Immunopathology and Infectious Disease

The Role of Platelets in Leukocyte Recruitment in Chronic Contact Hypersensitivity Induced by Repeated Elicitation

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Platelets have been shown to be important in inflammation, but their role in chronic allergic dermatitis remains unclear. To investigate the role of platelets in a mouse model of chronic contact hypersensitivity induced by repeated elicitation, mice were sensitized and repeatedly elicited in ears with hapten, with or without platelet depletion, by administering antiplatelet antibody or busulfan. Ear thickness, leukocyte infiltration, serum IgE, and scratching behavior significantly decreased in thrombocytopenic mice. cDNA microarray of ear tissue showed reduced gene expression associated with Th2 lymphocytes. Flow cytometry showed increased P-selectin expression on platelets and an increased number of platelet-leukocyte aggregates in blood of repeatedly elicited mice, compared with sham-sensitized mice. In thrombocytopenic mice, inflammation was restored by platelet infusion, which was blocked by platelets from P-selectin-deficient mice or by pretreating platelets with anti-P-selectin antibody. Moreover, injection of activated platelet supernatant into ears led to increased leukocyte infiltration, which was blocked by pretreating platelets with antiplatelet compounds or neutralizing several chemokines in the platelet supernatant. These results suggest that platelets induce leukocyte recruitment into skin by forming platelet-leukocyte aggregates via P-selectin in blood and secreting chemokines at inflamed sites. Therefore, controlling platelet activity may be useful for treatment of chronic allergic dermatitis. *(Am J Pathol 2007, 170:2019–2029; DOI: 10.2353/ajpath.2007.060881)*

The role of platelets in inflammatory processes is being increasingly recognized, in addition to their function in hemostasis and thrombosis.[1–8] Platelets accumulate in inflammatory sites concomitantly with leukocytes[1,2] and regulate a variety of inflammatory responses by secreting or activating adhesion proteins, growth factors, chemokines, cytokine-like factors, and coagulation factors. These proteins induce widely differing biological activities, including cell adhesion, chemotaxis, cell survival, and proliferation, all of which accelerate the inflammatory process.[3] Indeed, involvement of platelets has been demonstrated in the pathomechanisms of inflammatory disorders, including asthma and arthritis.[1,4–8] For example, in animal models of asthma, depletion of platelets in blood reduces infiltration of eosinophils and lymphocytes and decreases bronchial hyperresponsiveness.[6–8]

Chronic allergic dermatitis such as chronic contact dermatitis or atopic dermatitis (AD) is characterized by extreme pruritus and chronically relapsing inflammation. Chronic contact dermatitis includes occupational contact dermatitis and housewives' dermatitis and is characterized by chronic cutaneous inflammation due to repeated contact with allergen; such conditions are examples of recalcitrant skin diseases. Patients with awareness of their sensitivity may manage to avoid further allergen contact, but this is often difficult. AD is a chronic inflammatory skin disease associated with high serum IgE levels, positive immediate-type hypersensitivity to environmental allergens, and eosinophilia.[9] Th2-dominant immune responses to allergens in the skin based on undefined genetic predispositions are the central feature of AD.[10] In these diseases, scratching due to severe itch often results in excoriation and subsequent platelet aggregation at the inflamed lesion. Although platelets have been shown to play a role in cutaneous inflammation

through direct activation of local vascular capillary endothelial cells and attraction of effector T cells into the tissue,[11–15] the role of platelets in chronic allergic skin inflammation is poorly understood.

Many animal models of chronic allergic dermatitis have been reported, including a mouse model in which a chronic contact hypersensitivity reaction is induced by repeated epicutaneous application of hapten.[16] Chronic elicitation leads to a shift in the time course of chronic hypersensitivity responses from a typical delayed-type to an early-type response.[16] The development of a chronic phase results in a shift in the local cytokine pattern from a Th1-type to a Th2-type profile,[17] and the mouse model of chronic contact hypersensitivity is similar to many events seen in humans with chronic allergic dermatitis.

In the current study, we investigated the effects of platelet depletion and restoration on cutaneous inflammatory reaction and leukocyte recruitment in a mouse model of chronic contact hypersensitivity. We then evaluated the effects of repeated allergen exposure to skin on P-selectin expression on platelets and platelet-leukocyte complexes in the blood and the effects of deleting and blocking platelet P-selectin on leukocyte recruitment into skin. The effects of mediators released from activated platelets on leukocyte recruitment in the skin were also examined in this model. In addition, we examined whether treatment of platelets with antiplatelet compounds suppresses leukocyte recruitment to the skin.

Materials and Methods

Mice

Male BALB/c mice were obtained from Shimizu Laboratory Supplies (Kyoto, Japan) and used at the ages of 6 to 8 weeks. Male P-selectin knockout (P-selectin$^{-/-}$) mice and control wild-type C57/BL6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and used at the age of 6 to 8 weeks in studies of platelet restoration in platelet-depleted mice. P-selectin$^{-/-}$ mice were generated as described previously[18] and backcrossed between 10 and 12 generations onto the C57BL/6J genetic background. All mice were housed in a specific pathogen-free barrier facility and screened regularly for pathogens. This experimental procedure was approved by the Committee for Animal Research, Kyoto Prefectural University of Medicine.

Contact-Sensitizing Agent

2,4,6-Trinitro-1-chlorobenzene (TNCB) was obtained from Tokyo Kasei (Tokyo, Japan). TNCB was dissolved in acetone/olive oil (4:1) as a 1% solution and used for sensitization and elicitation.

Sensitization and Elicitation Procedure

BALB/c mice were sensitized with 20 μl (10 μl on the dorsal side and 10 μl on the ventral side) of 1% TNCB solution applied to the right ear as described previously.[16] Starting 7 days after sensitization, 20 μl of 1% TNCB

solution was repeatedly applied to the original sensitized right ear at 2-day intervals for 8 weeks until day 56. An identical amount of acetone/olive oil (4:1) was administered to the left ear as the control.

Ear thickness was measured with a dial thickness gauge (Shinwa Rules, Niigata, Japan) under light ether anesthesia every 7 days. The increase in ear thickness was defined as the difference between ear thickness before elicitation on day 0 and that on days 7, 14, 21, 28, 35, 42, 49, and 56. The ear swelling response was measured before and at 6-hour intervals after elicitation on days 0, 7, 14, 28, and 56 and was defined as the difference in ear thickness before and after elicitation.

Isolation of Murine Platelets

Blood was obtained by cardiac puncture from anesthetized mice, mixed with acid citrate dextrose (Sigma-Aldrich, Poole, UK) at a 9:1 ratio, and then centrifuged at 150 × g for 10 minutes. The platelet-rich plasma obtained was washed using the method of Mustard et al.[19] Washed platelets were suspended in minimal essential medium (Gibco, Grand Island, NY) containing 300 ng/ml prostaglandin I$_2$ (Sigma-Aldrich) and 10% fetal calf serum (ICN Biomedicals, Aurora, OH). The cellular content of the suspension was greater than 99% platelets, as demonstrated by flow cytometry light scattering analysis. Contamination with leukocytes and erythrocytes represented less than 1% of the cellular content (data not shown).

Platelet Depletion

Antiplatelet antisera (APAS) were prepared as previously described.[20] Washed platelets (3 × 10^9) were isolated from normal BALB/c mice. Platelets were homogenized in Freund's complete adjuvant and injected subcutaneously in rabbits. A second immunization was performed 10 days later by intramuscular injection of washed platelets in Freund's incomplete adjuvant. Thirty days after the second immunization, the rabbits were bled, and antiserum was inactivated at 56°C for 60 minutes and stored at −70°C. Nonimmune serum obtained from nonimmunized rabbits served as control serum (CS). Anesthetized BALB/c mice were injected intramuscularly every 3 days (from 24 hours before the first challenge to 30 minutes before the last challenge) with 0.1 ml of APAS or CS. Alternatively, busulfan (Sigma-Aldrich) was used to deplete platelets, as previously described.[21] Anesthetized BALB/c mice received busulfan or vehicle on days −4, −2, 1, and 8, and then every 2 weeks over the chronic challenge period (25 mg/kg, intraperitoneally).

Blood Sampling for Platelet, Leukocyte, and Serum IgE Profiles

Blood (50 μl) was collected from the lateral caudal vein and mixed with acid citrate dextrose at a 9:1 ratio. Platelets were counted using the method of Brecher and Cronkite.[22] In brief, blood was diluted 1:20 with 1% ammonium
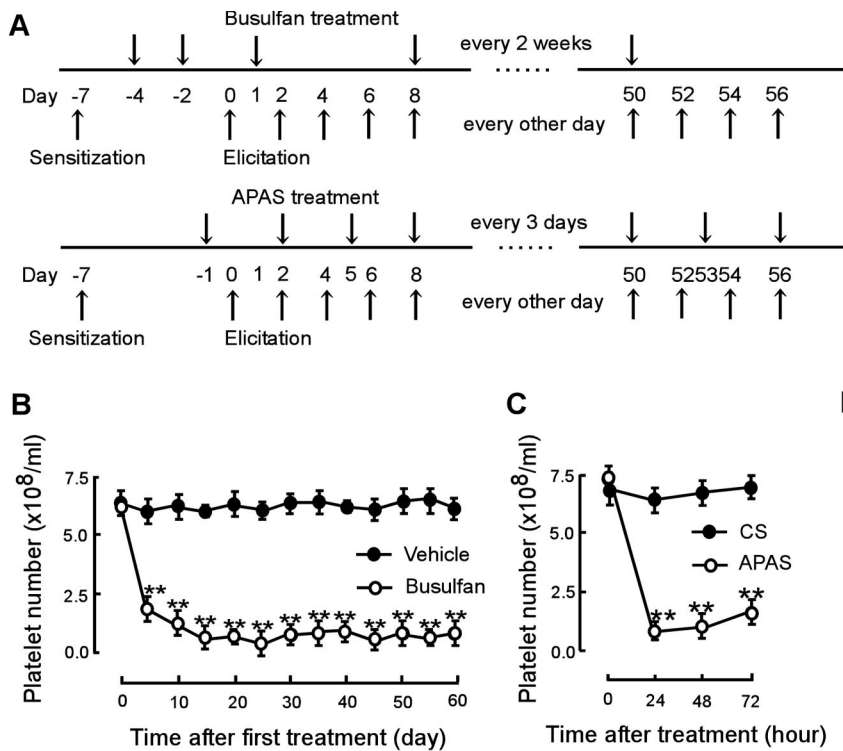
Figure 1. Platelet levels in peripheral blood of mice during chronic busulfan or APAS administration. **A:** Schemes showing the timing of busulfan (top panel) and APAS (bottom panel) administration and repeated hapten application. **B:** Mice were depleted of platelets by intraperitoneal busulfan administration. **C:** Mice were depleted of platelets for up to 72 hours after the first injection of APAS. **D:** Platelet depletion was maintained with APAS administration every 3 days over the chronic period. Data are expressed as the mean platelet number $\pm$ SD of five mice per group. $**P < 0.01$.

oxalate in saline solution to hemolyze erythrocytes. Platelets were counted under a hemocytometer using a thin coverslip (thickness 1) with a phase microscope, dark contrast, $\times 40$ objective after allowing the platelets to settle for 20 minutes. For total leukocyte counts, blood was mixed with hemolysis solution at a 9:1 ratio. The number of platelets and leukocytes was counted before and every 5 days after the first intraperitoneal injection in busulfan-treated mice or 24, 48, and 72 hours and every 5 days after the first intramuscular injection in APAS-treated mice. For measurement of serum total IgE, blood (0.5 ml) was taken from mice by cardiac puncture 6 hours after challenge on day 56. Levels of serum total IgE were analyzed using an IgE detection enzyme-linked immunosorbent assay kit (BD PharMingen, San Diego, CA).

Histological Examination

The ears were excised 6 hours after challenge on day 56, fixed with 10% formalin, and embedded in paraffin. Sections (5 μm) were cut using a microtome and stained with hematoxylin and eosin for general histological evaluation, toluidine blue for mast cell staining, and Luna solution for eosinophil staining. For immunohistochemistry, deparaffinized sections were autoclaved in citric buffer and incubated in phosphate-buffered saline (Sigma-Aldrich) containing 5% normal rabbit serum (10 minutes, 37°C) to block nonspecific staining. They were then incubated with rat anti-CD4 (clone GK1.5, 10 μg/ml; eBioscience, San Diego, CA) or rat anti-CD8 antibody (clone 53-6.7, 10 μg/ml; eBioscience) overnight at 4°C. Rat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) was used as a control for nonspecific staining. Sections were then incubated

sequentially (20 minutes, 37°C) with biotinylated rabbit anti-rat IgG (Vectastain ABC kit; Vector Laboratories, Burlingame, CA) and then horseradish peroxidase-conjugated avidin-biotin (Vectastain ABC kit; Vector Laboratories). Sections were developed with 3,3'-diaminobenzidine tetrahydrochloride and hydrogen peroxide and then counterstained with hematoxylin. Dermal infiltration of total leukocytes, CD4$^+$ T cells, CD8$^+$ T cells, eosinophils, and mast cells was evaluated by averaging the number of leukocytes present in 10 high-power fields (0.07 mm^2).

cDNA Microarray Analysis

The ears were removed 6 hours after challenge on day 56 and immediately homogenized. Total RNA was extracted with TRI Reagent (Sigma-Aldrich). Poly(A) messenger RNA (mRNA) was purified using oligo(dT) attached to magnetic beads and hybridized with a GEArray S Series Mouse Autoimmune and Inflammatory Response Gene Array (MM-602.3; SuperArray, Bethesda, MD) according to the manufacturer's instructions. Positive spots on the array were scanned and quantified using ScanAlyze and GEArray Analysis Suite software.

Flow Cytometry

Blood (0.5 ml) was obtained by cardiac puncture from anesthetized mice on citrate 6 hours after challenge on day 56 and incubated with fluorescein isothiocyanate-labeled rat anti-CD41 (clone MWReg30; Serotec, Oxford, UK) and rabbit polyclonal anti-CD62P (Chemicon Europe, Hampshire, UK), followed by secondary phyco-

erythrin-labeled goat anti-rabbit IgG. These samples were analyzed using a FACScan flow cytometer (Becton Dickinson, Heidelberg, Germany). In some experiments, blood was incubated with fluorescein isothiocyanate-labeled anti-CD41 and analyzed on a FACScan. Live gating was performed for leukocyte-sized events, the forward and side scattering characteristics of which exclude the presence of single platelets alone, as described previously.[23] Events in this region, which were positive for CD41, were considered to represent platelet-leukocyte aggregates.

Restoration of Platelet Population in Platelet-Depleted Mice

Washed platelets were isolated from the blood of repeatedly elicited or sham-sensitized BALB/c mice. The resulting platelet suspension or vehicle was injected intravenously into busulfan-treated mice 20 minutes before the final challenge. The skin tissue was examined 6 hours after challenge. The injection volume of 0.1 ml contained 5.5 to 6.0 × 10^8 platelets. Platelet reconstitution restored the circulating platelet population to 60 to 70% of the control level 20 minutes after injection of the platelet suspension. In some experiments, the platelet suspension obtained from normal BALB/c mice was treated with 100 μg of rat anti-P-selectin blocking antibody (clone RB40.34; Becton, Dickinson and Company, San Diego, CA) before injection. Irrelevant, isotype-matched, purified rat IgG served as the control (Jackson ImmunoResearch Laboratories).

In studies using P-selectin$^{-/-}$ mice, washed platelets were isolated from P-selectin$^{-/-}$ mice and control wild-type C57/BL6J mice. The resulting platelet suspension or vehicle was injected intravenously into busulfan-treated mice 20 minutes before the final challenge. The injection volume of 0.1 ml contained 5.5 to 6.0 × 10^8 platelets. Platelet reconstitution restored the circulating platelet population to 65 to 70% of the control level 20 minutes after injection of the platelet suspension.

Measurement of Chemokines in Platelet Supernatant

Washed platelets were isolated from the blood of normal BALB/c mice and suspended at a concentration of 1 × 10^9/ml. Platelets were stimulated with 0.2 U/ml thrombin for 15 minutes at 37°C. In some experiments, washed platelets were mechanically lysed by means of ultrasonic treatment. The levels of macrophage inflammatory protein-1α (MIP-1α), regulated on activation normal T expressed and secreted (RANTES), and thymus and activation-regulated chemokine (TARC) in the platelet supernatant and lysates were measured using enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, MN). Supernatant of platelets fixed with 2% paraformaldehyde without stimulation with thrombin served as the control.
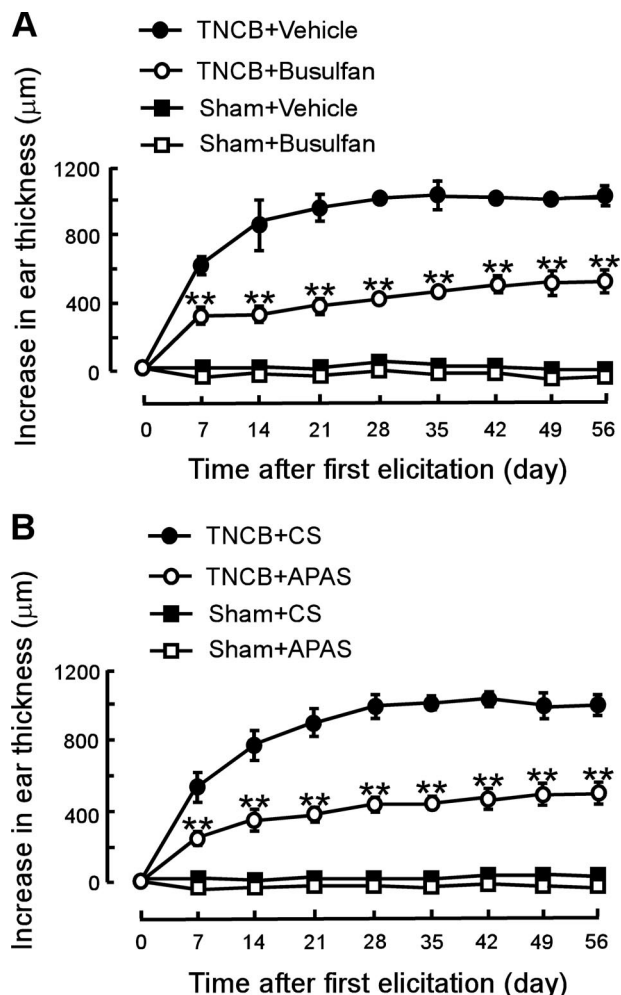

Figure 2. Increase in ear thickness following repeated application of TNCB. TNCB was repeatedly applied to the ears of mice at 2-day intervals for 8 weeks with administration of busulfan or vehicle (**A**) or APAS or CS (**B**). Repeated application of acetone/olive oil alone served as a control. Ear thickness was measured before challenge every 7 days. The increase in ear thickness is defined as the difference between ear thickness on day 0 and that on days 7, 14, 21, 28, 35, 42, 49, and 56. Data are expressed as the mean ± SD of five mice per group. **$P < 0.01$ versus vehicle or CS administration in repeatedly elicited mice.

Injection of Platelet Supernatant into Ears

Washed platelets were isolated from the blood of normal BALB/c mice and suspended at a concentration of 1 × 10^9/ml. Platelets were stimulated with 0.2 U/ml thrombin for 15 minutes at 37°C. Thrombin was then inactivated by addition of 10 U/ml hirudin (Sigma-Aldrich). To neutralize chemokines, the supernatant was treated with 10 μg/ml antibodies against MIP-1α (clone 39624, rat IgG; R&D Systems), RANTES (polyclonal rabbit IgG; ProSci, Poway, CA), and/or TARC (polyclonal goat IgG; R&D Systems) for 30 minutes at 37°C. Irrelevant, isotype-matched, purified IgG served as the control (Jackson ImmunoResearch Laboratories). After centrifugation, 50 μl of the supernatant was injected into the ears of busulfan-treated mice just before the final challenge. Supernatant of platelets fixed with 2% paraformaldehyde without stimulation with thrombin served as the control. The skin tissue was examined 6 hours after challenge.

Pretreatment with Antiplatelet Compounds

Washed platelets were treated with 1 mmol/L acetylsalicylic acid (aspirin; Sigma-Aldrich) or vehicle for 30 minutes at 37°C before stimulation with thrombin. In separate experiments, mice were orally dosed with clopidogrel (30 mg/kg/day; Sigma-Aldrich) or vehicle for 2 days, and blood was collected 1 hour after the final dose. Washed platelets were isolated, suspended, and then stimulated with thrombin.

Statistical Analysis

Data from all studies are expressed as means ± SD. Statistical significance was assessed using one-way analysis of variance followed by a Tukey multiple comparison test. A P value of less than 0.05 was considered significant.

Results

Maintenance of Thrombocytopenia

Mice were administered intraperitoneal injections of either busulfan or vehicle over the chronic challenge period, as shown in Figure 1A. This protocol maintained platelet depletion throughout the period of chronic challenge (Figure 1B). In separate experiments, mice were administered intramuscular injection of either APAS or CS on alternate upper hind leg regions every 3 days (Figure 1A). Mice were depleted of platelets for up to 72 hours after the first injection of APAS (Figure 1C), and platelet depletion was maintained throughout the period of chronic challenge (Figure 1D). Circulating leukocytes remained unaffected throughout the period of chronic challenge in busulfan-treated mice (before the first challenge:

$6.2 \pm 0.3 \times 10^6$; after the final challenge: $5.7 \pm 0.3 \times 10^6$) and in APAS-treated mice (before the first challenge: $6.7 \pm 0.5 \times 10^6$; after the final challenge: $7.0 \pm 0.7 \times 10^6$).

Reduction of Delayed-Type and Early-Type Contact Hypersensitivity Reactions in Platelet-Depleted Mice

First, we investigated the role of platelets in chronic allergic skin inflammation by estimating ear thickness after repeated elicitation in both thrombocytopenic mice and the controls. Repeated elicitation induced a dramatic increase in ear thickness in the control mice, and administration of busulfan (Figure 2A) or APAS (Figure 2B) significantly inhibited this increase. In addition, a detailed time course of ear swelling elicited by repeated TNCB challenge was assessed every 7 days; the swelling underwent a site-restricted shift from a typical delayed-type response (Figure 3A) to an early-type response that reached a peak 6 hours after challenge (Figure 3, B–E), as reported previously.[16] More interestingly, both delayed-type and early-type ear swelling responses significantly decreased in busulfan-treated mice compared with controls. These responses also decreased in APAS-treated mice compared with control mice (see Supplementary Figure S1 at *http://ajp.amjpathol.org*).

Histological examination of the ear skin of mice that received repeated elicitation revealed a hyperplastic epidermis, intense leukocyte infiltration, and dermal fibrosis 6 hours after elicitation on day 56, at the time when the early-type ear swelling response reached its peak (Figure 4, B and F). However, in busulfan-treated mice, only slight acanthosis with less cellular infiltration was observed (Figure 4, D and H). In addition, platelet depletion
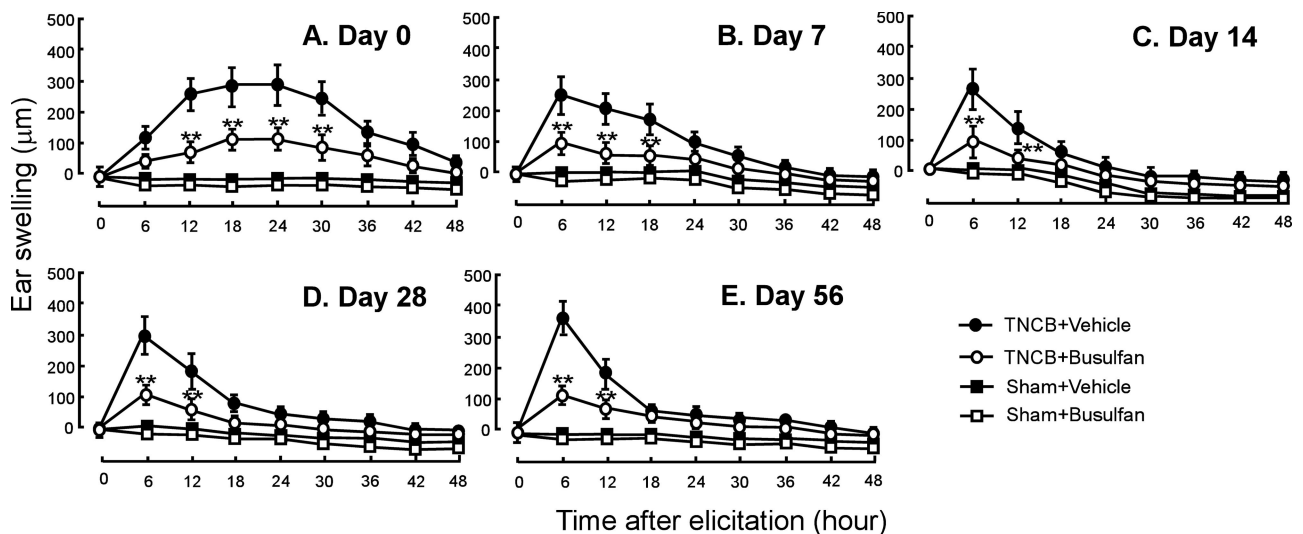

Figure 3. Time course of ear swelling responses after application of TNCB. TNCB was repeatedly applied to the ears of mice at 2-day intervals for 8 weeks with busulfan or vehicle administration. Repeated application of acetone/olive oil alone served as the control. Ear swelling responses were determined before and at 6-hour intervals after challenge on days 0 (**A**), 7 (**B**), 14 (**C**), 28 (**D**), and 56 (**E**), as the difference in ear thickness before and after elicitation. Data are expressed as the mean ± SD of five mice per group. **$P < 0.01$ versus vehicle administration in repeatedly elicited mice.

with busulfan significantly reduced the number of CD4^{+} T cells, CD8^{+} T cells, eosinophils, and mast cells in the skin after elicitation (Figure 4I). The thickness of the epidermis, leukocyte infiltration, and dermal fibrosis
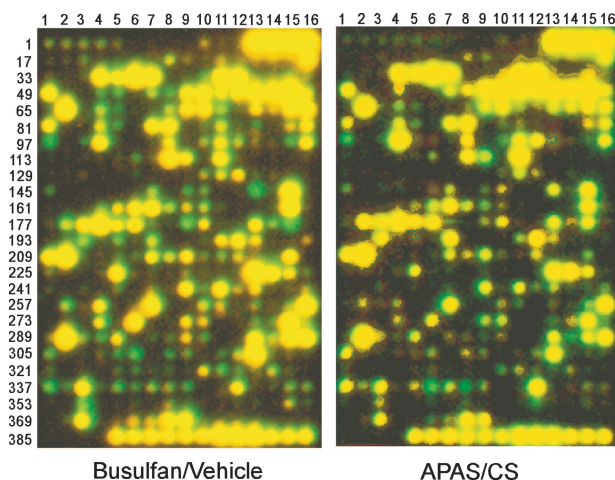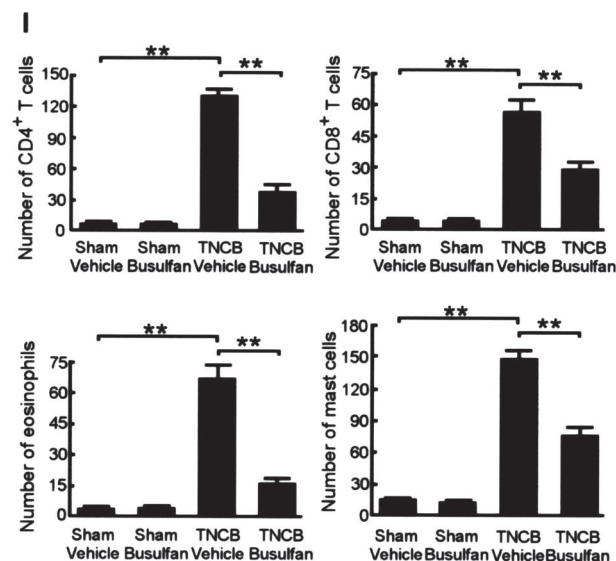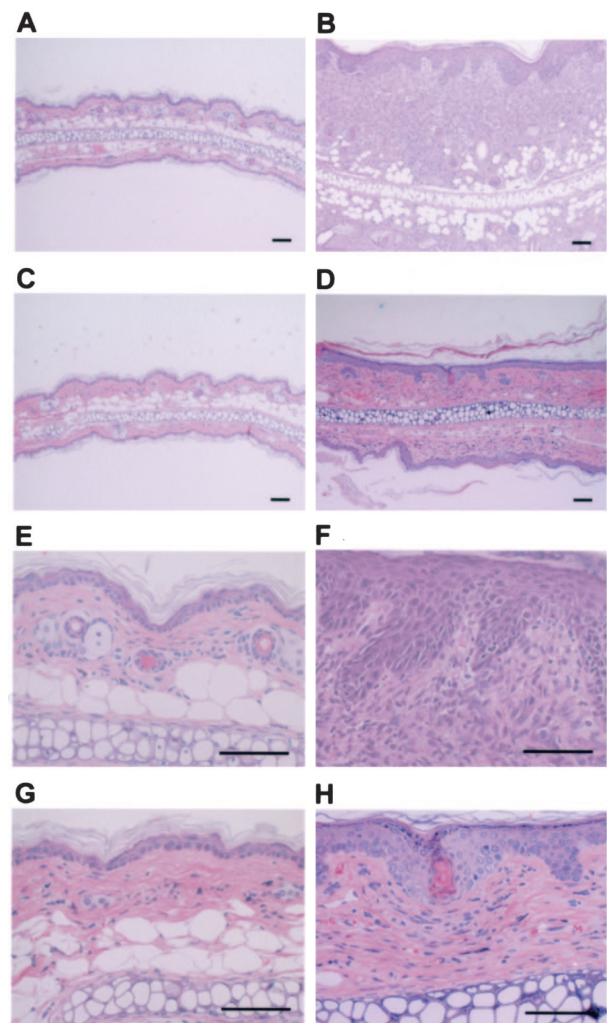

Figure 5. cDNA microarray analysis of gene expression in the ears of busulfan- and APAS-treated mice and controls. mRNA was prepared and hybridized with a GEArray S Series Mouse Autoimmune and Inflammatory Response Gene Array, as described in Materials and Methods. Gene expression levels in busulfan- and APAS-treated mice are represented as a gradation of red, and those in the controls are represented as a gradation of green. Genes expressed commonly in both appear in yellow ($n = 10$ mice per group).

also significantly decreased in APAS-treated mice compared with the controls (see Supplementary Figure S2 at *http://ajp.amjpathol.org*).

Repeated epicutaneous application of hapten in mice induces elevation of serum IgE, as often seen in patients with AD.[16] To investigate whether platelet depletion affects serum IgE levels, blood samples were collected 6 hours after elicitation on day 56, and serum IgE levels were examined by enzyme-linked immunosorbent assay. IgE levels were elevated in control mice and significantly reduced in busulfan-treated mice (5948.7 ± 2212.6 versus 668.7 ± 328.7 ng/ml, $P < 0.01$, $n = 4$ mice per group). The frequency of scratching behavior (measured over a 30-minute period from 6 hours after elicitation on day 56) accompanying repeated elicitation was also significantly reduced in busulfan-treated mice compared with the controls (91.7 ± 19.4 versus 239.2 ± 28.3, $P < 0.01$, $n = 6$ mice per group).

Expression of mRNA Associated with Activated Th2 Is Reduced in Platelet-Depleted Mice

A cDNA microarray analysis was performed to investigate the effects of platelet depletion on expression of genes related to molecular signaling in inflammation in mice treated chronically with hapten. Busulfan- and APAS-

Figure 4. Histology in the ears of busulfan-treated mice and controls. Skin tissue was excised 6 hours after the final application of TNCB with busulfan or vehicle administration, and formalin-fixed/paraffin-embedded sections were stained with hematoxylin and eosin. Repeated application of acetone/olive oil alone served as the control. **A** and **E:** Repeated application of acetone/olive oil alone with vehicle administration. **B** and **F:** Repeated application of TNCB with vehicle administration. **C** and **G:** Repeated application of acetone/olive oil alone with busulfan administration. **D** and **H:** Repeated application of TNCB with busulfan administration. Original magnifications: **A–D**, ×100; **E–H**, ×400. Scale bar = 50 μm. The numbers of CD4^{+} T cells, CD8^{+} T cells, eosinophils, and mast cells in the dermis per 10 high-power fields were counted (**I**). Data are expressed as the mean (per mm²) ± SD of six mice per group. **$P < 0.01$.

Table 1. Differential Expression of Genes Related to Molecular Signaling in Inflammation

Position	Gene	Density ratio of gene expression level	
		Thrombocytopenic mice/control	
		Busulfan/vehicle	APAS/CS
	Th1-typecytokines		
150	IFN-γ	0.850	1.008
183	IL-2	0.731	0.880
	Th2-type cytokines		
157	IL-10	0.427	0.857
166	IL-13	0.974	0.596
191	IL-4	0.420	0.778
193	IL-5	0.843	0.947
195	IL-6	0.520	0.776
	Chemokines		
43	Eotaxin	0.986	0.949
45	TARC	0.789	0.941
51	MDC	0.852	0.874
55	MIP-1α	0.610	0.624
57	RANTES	0.958	0.852
	Other cytokines		
179	IL-1α	0.971	0.801
180	IL-1β	0.982	0.899
190	IL-3	0.561	0.984
347	TNF-α	0.949	0.843

treated mice (shown in red in Figure 5) showed a remarkable decrease in expression of most genes in a visual comparison of expression levels with those in control mice (shown in green in Figure 5). The locations of genes related to skin inflammation are shown in Table 1. The expression levels of Th2-type cytokines greatly increased in a visual comparison with those of Th1-type cytokines in chronically challenged mice, as previously reported.[17] The mRNAs of Th2-type cytokines and other proinflammatory cytokines decreased in both busulfan- and APAS-treated mice compared with the control mice. Except for the results in Table 1, only the gene encoding involucrin (208) increased in busulfan-treated mice compared with control mice (density ratio: 1.163). The expression of positive control genes for ribosomal protein L13a (389, 390, 391), GAPDH (392, 393, 394), peptidylprolyl isomerase A (395, 396, 397), and β-actin (398, 399, 400) remained constant in treated and control mice.

Effect of Platelet Restoration on Ear Swelling and Leukocyte Recruitment in Skin

To confirm that platelets play a role in the induction of chronic hapten-induced dermatitis, the effects of platelet restoration were examined in busulfan-treated mice. Platelets from repeatedly elicited mice and sham-sensitized mice were injected intravenously into busulfan-treated mice before challenge on day 56, and, subsequently, TNCB was applied. The ear swelling response significantly increased after platelet restoration compared with that under thrombocytopenic conditions (Figure 6A). In addition, the levels of infiltrated CD4$^+$ T cells, CD8$^+$ T cells, eosinophils, and mast cells in the skin in response to allergen all increased after
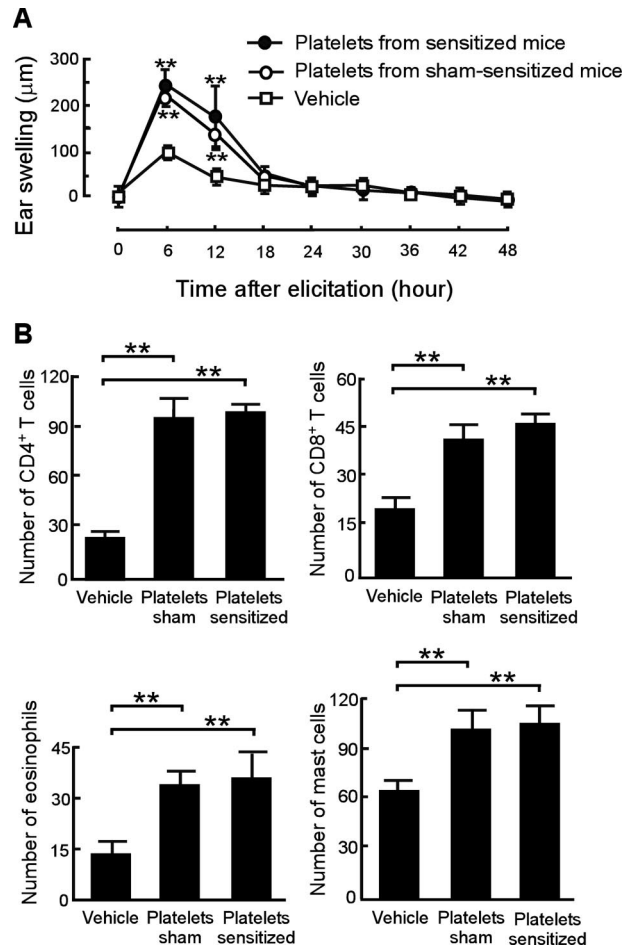

Figure 6. Effects of platelet restoration on ear swelling responses and leukocyte recruitment in the skin. Washed platelets were isolated from blood of repeatedly elicited mice and sham-sensitized mice. Busulfan-treated mice were injected intravenously with platelet suspension or vehicle before the final challenge. **A:** Ear swelling responses were determined before and at 6-hour intervals after challenge. **B:** The skin tissue was examined 6 hours after challenge. The numbers of CD4$^+$ T cells, CD8$^+$ T cells, eosinophils, and mast cells in the dermis per 10 high-power fields were counted. Data are expressed as the mean (per mm^2) $\pm$ SD of six mice per group. **$P < 0.01$ versus mice injected with vehicle.

platelet restoration (Figure 6B). No differences between repeatedly elicited mice and sham-sensitized mice as donors were observed in experiments with platelet restoration. These findings suggest that platelets play an important role in leukocyte recruitment in skin.

Leukocyte Recruitment Is Prevented by Deletion or Blockade of P-Selectin on Platelets

Leukocyte recruitment from the circulation to a site of inflammation is regulated by various mediators, including adhesion molecules.[3] In animal models of asthma, circulating platelets bind leukocytes via P-selectin, which induces pulmonary leukocyte recruitment.[24] Therefore, we examined the expression of P-selectin on platelets and the presence of platelet-leukocyte complexes in circulating blood of mice on day 56 after repeated hapten application. Expression of P-selectin on platelets significantly increased in mice that under-
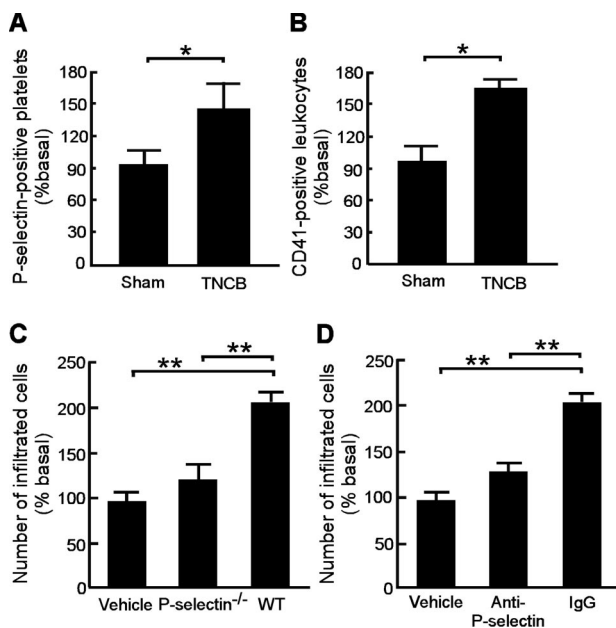
Figure 7. Platelet P-selectin is required for cutaneous leukocyte recruitment in chronically challenged mice. Blood was obtained from repeatedly challenged or sham-sensitized mice 6 hours after the final challenge and analyzed for P-selectin expression on platelets (**A**) and platelet-leukocyte complexes (**B**). Data are shown as the percentage of basal values in sham-sensitized mice. In studies of platelet restoration, washed platelets were isolated from P-selectin$^{-/-}$ mice and control wild-type C57/BL6J mice (**C**). In some experiments, washed platelets were isolated from normal BALB/c mice and treated with anti-P-selectin blocking antibody or isotype control antibody (**D**). Platelet suspension or vehicle was injected intravenously into busulfan-treated mice before the final challenge. Skin tissue was examined 6 hours after challenge. The number of infiltrated cells in the dermis per 10 high-power fields was counted. Data are shown as the percentage of the basal number of cells after injection of vehicle. Bars indicate the mean ± SD of five mice per group. $*P < 0.05$; $**P < 0.01$.

went repeated allergen challenge compared with sham-sensitized mice (Figure 7A). In addition, a significant increase in the percentage of leukocytes positive for the platelet-specific marker CD41 was found in blood taken from mice that received repeated hapten application compared with sham-sensitized mice (Figure 7B). To investigate the role of platelet P-selectin on leukocyte recruitment in the skin, a suspension of platelets obtained from P-selectin$^{-/-}$ mice or incubated with anti-P-selectin blocking antibody was injected intravenously into busulfan-treated mice before challenge, and, subsequently, TNCB was applied on day 56. Platelets obtained from P-selectin$^{-/-}$ mice (Figure 7C) or from incubation with anti-P-selectin blocking antibody (Figure 7D) suppressed leukocyte infiltration in the skin, whereas injection of platelets obtained from wild-type mice or incubated with a control IgG significantly increased leukocyte infiltration in the skin after hapten application. These results indicate that repeated epicutaneous allergen challenge induces P-selectin expression on platelets and formation of leukocyte-platelet complexes in the circulation in chronic allergic dermatitis. Moreover, platelet P-selectin was shown to be important for induction of leukocyte recruitment in the skin.

Effect of Platelet Supernatant on Leukocyte Recruitment in Skin

Recent studies have demonstrated that activated platelets can release chemokines and attract leukocytes to a site of injury and inflammation.[3,25] Minor hemorrhage caused by scratching and excoriation is often seen in the skin of mice following repeated hapten application, which raises the possibility that chemokines released from activated platelets after hemorrhage and subsequent aggregation in the cutaneous tissue may contribute to establishment of chronic dermatitis. Therefore, we assessed the ability of platelet-derived soluble factors to attract leukocytes into the skin by injection of the supernatant of thrombin-activated platelets into the ear skin of busulfan-treated mice before challenge. First, we examined the release of MIP-1α, RANTES, and TARC from activated platelets, since these chemokines have been reported to play a role in the pathogenesis of chronic allergic dermatitis in humans.[9,13,26] The platelets secreted high levels of these chemokines following stimulation with thrombin compared with unstimulated platelets (Figure 8A). As shown in Figure 8B, injection of supernatant of activated platelets into the ear skin significantly increased leukocyte infiltration in the dermis after hapten application. Pretreatment of the supernatant with neutralizing antibodies against MIP-1α, RANTES, and/or TARC significantly suppressed the induction of dermal infiltration after challenge, suggesting that these chemokines are released in cutaneous tissue and contribute to recruitment of inflammatory cells in chronic allergic dermatitis.

Antiplatelet drugs including aspirin and clopidogrel are widely used in the treatment and prevention of vascular diseases such as stroke. To examine whether antiplatelet drugs inhibit release of chemotactic factors from platelets, the platelets were pretreated with aspirin or clopidogrel and then stimulated with thrombin. As shown in Figure 8A, the release of MIP-1α, RANTES, and TARC was largely prevented in platelets exposed to antiplatelet compounds. Finally, we injected the supernatant of thrombin-stimulated platelets treated with antiplatelet compounds into the ear skin of busulfan-treated mice. As expected, pretreatment with antiplatelet compounds resulted in a significant reduction of recruitment of leukocytes into the dermis after hapten application (Figure 8C).

Discussion

Repeated epicutaneous hapten application led to a shift in the time course of chronic hypersensitivity responses from a typical delayed-type to an early-type response and induced a dominant Th2-type cytokine pattern in inflamed sites, as previously reported.[16,17] In this study, depletion of more than 80% of circulating platelets in mice significantly reduced both delayed-type and early-type contact hypersensitivity reactions. In accordance with the decreased number of dermal CD4$^+$ T cells in thrombocytopenic mice, cDNA microarray analysis showed a decrease in expression of genes encoding Th2-type cytokines such as interleukin (IL)-4, IL-5, IL-6,
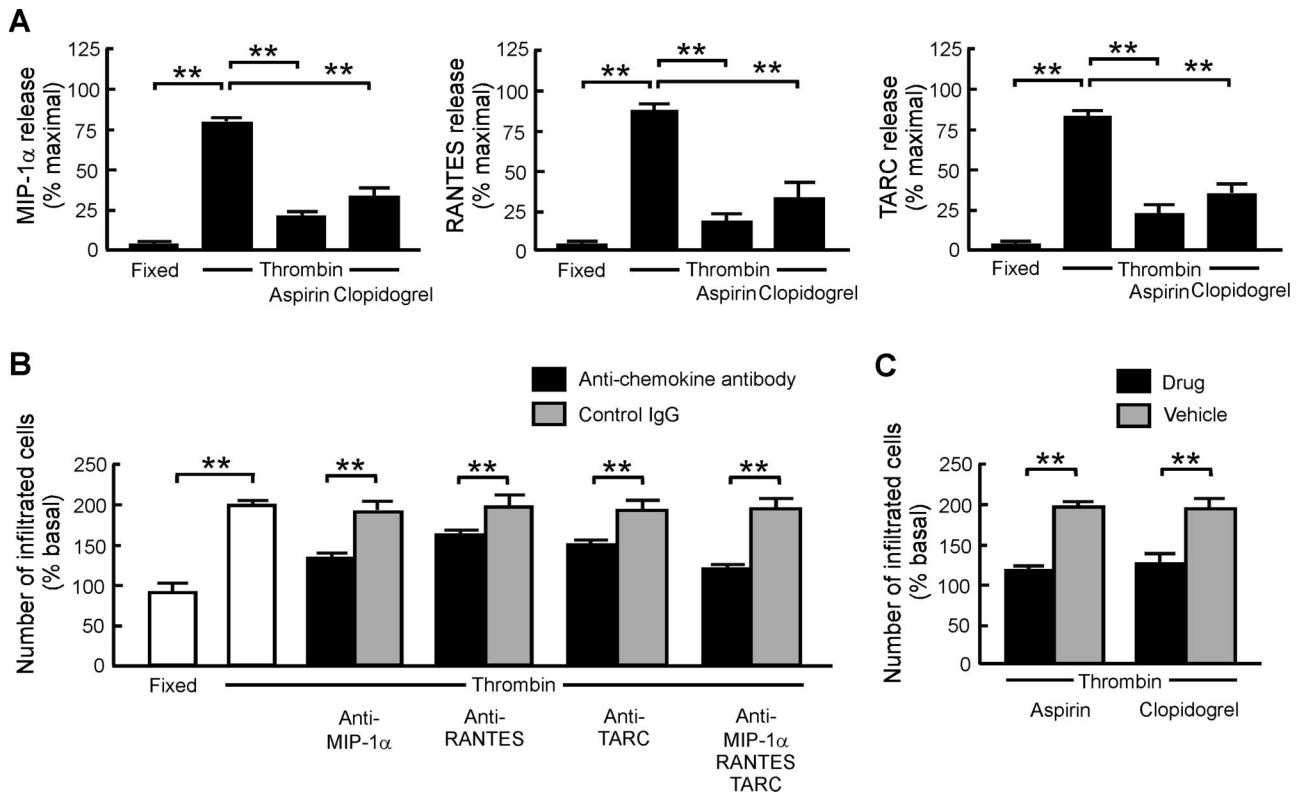
Figure 8. Effects of platelet supernatant on leukocyte recruitment in the skin. Washed platelets were isolated from blood of normal mice and stimulated with thrombin. In studies using antiplatelet drugs, washed platelets were pretreated with 1 mmol/L aspirin or vehicle before stimulation with thrombin. In some experiments, mice were orally dosed with clopidogrel (30 mg/kg/day) or vehicle for 2 days, and blood was collected 1 hour after the final dose. Washed platelets were isolated and stimulated with thrombin. In further studies, washed platelets were mechanically lysed by means of ultrasonic treatment. The supernatant of platelets fixed without stimulation with thrombin served as the control. **A:** The levels of MIP-1α, RANTES, and TARC were measured in platelet supernatant and lysates. Data are shown as the percentage of the maximum number of chemokines in platelet lysates. Bars indicate the mean ± SD of four independent experiments. **B:** Platelet supernatant was injected into the ears of busulfan-treated mice just before the final challenge, after adding anti-MIP-1α, anti-RANTES, anti-TARC, or isotype control antibody to the supernatant. **C:** The supernatant of platelets treated with aspirin, clopidogrel, or vehicle was injected into the ears of busulfan-treated mice just before the final challenge. Skin tissue was examined 6 hours after challenge. The number of infiltrated cells in the dermis per 10 high-power fields was counted. Data are shown as the percentage of the basal number of cells after injection of fixed platelet supernatant. Bars indicate the mean ± SD of five mice per group. **P < 0.01.

IL-10, and IL-13 in these mice. Expression of genes encoding Th1-type cytokines such as IL-2 and interferon-γ also decreased in thrombocytopenic mice, although expression of the gene encoding interferon-γ slightly increased in APAS-treated mice. The greater decrease of activated Th2 cells in platelet-depleted mice probably accounts for the observed reduction of serum IgE levels. CC chemokines such as macrophage-derived chemokine and TARC and other proinflammatory cytokines such as tumor necrosis factor-α, IL-1, and IL-3 are increased in skin lesions of chronic allergic dermatitis including AD,[9,17] whereas the levels of similar cytokines were decreased in thrombocytopenic mice. The diminished number of mast cells and platelets in the dermis is likely to account for the significantly lower frequency of scratching behavior in busulfan-treated mice, because these cells contain various pruritogenic mediators such as histamine and serotonin. Although a possible effect of busulfan on IgE production and scratching behavior are not excluded, other findings shown in busulfan- and APAS-treated mice strongly suggest that platelets play an important role in chronic allergic dermatitis.

Activated platelets have been demonstrated to adhere to monocytes, neutrophils, eosinophils, basophils, and T cells.[27–30] The interaction with leukocytes occurs via P-selectin, which is expressed on the surface of activated platelets in the circulation, and leads to formation of aggregates that roll along the endothelium.[31] This phenomenon enables leukocytes to transmigrate into subendothelial tissue. Indeed, activated platelets have been reported to increase leukocyte rolling in murine skin, and P-selectin expression on platelets increases in the blood of patients with psoriasis in parallel with disease severity.[11] In this study, P-selectin expression on platelets and the number of platelet-leukocyte aggregates significantly increased in the blood of mice with chronic hapten-induced allergic dermatitis. Moreover, depletion or blockade of P-selectin expression on platelets decreased leukocyte recruitment in the skin. These results suggest that elicitation of chronic allergic dermatitis in the earlobe induces P-selectin expression on circulating platelets, which contributes to further leukocyte recruitment into the site of inflammation. Although the precise mechanism through which circulating platelets are activated in chronic allergic dermatitis remains to be elucidated, endothelial cells activated by proinflammatory cytokines and platelet-activating factor released during skin inflammation may be involved.[32] No difference between chron-

ically challenged mice and sham-sensitized mice as donors was observed in experiments with platelet restoration, suggesting that leukocyte recruitment is dependent simply on the presence of platelets as circulating blood elements. With activation of the immune system, various proinflammatory mediators may induce concomitant activation of platelets.

Platelets contain various mediators that attract leukocytes,[3] and mRNA for several chemokines has been identified in platelets.[25] These chemokines, which not only attract leukocytes but also stimulate them and further activate other platelets, have been shown to be important for the pathogenesis of chronic allergic dermatitis such as AD.[13,26] In patients with AD, platelets contain high levels of TARC,[13] which may result in extravasation of Th2 cells and skin-homing memory T cells expressing CC chemokine receptor 4 and further activation of other platelets.[33,34] RANTES and MIP-1α, which are powerful chemoattractants for T lymphocytes, monocytes, and eosinophils, have been shown to be present in α-granules in platelets and to be released from platelets stimulated with thrombin.[35,36] In this study, we confirmed the release of MIP-1α, RANTES, and TARC from thrombin-stimulated murine platelets. In addition, we demonstrated for the first time that the injection of activated platelet supernatant into the skin tissue of busulfan-treated mice can restore allergen-induced cutaneous leukocyte recruitment. However, this infiltration was much less than that under nonthrombocytopenic conditions, which indicates that P-selectin expression on platelets and platelet-derived soluble factors are both necessary for cutaneous leukocyte recruitment. In this experiment, platelet-releasing factors seem to have attracted leukocytes to the injected area via P-selectin on few circulating platelets. More interestingly, this effect was blocked by neutralization of MIP-1α, RANTES, and TARC in the supernatant, suggesting that chemokines released from activated platelets in the cutaneous tissue may induce leukocyte recruitment at sites of skin inflammation.

Our results suggest that control of platelet activity may lead to regulation of cutaneous allergic inflammation. Antiplatelet drugs, especially aspirin and clopidogrel, have been widely used for preventing thrombotic complications of atherosclerosis. Aspirin preferentially inhibits cyclooxygenase in platelets, which metabolizes arachidonic acid to a variety of prostanoids, including thromboxane A$_2$.[37] Clopidogrel is a thienopyridine inhibitor of adenosine diphosphate-induced platelet aggregation.[38] It has been reported that clopidogrel is activated by cytochrome P450 to give a metabolite that acts as an irreversible P2Y$_{12}$ receptor antagonist.[39] Recently, clopidogrel has also been shown to reduce thrombin-induced thromboxane A$_2$ generation in platelets,[40] suggesting that thrombin-induced platelet activation is inhibited not only by aspirin but also by clopidogrel. In this study, pretreatment of platelets with aspirin and clopidogrel inhibited the release of mediators that attract leukocytes. Therefore, these findings suggest that antiplatelet drugs may be useful for treatment of chronic allergic skin inflammation. However, suppressing platelet activity in the whole body can induce spontaneous or excessive bleeding in various organs, because the functions of platelets in hemostasis and inflammation have much in common.[3] Therefore, topical application of antiplatelet drugs may be useful for suppression of platelet function at a cutaneous inflammatory site with lesser effects on other organs, and we intend to investigate the effect of epicutaneous application of antiplatelet drugs on chronic allergic skin inflammation.

In conclusion, our data indicate that platelets are important for developing murine chronic allergic skin inflammation, which shares many characteristics with human chronic allergic dermatitis, including frequent scratching, a high serum IgE titer, and delayed-type and early-type inflammatory responses. We have also provided evidence that platelets induce leukocyte recruitment into skin by forming platelet-leukocyte aggregates via P-selectin in blood and secretion of chemokines at inflamed sites. Therefore, control of platelet activity may offer a new strategy for treatment of chronic allergic skin inflammation.

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