Anaphylaxis-induced mesenteric vascular permeability, granulocyte adhesion, and platelet aggregates in rats

GEoffrey D. Withers, Paul Kubes, Geoffrey Ibbotson, and R. Brent Scott. Anaphylaxis-induced mesenteric vascular permeability, granulocyte adhesion, and platelet aggregates in rats. Am. J. Physiol. 275 (Heart Circ. Physiol. 44): H274–H284, 1998.—This study investigates the response of small venules to IgE-dependent, antigen-mediated mast cell activation. Intravital microscopy was utilized to visualize 25- to 40-µm mesenteric venules, mast cell degranulation (on-line detection), vascular permeability changes (albumin leakage), leukocyte adhesion, and the formation of platelet aggregates in rats sensitized with 10 µg of intraperitoneal egg albumin (EA) in saline or sham-sensitized (saline alone) rats. Sensitized rats challenged with EA (1 mg/ml superfusing mesentery), but not sensitized rats challenged with BSA or sham-sensitized rats challenged with EA, exhibited mast cell degranulation with significant time-dependent increases in vascular permeability (inhibited by diphenhydramine, salbutamol, and indomethacin), leukocyte adhesion (inhibited by Web-2086), and the formation of cellular aggregates (platelet), which were associated with intermittent obstruction of venular flow. Anti-platelet antibody, but not anti-neutrophil antibody or fucoidin (selectin antagonist), prevented platelet aggregate formation. Compound 48/80-induced mast cell degranulation caused similar changes in permeability (via different mediators) and leukocyte adhesion but did not induce platelet aggregation. EA-induced platelet aggregation was not inhibited by any of the mediators tested, and platelets isolated from sensitized rats failed to aggregate in response to direct EA challenge, suggesting release of an unidentified inflammatory mediator as the factor initiating platelet aggregation.

Mast cells; neutrophils; anaphylaxis; allergy

MAST CELL-MEDIATED hypersensitivity reactions to allergens are involved in the pathogenesis of asthma, allergic rhinitis, drug allergy, urticaria, and food allergy. Mast cells express the receptor FcRI that binds the Fc portion of the IgE antibody with high affinity (13). Subsequent cross-linking of the membrane bound IgE with antigen results in mast cell activation and the release of preformed and newly synthesized mediators responsible for the immediate- and late-phase inflammatory response (7, 13).

Mast cell activation may occur in response to stimuli other than the classic IgE-dependent, antigen-mediated pathway including ischemia-reperfusion (16), bacterial toxin (21, 32), Helicobacter pylori (20), and chemical stimulants including compound 48/80, concanavalin A, and the calcium ionophores (8, 28, 34). The use of intravital microscopy allows direct observation of the early events of mast cell degranulation and its effect on the microvasculature. For example, activation of rat peritoneal mast cells with compound 48/80 is followed by increased leukocyte rolling, increased leukocyte adhesion, and increased vascular permeability in post-capillary venules, responses that are inhibited by the use of specific antagonists of known mast cell mediators including histamine, serotonin (5-hydroxytryptamine, 5-HT), and platelet-activating factor (PAF) (8, 18). However, direct visualization of the microcirculation in immediate hypersensitivity has never been undertaken, and indirect evidence suggests that IgE may cause very different vascular disturbances relative to other stimuli. Indeed, the literature suggests that the type and amount of mediators released from mast cells depends on the activating stimulus. Pretreatment with the tricyclic antidepressant amitriptyline modulates the proportions of histamine and 5-HT released from sensitized mast cells (38), whereas pretreatment with misoprostol or PGE2 inhibits histamine release from peritoneal mast cells stimulated by ionophore but not by anaphylaxis (11).

It is therefore conceivable that the microcirculatory changes associated with antigen-induced, IgE-mediated mast cell activation, the event responsible for the anaphylaxis, might differ from the previously described response to compound 48/80-induced mast cell activation (8, 11). To test this hypothesis, the Hooded-Lister rat model of anaphylaxis (25, 29, 33, 34, 35) and intravital microscopy were used to systematically study the acute microvascular changes that occur in response to IgE-dependent, antigen-mediated mast cell activation compared with chemical activation with compound 48/80.

Our data are unique in that they demonstrate rapid formation (within 3–5 min) of platelet aggregates during an immediate hypersensitivity reaction in an in vivo model. These were homogeneous platelet aggregates that were associated with profound microvascular changes. Aggregate formation could not be inhibited by blocking histamine, 5-HT, prostaglandins, PAF, leukotriene synthesis, and selectins or by employing agents that “stabilize” mast cells and prevent degranulation. Moreover, our data reveal a selective profile for IgE-dependent alterations in vascular permeability that differs significantly from chemical activation of mast cells with compound 48/80 and suggests that different stimuli invoke unique mast cell responses. The clinical significance is most relevant for immediate hypersensitivity-mediated diseases such as asthma in which platelet aggregation could significantly amplify the inflammatory response and affect local blood flow.

MATERIALS AND METHODS

Animal model, sensitization, and determination of IgE antibody levels. Experimental procedures were approved by the University of Calgary Animal Care Committee. Hooded-
Litter rats weighing 120–200 g were maintained on a purified laboratory diet. On day 1 of the protocol, rats were sensitized by intraperitoneal injection of 10 µg of chicken egg albumin (EA) and 10 mg of aluminum hydroxide as adjuvant in saline (33).

Thirteen days after sensitization, animals were bled via cardiac puncture to determine EA antibody titers via passive cutaneous anaphylaxis (1, 33). Briefly, duplicate dilutions of serum (1:8–1:64) were injected intradermally in Sprague-Dawley rats weighing 200–300 g. Seventy-two hours later, 2.5 mg of EA and 0.5 ml of 1% Evans blue were injected intravenously, and skin reactions were read after 60 min. Titers were recorded as the greatest dilution of serum producing a colored reaction measuring ≥5 mm in diameter. Sensitized animals had antibody titers of ≥1:64, whereas sham-sensitized animals had none.

Intravitral microscopy. On day 14 after sensitization and after an 18-h fast, animals were prepared for intravitreal microscopy. Rats were anesthetized with pentobarbital sodium (65 mg/kg body wt). The right jugular vein was cannulated for drug and additional anesthetic administration. Systemic arterial pressure was monitored via cannulation of the right carotid artery with a Statham P23 XL pressure transducer and Grass physiological recorder. A midline abdominal incision was made and the rats were placed in a supine position on an adjustable Plexiglas microscope stage. A segment of jejunum was exteriorized through the abdominal incision, and the mesentery was draped over an optically clear viewing pedestal that allows for transillumination of a 2-cm² segment of tissue as previously described (8). All exposed tissue was covered with saline-soaked gauze to minimize tissue dehydration. The temperature of the pedes-

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concentration of EA. The optimal dose of EA was determined in preliminary experiments at three different concentrations (0.1, 1.0, and 10 mg/ml), and a concentration of 1 mg/ml was selected as the minimal consistently effective dose. Video recordings were made before challenge and at 15-min intervals after the onset of challenges.

To compare the anaphylaxis-mediated response with pharmacologically induced mast cell activation, an additional group of EA-sensitized animals was challenged with compound 48/80 (activates connective tissue-type mast cells; Sigma) added to the superfusion solution at a dose of 15 µg and given at a concentration of 1 µg/ml over 15 min. Previous experiments in our laboratory have shown this concentration to be maximally effective (8). It should be noted that the animals did not need to be sensitized for responses to compound 48/80 to occur.

To test the hypothesis that the aggregates formed in response to EA challenge were actually platelet aggregates and that platelet aggregation was selectin mediated, EA-sensitized animals were challenged with EA (1 mg/ml) until aggregates formed. At this time, animals were administered anti-rat platelet antibody (0.5 ml/kg, Accurate Chemical and Scientific, Westbury, NY), anti-rat neutrophil antibody (0.5 ml/kg, Accurate Chemical and Scientific, Westbury, NY), the selectin antagonist fucoidan (fucoidan, 20 mg/kg, Sigma) given by slow intravenous injection over 10 min to minimize hypotensive effects. Antigen challenge continued during and after the treatments, and recordings were made before treatment and every 10 min for 20 min after onset of the intravenous injection. In additional experiments, a pretreatment regimen was used consisting of anti-rat platelet antibody (0.5 ml/kg iv), anti-rat neutrophil antibody (0.5 ml/kg iv), and fucoidan (20 mg/kg iv) to determine whether aggregate formation could be prevented. These experiments were performed to determine whether aggregate formation required an initial platelet-neutrophil mechanism before possible permanent formation of aggregates. To determine the specificity and effectiveness of the anti-rat platelet and neutrophil antibodies, additional animals were given equivalent doses of antibody, and blood was taken both before and at the end of the intravenous injection for counting of platelets by manual examination of blood smears and counting by Coulter counter.

To determine whether the antigen-induced alterations in microvascular function could be inhibited by preventing mast cell activation-degranulation, the mast cell stabilizing agents disodium cromoglycate and doxantrazole and the β-sympathomimetic agonist salbutamol were used. Animals were treated with disodium cromoglycate 25 mg/kg (by iv bolus injection before exteriorization of bowel, and 0.33 mg/kg was added to bicarbonate buffer superfusing mesentry; Sigma). This concentration has been shown to be effective in blocking compound 48/80-induced mast cell activation in previous experiments (18). Doxantrazole (50 mg/kg, Aldrich, Milwaukee, WI) was given by intraperitoneal injection 30 min before anesthetic. This dose has been shown to be effective in preventing EA-induced, IgE-dependent, mast cell-mediated alterations of intestinal motility in the rat model of anaphylaxis (34). Salbutamol (albuterol, 50 mg/kg, Sigma) is relatively nonspecific as a mast cell stabilizer but has been previously demonstrated to inhibit the edema of mast cell activation in the rat when given in high concentrations (4) and has been commonly used in the treatment of asthma.

To identify the effects of mast cell mediators in this model, antagonists were given to putative mediators. To determine the major mediator associated with the increase in vascular permeability, diphenhydramine (H1-receptor antagonist; 30 mg/kg ip injection, Sigma) and/or methysergid (1 mg/kg ip injection, Sandoz, Dorval, QB, Canada) were given 20 min before anesthetic. To determine the importance of PAF, leukotrienes, and prostaglandins in the model, Web-2086 (PAF-receptor antagonist, 10 mg/kg ip injection, Boehringer Ingelheim, Ingelheim, Germany), the leukotrienes synthesis inhibitor indomethacin (5 mg/kg ip 20 min before anesthetic) were administered before antigen challenge. All dosages of inhibitors had previously been shown to be effective (18, 34).

Platelet aggregation bioassay. The response of platelets collected from sensitized or sham-sensitized rats to EA (sensitizing antigen) and BSA (control) was assessed using an in vitro platelet aggregation assay (17). Approximately 10 ml of blood were collected from each animal into 15-ml polypropylene test tubes containing 1 ml of 3.15% trisodium citrate and then gently mixed. The blood sample was centrifuged at 120 g for 10 min. The supernatant of platelet-rich plasma was transferred into labeled 15-ml polypropylene test tubes. The platelet-rich solution was allowed to stand at 22°C for ~30 min to stabilize. Approximately 2–3 ml of platelet-rich plasma were obtained from each rat, and each sample was tested individually.

The platelet aggregation bioassay was performed using a Payton dual-channel aggregometer connected to a two-pen chart recorder. Thrombin (1 IU, Sigma) was added to 0.5-ml aliquots of platelet-rich plasma in the aggregometer and used as a positive control to determine the amount of platelet aggregation resulting from thrombin. Platelet-rich plasma from sensitized and sham-sensitized rats was then challenged with 10 µg of EA to give a total concentration of 1 mg/ml. In the absence of EA-induced platelet aggregation, thrombin was added to the platelet-rich plasma 5 min after antigen challenge to confirm platelet aggregability. Subsequent samples of the platelet-rich plasma were challenged with BSA, and in the absence of platelet aggregation, this was followed by the addition of thrombin as above. Platelet aggregation was measured qualitatively as a positive or negative response to antigen or thrombin.

Statistical analysis. To compare baseline or posttreatment data at multiple time points within and between treatment groups, measured data points for each animal in the treatment group were converted to a linear graphic display and the area under the curve was then converted back to an average response over the 60 min of the experiment. All results are expressed as means ± SE, where n is the number of animals. Statistical significance of the difference between means was determined at P < 0.05 by using the unpaired Student's t-test (2 sided unless specified) for comparison of two means or ANOVA for three or more means.

RESULTS

Effect of sensitization on systemic blood pressure and microvascular hemodynamics. There were no significant differences in the mean body weights, mean systemic blood pressures, venular diameters, baseline mean venular shear rates, or average mean venular shear rates in the sham-sensitized rats subsequently challenged with EA (n = 7) and sensitized rats subsequently challenged with BSA (n = 4), EA (n = 7), or compound 48/80 (n = 9).
Immune-mediated mast cell activation. Mast cell staining was seen in the EA-sensitized group challenged with compound 48/80 (50.0 ± 15.0 and 57.4 ± 10.2% of mast cells stained), whereas there was no staining in sham-sensitized animals challenged with EA (P < 0.05 vs. sensitized animals challenged with EA or compound 48/80) or EA-sensitized animals challenged with BSA (P < 0.05 vs. sensitized animals challenged with EA or compound 48/80). Because this is a subjective or qualitative measure, we used the intensity of ruthenium red staining as an objective, quantitative index of activation (Fig. 1). Mast cell activation was measured on-line at time 0 (before) and at 15-min intervals after challenge of sham-sensitized rats with EA or of sensitized rats with EA, BSA, or compound 48/80. There was no increase in the average intensity of ruthenium red staining in sham-sensitized animals challenged with EA; however, there was a significant (P < 0.05) increase in average mast cell staining in sensitized animals after exposure to EA or compound 48/80 (Fig. 1). Measured in this fashion, the magnitude and time course of mast cell activation was similar in response to challenge with either EA or compound 48/80, and staining continued to increase over the course of the experiment (0–60 min) despite cessation of challenge at 15 min. The mast cell degranulation observed in animals sensitized to EA and then challenged with EA was immune specific, since there was no increase in average mast cell staining or in the percentage of mast cells taking up the dye in sensitized animals challenged with BSA (1 mg/ml at 1 ml/min for 15 min).

**Fig. 1.** Intensity of ruthenium red staining of mast cells (an index of mast cell activation) was measured as mast cell absorbance using computer-assisted digital imaging at time 0 (before) and at 15-min intervals after challenge of sensitized rats with chicken egg albumin (EA, 1 mg/ml for 15 min, n = 7), compound 48/80 (1 mg/ml for 15 min, n = 9) or BSA (1 mg/ml for 15 min, n = 4) and of sham-sensitized rats with EA (1 mg/ml for 15 min, n = 7). *P < 0.05 for means ± SE of area under curve for sensitized animals challenged with EA or compound 48/80 compared with sensitized animals challenged with BSA or sham-sensitized animals challenged with EA.

Altered microvascular permeability. An index of vascular permeability was measured as an index of leakage of FITC-BSA from the venule into the interstitium (Fig. 2). In sham-sensitized animals challenged with EA, there was a slight gradual increase in leakage of fluorescent albumin with time. In contrast, there was a marked and significantly (P < 0.05) greater rise of permeability to near-maximal values (100) at the end of challenge, followed by a slow decline over the course of the next 45 min in sensitized animals exposed to EA or compound 48/80 for the first 15 min. The increase in permeability observed in animals sensitized to EA and challenged with EA was immune specific and did not occur in sensitized animals challenged with BSA. The average permeability index was significantly (P < 0.05) greater over the 60 min of the experiment in sensitized animals challenged with EA (70.0 ± 6.2) or 48/80 (72.3 ± 7.5) compared with sham-sensitized animals challenged with EA (29.3 ± 11) or sensitized animals challenged with BSA (32.0 ± 4.1).

Leukocyte flux, adhesion, and emigration. In sham-sensitized animals challenged with EA and sensitized animals challenged with BSA, there was a small but significant (P < 0.05) increase in venular leukocyte adhesion compared with baseline (Fig. 3). However, the leukocyte adhesion in response to EA or 48/80 challenge was significantly (P < 0.05) increased above that observed in sensitized animals challenged with BSA or sham-sensitized animals challenged with EA. The magnitude of the increase after EA or compound 48/80 challenge of sensitized animals was similar, and on the video image leukocytes ap-
Peared to almost completely line the vessel wall. There was also a significant (P < 0.05) difference in venular leukocyte emigration at 60 min after onset of challenge in EA-sensitized animals challenged with EA (1.7 ± 0.47) or compound 48/80 (1.9 ± 0.26) compared with sham-sensitized animals challenged with EA (0.3 ± 0.3) or EA-sensitized animals challenged with BSA (0.25 ± 0.25). Although there were differences in venular leukocyte adhesion and leukocyte emigration during the experiments in the groups described above, they were not attributable to differences in leukocyte rolling flux, since there were no significant differences in leukocyte rolling flux between the groups (average leukocyte rolling flux for EA-sensitized animals challenged with EA, compound 48/80, and BSA and sham-sensitized animals challenged with EA were 13.9 ± 2.8, 20.5 ± 3.4, 16.7 ± 3.6, and 18.0 ± 4.6 leukocytes/min respectively).

Formation of platelet aggregates. The formation of what appeared on the video monitor to be platelet aggregates occurred in response to EA challenge of sensitized rats and was not seen after challenge of sham-sensitized animals with EA or challenge of sensitized animal with BSA or compound 48/80. These aggregates were first noted 3–8 min after the onset of EA challenge of sensitized animals, and in some animals, their presence was associated with rolling of individual platelets along the venular wall. The flux of these aggregates was maximal 15 min after the onset of challenge and waned with time thereafter (Fig. 4). Larger aggregates stripped adherent neutrophils from the venular wall and were associated with temporary slowing or, on occasion, temporary cessation of venular blood flow.

To determine whether the aggregates that formed in response to EA challenge were actually platelet aggregates (and not leukocyte or leukocyte-platelet aggregates) and if aggregation was selectin mediated, groups of experiments were performed using anti-rat platelet antibody, anti-rat leukocyte antibody and fucoidin. The specificity and effectiveness of anti-rat platelet and anti-rat neutrophil antibody were first confirmed by performing a complete blood count on rats (n = 5) before and after treatment. There was no change in hemoglobin in response to treatment with anti-platelet antibody (133 ± 5 before vs. 133 ± 4 g/l after) or anti-leukocyte antibody (132 ± 3 before vs. 139 ± 9 g/l after). The leukocyte count was significantly (P < 0.05) reduced by treatment with anti-platelet antibody (0.6 ± 0.6 × 10⁹/l before vs. 0.2 ± 0.1 × 10⁹/l after), but the anti-leukocyte antibody was an order of magnitude more effective (0.4 ± 0.3 × 10⁹/l before vs. 0.03 ± 0.05 × 10⁹/l after). The platelet count was significantly (P < 0.05) reduced by treatment with both anti-platelet antibody (854 ± 228 × 10⁹/l before vs. 18 ± 9 × 10⁹/l after) and anti-leukocyte antibody (948 ± 123 × 10⁹/l before vs. 313 ± 232 × 10⁹/l after), but again the anti-platelet antibody was an order of magnitude more effective. It is possible that the more subtle nonspecific effects of these antibodies are related to some platelet-leukocyte interactions after administration of antibody.

The administration of anti-platelet antibody, anti-leukocyte antibody and fucoidin was associated with significant (P < 0.05 compared with baseline) reductions in mean arterial blood pressure of ~20–30%.

In experiments in which anti-platelet antibody, anti-neutrophil antibody, or fucoidin (selectin antagonist) were administered after EA challenge had already

Fig. 3. Number of adherent polymorphonuclear leukocytes (PMN)/100-μm length of mesenteric venule was counted as a measure of leukocyte adhesion at time 0 (before) and at 15-min intervals after challenge of sensitized rats with EA (1 mg/ml for 15 min, n = 7), compound 48/80 (1 mg/ml for 15 min, n = 9), or BSA (1 mg/ml for 15 min, n = 4) and of sham-sensitized rats with EA (1 mg/ml for 15 min, n = 7). *P < 0.05 for means ± SE of area under curve for sensitized animals challenged with BSA or sham-sensitized animals challenged with EA.

Fig. 4. Number of platelet aggregates passing a reference point in mesenteric venule/min were counted at time 0 (before) and at 15-min intervals after challenge of sensitized rats with EA (1 mg/ml for 15 min, n = 7), compound 48/80 (1 mg/ml for 15 min, n = 9), or BSA (1 mg/ml for 15 min, n = 4) and of sham-sensitized rats with EA (1 mg/ml for 15 min, n = 7). *P < 0.05 for means ± SE of area under curve for sensitized animals challenged with EA compared with sensitized animals challenged with BSA or compound 48/80 or sham-sensitized animals challenged with EA.
initiated the formation of what appeared to be platelet aggregates, the number of aggregates observed per minute was significantly reduced by anti-platelet antibody (25.5 ± 6.8 before vs. 1.4 ± 0.5 aggregates/min after, P < 0.05) but not affected by anti-leukocyte antibody (22.5 ± 6.5 before vs. 37.5 ± 9.3 after, not significant (NS)) or fucoidin (25.0 ± 3.8 before vs. 26.4 ± 1.7 after, NS). Anti-neutrophil antibody and fucoidin, but not anti-platelet antibody, caused a significant reduction (P < 0.05 for number after EA challenge alone vs. treatment with antibody or fucoidin after EA challenge) in the number of rolling leukocytes/min at 20 min after treatment (Table 1). The number of adherent leukocytes/100 µm was significantly reduced (P < 0.05 for the number after EA challenge alone vs. treatment with antibody or fucoidin after EA challenge) by anti-neutrophil antibody but was unaffected by anti-platelet antibody or fucoidin (Table 1). The lack of effect of fucoidin as a posttreatment on adhesion is consistent with adhesion being dependent on CD18.

When anti-platelet antibody, anti-leukocyte antibody, or fucoidin were administered before EA challenge, only pretreatment with anti-platelet antibody prevented aggregate formation. Anti-neutrophil antibody and fucoidin were ineffective in preventing aggregate formation (Fig. 5). Pretreatment with anti-neutrophil antibody (an average over 60 min of 6.1 ± 1.6 leukocytes/min, P < 0.05 vs. EA alone) and fucoidin (2.4 ± 1.0, P < 0.05 vs. EA alone), but not anti-platelet antibody (19.6 ± 3.8, NS vs. EA alone), significantly inhibited the average number of rolling leukocytes/min observed in response to challenge with EA alone (21.6 ± 6.1, P < 0.05). Anti-neutrophil antibody (6.4 ± 2.2 leukocytes/100 µm as an average over 60 min) and fucoidin (5.4 ± 1.3) also significantly (P < 0.05) inhibited leukocyte adhesion compared with EA alone (22.3 ± 5.2), whereas anti-platelet antibody (18.6 ± 4.6) was ineffective.

Inhibition of mast cell activation and microvascular response. Disodium cromoglycate was the only agent that was effective in significantly reducing mast cell staining in response to EA (Fig. 6A), and this inhibition was of the order of ~50%. Both disodium cromoglycate and salbutamol were effective at significantly inhibiting vascular permeability changes, and the inhibition was maximal in the first 30 min after antigen challenge (Fig. 6B). Pretreatment with doxantrazole was associated with a significant (P < 0.05) increase in baseline vascular permeability to values exceeding 40% (normal range 10–15% in all other groups), which precluded using this parameter as an index of whether the agent stabilized mast cells and prevented the release of mediators causing an increase in vascular permeability. Although pretreatment of EA-sensitized animals challenged with EA with the β2-adrenergic blocker salbutamol was associated with a significant decrease in baseline and mean blood pressure compared with untreated EA-sensitized animals challenged with EA, pretreatment with disodium cromoglycate, doxantrazole and salbutamol was not associated with changes in venular shear. No mast cell stabilizer significantly inhibited the magnitude of leukocyte adhesion (Fig. 6C) or the number of platelet aggregates in response to EA challenge (Fig. 6D).

Effect of inhibition of putative mast cell mediators. None of the inhibitors of mast cell mediators were associated with a significant inhibition of mast cell staining with ruthenium red when either the percentage of mast cells taking up the dye or the average staining of mast cells was utilized as the parameter for comparison.

Significant inhibition of the anaphylaxis-induced increase in vascular permeability was achieved by pretreatment with the H1-receptor antagonist diphenhydramine and the prostaglandin synthesis inhibitor indomethacin (P < 0.05 vs. EA challenge of sensitized rats not pretreated), whereas methysergide, MK-886, and Web-2086 had no effect (Fig. 7, A and B). The combination of pretreatment with both diphenhydramine and methysergide offered no benefit over pretreatment with diphenhydramine alone, suggesting that the

Table 1. Effect of anti-platelet or anti-neutrophil antibody or fucoidin on leukocyte rolling and adhesion

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<tr>
<th>Treatment</th>
<th>Rolling Leukocytes/min</th>
<th>No. of Adherent Leukocytes/100 µm</th>
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<tbody>
<tr>
<td>Anti-platelet Ab</td>
<td>19.3 ± 1.9</td>
<td>11.3 ± 3.1</td>
</tr>
<tr>
<td>Anti-neutrophil Ab</td>
<td>21.4 ± 4.1</td>
<td>12.7 ± 2.2</td>
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<tr>
<td>Fucoidin</td>
<td>20.8 ± 1.5</td>
<td>15.3 ± 2.3</td>
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Values are means ± SE for values after egg albumin (EA) alone vs. 20 min after EA challenge of sensitized rats has initiated the formation of what appear to be platelet aggregates. Ab, antibody. *P < 0.05 for an effect of treatment on condition initiated by EA alone.
release of 5-HT did not contribute significantly to the anaphylaxis-induced increase in vascular permeability.

Inhibition of leukocyte adhesion occurred only in the group pretreated with the PAF antagonist Web-2086 (average leukocyte adhesion over 60 min 7.9 ± 3.1 vs. 15.1 ± 3.7 for EA challenge of sensitized rats not pretreated, P < 0.05), whereas diphenhydramine (19.3 ± 3.6), methysergide (11.1 ± 1.2), indomethacin (13.5 ± 2.1), and MK-886 (11.4 ± 1.9) were ineffective in inhibiting leukocyte adhesion. Significant inhibition of leukocyte emigration also occurred only in the Web-2086-treated group (0.2 ± 0.1 emigrated leukocytes/100 µm at 60 min vs. 1.7 ± 0.5 for EA challenge alone), whereas diphenhydramine (0.8 ± 0.4), methysergide (1.2 ± 0.2), MK-886 (1.1 ± 0.6), and indomethacin (1.2 ± 0.7) were ineffective in inhibiting leukocyte emigration. None of Web-2086, diphenhydramine, methysergide, MK-886 or indomethacin had any effect on the magnitude of leukocyte rolling flux observed after challenge with EA alone (an average over 60 min of 13.9 ± 2.8 leukocytes/min).

The formation of platelet aggregates was not inhibited by pretreatment with any pharmacological agent (an average over 60 min of 6.1 ± 1.1 platelet aggregates/min for diphenhydramine, 5.8 ± 2.4 for methysergide, 8.4 ± 3.0 for indomethacin, 4.6 ± 1.2 for MK-886, and 5.6 ± 1.0 for Web-2086) compared with the response to EA challenge alone (an average over 60 min of 7.7 ± 2.7 platelet aggregates/min).

In vitro platelet response to sensitizing antigen. Platelet aggregation invariably occurred in response to the positive control of thrombin (1 IU), thus verifying the
potential for platelet aggregation in these preparations of platelet-rich plasma. However, platelet aggregation was not observed in any of the samples of platelet-rich plasma after challenge with the sensitizing antigen EA or with BSA.

**DISCUSSION**

By use of the Hooded-Lister rat model of intestinal anaphylaxis (25, 29, 33, 34, 35) and the technique of intravital microscopy, this study has documented the acute effects of IgE-dependent, antigen-mediated anaphylaxis, including mast cell activation, a rapid histamine-dependent increase in vascular permeability, a PAF-dependent increase in venular neutrophil adhesion and emigration, and the formation of platelet-only aggregates in the microvasculature. The model highlights important similarities and differences in the microvascular response to chemical mast cell activation with compound 48/80 vs. IgE-dependent, antigen-mediated anaphylaxis, most notably, the finding that immediate hypersensitivity in this model is characterized by very profound platelet aggregation that is not selectin mediated or dependent on the presence of circulating neutrophils.

As mentioned previously, the mast cell has been shown to be an important player in the inflammatory response to various stimuli including chemical activation with compound 48/80, ischemia-reperfusion, bacteria, and bacterial toxin (8, 16, 20, 21, 28, 32, 34, 37). Past work in our laboratory using intravital microscopy has allowed examination of the acute stages of mast cell activation secondary to compound 48/80, which initiates a rapid degranulation of mast cells, an increase in vascular permeability and increased leukocyte venular adhesion (8). In ischemia-reperfusion-induced (17) and H. pylori-induced (20) mast cell activation, increased vascular permeability has been shown to be associated with increased leukocyte venular adhesion and emigration. This paper demonstrates that activation of mast cells via an IgE-dependent, Ag-mediated mechanism also results in increased vascular permeability and leukocyte adhesion, but there are some important differences. In compound 48/80-mediated mast cell activation, vascular permeability changes have been shown to be secondary to release of the amine 5-HT for the first 30 min after onset of stimulation. After 30 min, vascular permeability changes are dependent on leukocyte adhesion as inhibition of leukocyte adhesion inhibits permeability changes after this time (18). In the present study, histamine, not 5-HT, was the mediator responsible for altered vascular permeability, and the effect lasted up to 1 h after the onset of mast cell activation. This suggests that either different stimuli result in differential secretion of mast cell mediators, specifically the vasoactive amines histamine and 5-HT, or that antigen and compound 48/80 differentially activate other cell types that contribute to the spectrum of mediator release. Last, inhibition of leukocyte adhesion with Web-2086 (PAF antagonist) or anti-neutrophil antibody and fucoidin (selectin antagonist) did not inhibit the anaphylaxis-induced increases in vascular permeability beyond 30 min, suggesting that histamine-induced vascular changes predominate over the late effect that leukocyte adhesion can exert on permeability.

Inhibition of vascular permeability changes also occurred with the prostaglandin inhibitor indomethacin and the β-sympathomimetic salbutamol, raising the possibility of prostaglandin- or adrenergic-induced permeability changes. Mast cells are known to release PGD2 after activation (7), which could contribute to the
increase in permeability at this time. An alternative explanation is that indomethacin is exerting nonspecific effects and inhibiting histamine-induced permeability changes. Indomethacin has previously been shown to inhibit histamine-induced changes in smooth muscle contractility in the Hooded-Lister rat (34). The inhibitory effect of indomethacin was not due to mast cell stabilization, since it did not affect mast cell activation as measured by ruthenium red staining. The ability of salbutamol to decrease vascular permeability is probably secondary to a direct action on the venule itself (12).

Activation of mast cells in this study resulted in increased leukocyte adhesion and emigration. The response was inhibited by the PAF-receptor antagonist Web-2086, which highlights the importance of PAF in mediating leukocyte venular adhesion, an observation consistent with findings reported for mast cell activation with compound 48/80 and H. pylori models of mast cell activation (8, 20). In the present study and in models of compound 48/80 (8) and H. pylori (20)-induced mast cell activation, inhibition of leukotriene synthesis was ineffective in attenuating leukocyte venular adhesion in the first 60 min after mast cell activation. This lack of effect of leukotrienes is probably a site-specific difference in mast cell populations, since rat peritoneal mast cells, in contrast to rat mucosal mast cells, do not produce leukotrienes (9).

The failure of the mast cell antagonist doxantrazole to prevent mast cell activation and the subsequent changes in leukocyte adhesion and vascular permeability was unexpected, since it has previously been shown to be effective in inhibiting mast cell-mediated alterations in intestinal secretion (29), motility and diarrhea in the Hooded-Lister rat (35) and activation of peritoneal mast cells via neuropeptides (36) and IgE-dependent, antigen-mediated allergic responses in humans (2). Disodium cromoglycate significantly, but only partially, inhibited mast cell activation and vascular permeability changes in this model but was ineffective at blocking leukocyte adhesion or platelet aggregation. This is consistent with previously documented partial inhibition of mast cell activation (8, 36), permeability changes (16, 18), or histamine release (26). It is also possible that, in the present study, the concentration of albumin employed for the challenge (1 mg/ml) represents a near-maximal stimulation of peritoneal mast cells bearing anti-EA IgE on their surface. Nevertheless, these data clearly demonstrate that available mast cell stabilizers are less than effective in preventing critical alterations at the level of the microvasculature in response to allergen and that a need exists for more efficacious drugs.

The most exciting observation in this study is the demonstration that, in this model of intestinal anaphylaxis, allergens induce platelet aggregation in the microcirculation. In this study, anti-platelet antibody was effective in preventing the formation of the aggregates, whereas anti-neutrophil serum was not, suggesting that, in this model, platelets but not neutrophils are an integral part of aggregate formation. By contrast, in other inflammatory conditions [H. pylori infection (20), Clostridium difficile toxin A (21), and ischemia-reperfusion (22)], neutrophil-platelet aggregation has been noted and could be inhibited by the use of P-selectin antagonists, suggesting the platelet-neutrophil interaction is P-selectin dependent. Yet, in our model, the selectin antagonist fucoidin was ineffective in preventing the formation of platelet aggregates, which are thought to be Gp IIb/IIIa dependent, thus providing further indirect evidence against leukocytes being an integral part of aggregate formation in this instance. Intravascular aggregation of platelets has previously been demonstrated in IgE-dependent, systemic anaphylactic shock in the rabbit. In that model of immediate hypersensitivity to horseradish peroxidase, the IgE-dependent platelet alterations were mediated by basophil- and mast cell-derived PAF. Platelet aggregates were sequestered in the pulmonary microcirculation, thus contributing to profound alterations in cardiovascular and pulmonary function (31).

Platelets have previously been described to contain IgE on the surface of their plasma membranes, and activation of platelet-rich serum from sensitized animals and humans has been documented in response to antigen challenge in vitro (5, 14, 15). However, this did not appear to be the mechanism of platelet activation in our model, since platelet activation did not occur with the addition of antigen to platelet-rich serum from sensitized animals. It is probable that, in our experiments, platelet activation is secondary to the release of a specific mediator(s) from mast cells (although one that none of the so-called mast cell stabilizers used therapeutically could inhibit) or other cell types known to express high- and low-affinity receptors for IgE, including basophils, monocytes, eosinophils, and platelets. The mast cell releases a vast array of preformed inflammatory mediators including histamine, 5-HT, lysosomal enzymes, superoxide anions, and proteoglycans and newly synthesized mediators including leukotrienes, prostaglandins, thromboxanes, platelet-activating factor (PAF) and various cytokines including interleukins-1 to -6, interferon-γ, and tumor necrosis factor-α (7). Platelets are known to express receptors to 5-HT, thromboxane A2, PGD2, and PAF on their external membrane (3) [but PAF receptors are not present on rat platelets (19)], making activation via a mast cell mediator feasible. However, in this study, histamine, 5-HT, prostaglandins, leukotrienes, and PAF had no role, suggesting an as yet unidentified mediator or mechanism. Further studies evaluating additional mediators and other cell types are necessary.

The likely consequences of platelet activation and aggregation as part of the intestinal anaphylactic response may be of critical clinical importance, including amplification of the inflammatory response and effects on local blood flow. Platelets contain dense α- and lysosomal granules that contain potent proinflammatory agents (27), including the vasoactive amine 5-HT, ADP, ATP, procoagulants fibrinogen and von Willebrand factor, chemotactic factors including platelet factor 4 and platelet-derived growth factor, and en-
zymes including elastase, collagenase, β-glucuronidase, arylsulfatase, and heparinase. Platelets interact with neutrophils in the formation of leukotrienes (24) and PAF (6), thereby amplifying the inflammatory response and increasing leukocyte migration to the site of inflammation. Indeed, removal of the platelet aggregates in this study eliminated the interruption in microvascular blood flow.

The clinical impact of platelet activation as part of the IgE-dependent, antigen-mediated immune response derives principally from those diseases in which the anaphylactic response is well documented, including asthma, allergic rhinitis, bee sting, and food allergy. Evidence for platelet activation has best been documented in asthma, in which thrombocytopenia, circulating platelet aggregates, morphological and biochemical evidence of activated platelets, and a shortened survival time (10) have been reported. In humans, in contrast to rats, platelets do possess receptors to PAF, PAF has been documented to be elevated in the serum of patients with severe asthma, and PAF-induced platelet activation-aggregation may play a pathophysiologic role (10, 39). Clearly, drugs aimed at preventing platelet activation-aggregation may play a pathophysiological role in patients with severe asthma, and PAF-induced platelet activation-aggregation may play a pathophysiologic role (10, 39). Clearly, drugs aimed at preventing platelet aggregation could conceivably improve the management of the aforementioned disease states.

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