to tissue culture plastic, indicating a role for endothelial cells in the observed phenomenon (Fig 7, C). Furthermore, preincubation of platelets with wortmannin at a concentration of 100

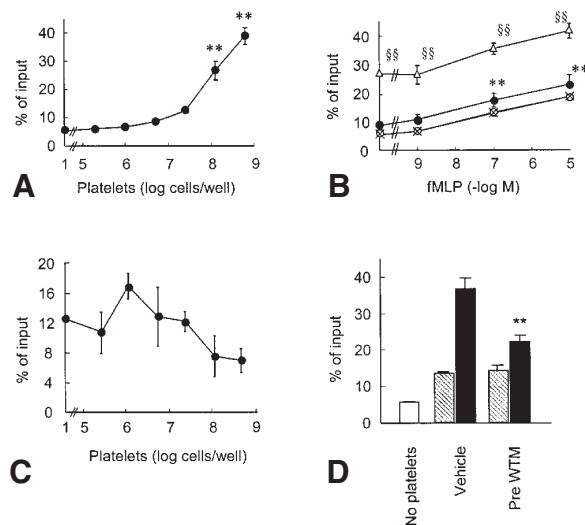


FIG 7. Effect of platelets on PMN adhesion to HUVECs. **A**, Adhesion of chromium 51-labeled PMNs to HUVEC monolayers was assessed in the absence and presence of autologous platelets. $**P < .01$. **B**, fMLP-stimulated PMN adhesion to HUVECs was examined with and without platelets. $**P < .01$ represents the significance of the effect of fMLP in the absence of platelets (X). $§§P < .01$ represents the significance of the effect of platelets (2×10^5 [open circles], 5×10^6 [filled circles], or 1.1×10^8 [triangles] per well) in the presence of indicated concentrations of fMLP. **C**, The effect of platelets on adhesion of PMNs to tissue-culture plastic (ie, in the absence of HUVECs) was tested. **D**, Platelets were pretreated with wortmannin and then washed before being introduced (2.3×10^7 [hatched bars] or 5.6×10^8 [filled bars] per well) to the adhesion assay. The effects of platelets, both untreated and wortmannin treated, on PMN adhesion to HUVECs are shown ($**P < .01$). In all cases data represent the percentage of PMNs added (2×10^5 cells per well) adherent to HUVECs at the end of the assay and are expressed as mean $\pm$ SE values from 4 to 6 separate experiments, each carried out in triplicate.

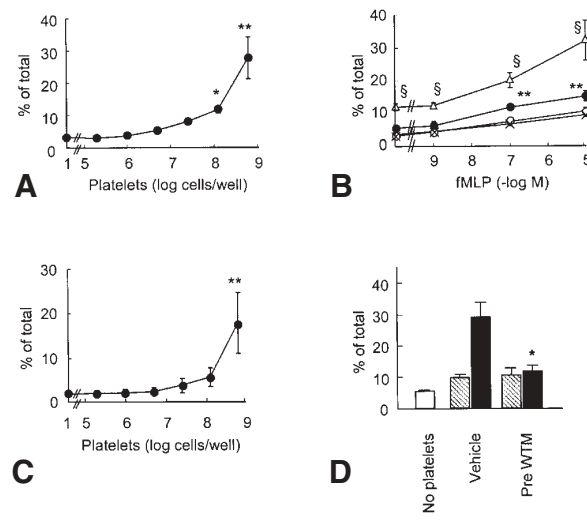


FIG 8. Effect of platelets on PMN activation. **A**, The effect of platelets on MPO release from PMNs was assessed in the presence of HUVEC monolayers. $*P < .01$, $**P < .01$. **B**, MPO release from fMLP-stimulated PMNs, again in the presence of HUVECs, was examined with and without platelets. $**P < .01$ represents the significance of the effect of fMLP in the absence of platelets (X). $§P < .05$ represents the significance of the effect of platelets (2×10^5 [open circles], 5×10^6 [filled circles], or 1.1×10^8 [triangles] per well) in the presence of indicated concentrations of fMLP. **C**, The effect of platelets on MPO release from PMNs incubated on tissue-culture plastic (ie, in the absence of HUVECs) was examined. $*P < .05$. **D**, Platelets were pretreated with wortmannin and then washed before being introduced (2.3×10^7 [hatched bars] or 5.6×10^8 [filled bars] per well) to the mixed cell assay. The effects of platelets, both untreated and wortmannin treated, on MPO release are shown ($*P < .05$). In all cases MPO release is expressed as a percentage of the total MPO remaining at the end of the assay in identical wells incubated in the same manner before lysis of the total content and measurement of MPO. Data shown are mean $\pm$ SE values from 4 to 6 separate experiments, each carried out in triplicate.

nmol/L attenuated the effect of platelets on PMN adhesion, suggesting also that platelet activation contributes to the response (Fig 7, D). Activation of PMNs was assessed under the same assay conditions by means of measurement of MPO release. Increasing the concentration of platelets led to increased release of MPO (Fig 8, A), an effect that again was augmented in an additive manner by the presence of fMLP (Fig 8, B). However, in the absence of endothelial cells, MPO release was significantly increased in the presence of high concentrations of platelets (Fig 8, C), despite the fact that adhesion was unaltered. As with adhesion, the effect of platelets on release of MPO by PMNs was inhibited by pretreating platelets with the phosphatidylinositol 3 kinase inhibitor wortmannin (100 nmol/L; Fig 8, D).

DISCUSSION

Our results demonstrate an essential role for platelets in the recruitment of leukocytes to the lung in a mouse model of allergic inflammation in that, in accordance with previous studies,^{7,8} depletion of more than 90% of circulating platelets significantly reduced the accumula-

tion of leukocytes in BAL fluid and into airway tissue after exposure of animals to specific allergen. Platelet depletion in these studies did not affect circulating leukocyte numbers or serum IgE levels, suggesting that these methods were nontoxic to leukocytes and did not affect the sensitization process. Moreover, we demonstrate, for the first time, that transfusion of platelets to thrombocytopenic animals is able to restore allergen-induced pulmonary leukocyte recruitment. When platelets from allergen-sensitized animals were used to partially restore the platelet population in thrombocytopenic allergic animals, the response to allergen inhalation in the recipient mice was restored to that of the allergic control animals, although platelets obtained from naive mice were found to only partially restore the inflammatory response in the lungs of sensitized mice. These latter data imply that at least part of the response is dependent simply on the presence of platelets as circulating blood elements. This hypothesis is supported by the fact that PPP from sensitized animals administered to thrombocytopenic sensitized animals was unable to reestablish the response to allergen. Therefore it is unlikely that the restorative effect

of PRP from these animals is due to the presence of inflammatory mediators in the plasma, platelet derived or otherwise, but more likely involves contact-dependent mechanisms that require the presence of intact platelets, which, in addition to releasing an array of inflammatory mediators, possibly in part through an IgE-dependent process, might interact directly with blood leukocytes, vascular endothelial cells, or both. Indeed, cellular cooperation has been observed between platelets and leukocytes in a range of conditions, including cardiovascular disease³⁸⁻⁴⁰ and tumor metastasis.⁴¹ Activated platelets have been demonstrated to bind monocytes, PMNs, eosinophils, basophils, and T cells,^{40,42-44} and in the present study we have found that platelet-leukocyte aggregates are present in the blood of mice under allergic inflammatory conditions.

We have also demonstrated circulating platelet-leukocyte aggregates in human subjects with allergic asthma undergoing a LAR to allergen, which is known to be associated with an infiltration of leukocytes into the lung.¹ These data provide further evidence that platelets are activated in human allergic inflammation and, in light of our findings in allergic mice, suggest a fundamental requirement for platelets in cellular recruitment into the lungs, as shown by the results of our platelet depletion and reinfusion studies, that could well extend to human pathophysiology. We have found that leukocytes involved in interactions with platelets in circulating blood of allergic asthmatic patients undergoing a LAR express greater surface levels of CD11b, whereas leukocytes lacking bound platelets do not differ in CD11b expression from those obtained from patients not undergoing allergen challenge. Furthermore, allergic mice depleted of platelets showed a reduction in CD11b expression compared with that seen in OVA-sensitized control animals, and although it is possible that the administration of APAS affected CD11b expression on leukocytes directly, other studies using APAS have demonstrated no direct interference with neutrophil adhesion to damaged arterial walls³² and therefore suggest that the effects of APAS in our study are likely through platelet depletion. Although our data do not allow us to differentiate the types of leukocytes involved in interactions with platelets, with CD11b being present on more than one cell type, they do indicate the possibility that by platelets aggregating to leukocytes, they could act to prime leukocytes for subsequent adhesion to and transmigration across the vascular endothelium.

To further investigate the possibility that platelets facilitate leukocyte-endothelial adhesion, we carried out *in vitro* cell-adhesion studies to examine the effect of the presence of platelets in this assay. PMNs, for which CD11b/CD18 (Mac-1) is an essential molecule for firm adhesion and transendothelial migration,⁴⁵ were found to adhere to cultured endothelial cells more readily in the presence, rather than in the absence, of autologous platelets and as a function of the density of platelets in the assay well. Interestingly, this effect of platelets was observed in the absence of any deliberate stimulation of

either PMNs or endothelial cells, although in parallel experiments this effect was found to be additive to the proadhesive effects of fMLP. PMN activation, as measured by the presence of the azurophilic granule component MPO in the assay medium, was found to correlate with the increase in adhesion elicited by the presence of platelets and again was found to be further enhanced by means of stimulation with fMLP. In addition to PMN activation, our data provide evidence for platelet activation in the mixed cell system in that platelets treated with wortmannin, an irreversible inhibitor of phosphatidylinositol 3 kinase, and then washed and introduced to the adhesion assay had a much reduced effect on PMN adhesion and activation, although neither parameter was completely inhibited. Our results suggest the possible involvement of activation pathways aside of phosphatidylinositol 3 kinase or, alternatively, that the simple presence of intact platelets is sufficient to augment leukocyte-endothelial interactions. Nonetheless, the fact that irreversible inhibition of the phosphatidylinositol 3 kinase system, through which several major platelet-activating substances, such as thrombin and platelet-activating factor, operate,⁴⁶ should limit the effect of platelets in our assay suggests that an element of platelet activation is involved, although it is not clear from our study which specific mechanisms are involved. It is noteworthy, however, that platelets did not enhance the adhesion of PMNs to empty tissue-culture wells, despite the fact that PMN activation was enhanced, suggesting the vascular endothelium to be a necessary platform for these actions to occur.

In summary, we have provided evidence that platelets are essential for leukocyte trafficking from blood to tissue in allergic inflammation. Moreover, our data from human asthmatic patients, as well as from *in vitro* functional assays, indicate that interactions between platelets, leukocytes, and the vascular endothelium within the circulation are important in human subjects.

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