

Take the Road Less Traveled.

**Bioactive Recombinant
Cytokines & Chemokines**

• Manufacturer of 170+ Proteins • Functional Testing on Every Lot



Aspirin Activation of Eosinophils and Mast Cells: Implications in the Pathogenesis of Aspirin-Exacerbated Respiratory Disease

This information is current as of June 12, 2014.

John W. Steinke, Julie Negri, Lixia Liu, Spencer C. Payne and Larry Borish

J Immunol published online 2 June 2014

<http://www.jimmunol.org/content/early/2014/05/31/jimmunol.1301753>

-
- Subscriptions** Information about subscribing to *The Journal of Immunology* is online at: <http://jimmunol.org/subscriptions>
- Permissions** Submit copyright permission requests at: <http://www.aai.org/ji/copyright.html>
- Email Alerts** Receive free email-alerts when new articles cite this article. Sign up at: <http://jimmunol.org/cgi/alerts/etoc>

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
9650 Rockville Pike, Bethesda, MD 20814-3994.
Copyright © 2014 by The American Association of
Immunologists, Inc. All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.



Aspirin Activation of Eosinophils and Mast Cells: Implications in the Pathogenesis of Aspirin-Exacerbated Respiratory Disease

John W. Steinke,^{*,†} Julie Negri,^{*,†} Lixia Liu,^{*,†} Spencer C. Payne,^{*,†,‡} and Larry Borish^{*,†}

Reactions to aspirin and nonsteroidal anti-inflammatory drugs in patients with aspirin-exacerbated respiratory disease (AERD) are triggered when constraints upon activated eosinophils, normally supplied by PGE₂, are removed secondary to cyclooxygenase-1 inhibition. However, the mechanism driving the concomitant cellular activation is unknown. We investigated the capacity of aspirin itself to provide this activation signal. Eosinophils were enriched from peripheral blood samples and activated with lysine ASA (LysASA). Parallel samples were stimulated with related nonsteroidal anti-inflammatory drugs. Activation was evaluated as Ca²⁺ flux, secretion of cysteinyl leukotrienes (CysLT), and eosinophil-derived neurotoxin (EDN) release. CD34⁺ progenitor-derived mast cells were also used to test the influence of aspirin on human mast cells with measurements of Ca²⁺ flux and PGD₂ release. LysASA induced Ca²⁺ fluxes and EDN release, but not CysLT secretion from circulating eosinophils. There was no difference in the sensitivity or extent of activation between AERD and control subjects, and sodium salicylate was without effect. Like eosinophils, aspirin was able to activate human mast cells directly through Ca²⁺ flux and PGD₂ release. AERD is associated with eosinophils maturing locally in a high IFN-γ milieu. As such, in additional studies, eosinophil progenitors were differentiated in the presence of IFN-γ prior to activation with aspirin. Eosinophils matured in the presence of IFN-γ displayed robust secretion of both EDN and CysLTs. These studies identify aspirin as the trigger of eosinophil and mast cell activation in AERD, acting in synergy with its ability to release cells from the anti-inflammatory constraints of PGE₂. *The Journal of Immunology*, 2014, 193: 000–000.

Aspirin-exacerbated respiratory disease (AERD) is a distinct syndrome characterized by asthma, chronic sinusitis, nasal polyposis, and sensitivity to aspirin and other nonselective inhibitors of cyclooxygenase (COX) (1, 2). Patients with aspirin sensitivity experience a wide variety of symptoms ranging from nasal congestion, rhinorrhea, and wheezing to life-threatening asthma attacks within 2–3 h of ingesting aspirin or other nonsteroidal anti-inflammatory drugs (NSAIDs) (3–5). These reactions do not reflect an IgE-mediated allergic process. Instead, it is recognized that constitutive (COX-1–derived) PGE₂ acts as an inhibitor of eosinophils and possibly mast cell activation, and it is the removal of this “brake” by COX-1 inhibitors that permits cellular activation. Support of this concept is derived from recognition that exogenously administered PGE₂ can protect the airway from reactivity to aspirin (6).

In particular, ingestion of NSAIDs leads to a surge in secretion of cysteinyl leukotrienes (CysLTs), reflecting the dramatic overexpression of leukotriene C₄ synthase (LTC₄S) by sinonasal and airway eosinophils, including an additional contribution by LTC₄S-expressing platelets that are adherent to eosinophils and neutrophils in AERD (7–10). It is this surge in CysLT production that primarily drives the bronchospasm, as shown by the ability of LT modifiers to attenuate these reactions (11).

The role of concomitant mast cell activation in AERD is less clear than that of eosinophil activation. Studies, including ours, have shown variable expression of mast cells in AERD tissue (8, 12, 13). In contrast, the intensity of the infiltration of the sinuses and lungs with eosinophils is a defining feature of this disease and comprehensively distinguishes AERD from either aspirin-tolerant asthma or (aspirin-tolerant) eosinophilic sinusitis (13–15). Furthermore, when identified, the mast cells observed in AERD modestly express LTC₄S (8) and, as such, are likely minor contributors to the surge in CysLT production that defines these reactions. However, in addition to production of CysLTs and eosinophil-derived cationic peptides in AERD, NSAID challenges are associated with secretion of PGD₂ and histamine, indicative to some degree of mast cell activation (7, 16).

The traditional explanation that a decline in PGE₂ concentrations drives the hypersensitivity reaction in AERD, however, is incomplete. In the absence of robust LTC₄S expression, CysLT secretion would be minimal in asthmatics without AERD and in healthy controls. However, an increase in other eosinophil degranulation products (e.g., cationic protein and eosinophil derived neurotoxin [EDN]) upon a decline in PGE₂ would be expected. As such, the lack of such a response in these populations suggests the need for additional mechanisms driving the activation process.

^{*}Asthma and Allergic Disease Center, Department of Medicine, University of Virginia Health System, Charlottesville, VA 22908; [†]Carter Immunology Center, University of Virginia Health System, Charlottesville, VA 22908; and [‡]Department of Otolaryngology–Head and Neck Surgery, University of Virginia Health System, Charlottesville, VA 22908

Received for publication July 2, 2013. Accepted for publication May 3, 2014.

This work was supported by National Institutes of Health Grants R01-AI47737 and P01-AI50989.

Address correspondence and reprint requests to Dr. Larry Borish, Asthma and Allergic Disease Center, Box 801355, Charlottesville, VA 22903. E-mail address: lb4m@virginia.edu

Abbreviations used in this article: AERD, aspirin-exacerbated respiratory disease; COX, cyclooxygenase; CysLT, cysteinyl leukotriene; EDN, eosinophil-derived neurotoxin; EGTA, ethylene glycol-bis(β-aminoethyl ester)-N,N,N',N'-tetraacetic acid; LTC₄S, leukotriene C₄ synthase; LysASA, lysine aspirin; NaSal, sodium salicylate; NSAID, nonsteroidal anti-inflammatory drug; SCF, stem cell factor.

Copyright © 2014 by The American Association of Immunologists, Inc. 0022-1767/14/\$16.00

Although the defining infiltration of eosinophils suggests a Th2 cytokine milieu, our recent studies demonstrated that AERD is also characterized by the expression of IFN- γ and that the eosinophils themselves are the primary source of this cytokine (17). Significantly, we demonstrated that among the classic signature Th1 and Th2 cytokines, only IFN- γ was able to upregulate LTC₄S in eosinophils developing from hematopoietic progenitor cells, a feature not shared with IL-4, IL-5, IL-13, or GM-CSF (17). As upregulated LTC₄S in AERD is primarily a feature of sinonasal and lung—but not circulating—eosinophils (8, 15; J.W. Steinke and L. Borish, unpublished observations), we argued that this was a feature of eosinophils differentiating locally in the airway from the exuberant population of infiltrating precursors (CD34⁺IL-5R α ⁺ cells) (18, 19).

The current studies were therefore designed to assess direct activation of eosinophils and mast cells by aspirin and other NSAIDs through one of the off-target pathways ascribed to them (20, 21). In addition, we investigated the capacity of IFN- γ to license the ability of newly differentiated eosinophils to respond to aspirin (and other NSAIDs) with CysLT release.

Materials and Methods

Subjects

The study was approved by the University of Virginia Institutional Review Board for Health Science Research, and all subjects gave their informed consent. Subjects with and without AERD were recruited for this study. AERD was diagnosed based upon clinical criteria and was defined by the presence of asthma and at least one hypersensitivity reaction, including urticaria, nasal congestion, or shortness of breath within 2–3 h of ingestion of either aspirin or another nonsteroidal anti-inflammatory drug.

Reagents

Ketorolac was purchased from Hospira (Lake Forest, IL), sodium salicylate (NaSal) from Acros Organics (Geel, Belgium), lysine aspirin (LysASA) from Sanofi-Aventis (Athens, Greece), and celecoxib from Pfizer (New York).

Cell culture

PBMCs were isolated through Ficoll-Hypaque (Sigma-Aldrich, St Louis, MO) density centrifugation from blood obtained from subjects enrolled in the study. Eosinophils were enriched from granulocytes using negative magnetic affinity column purification (CD16[−]; Miltenyi Biotec, Auburn, CA) with >95% purity. Cells were suspended in complete RPMI 1640 (Invitrogen, Carlsbad, CA) medium supplemented with 10% autologous serum.

Cellular activation

For all activation experiments, 1×10^6 cells in 0.5 ml were exposed to LysASA, NaSal, ketorolac, or celecoxib at concentrations ranging from 0.3 to 10 mM for 30 min, after which supernatants were collected for ELISAs.

ELISA

PGD₂ (Cayman), CysLT (Assay Designs, Ann Arbor, MI), and EDN (MBL, Nagoya, Japan) concentrations in culture supernatants were quantified by ELISA according to the manufacturer's instructions. The detection limits for the assays were 55.0 pg/ml for PGD₂, 26.6 pg/ml for CysLT, and 0.62 ng/ml for EDN.

Ca²⁺ flux

Eosinophils were aliquoted in a 96-well dark-walled plate at 125,000 cells per well in 50 μ l and allowed to settle for 60 min. Cells were either left untreated or stimulated with LysASA, NaSal, or ketorolac at concentrations ranging from 0.3 to 10 mM at 37°C. The calcium flux assay was performed using the Fluo-4 NW Calcium Assay Kit following the manufacturer's instructions (Molecular Devices, Sunnyvale, CA). Briefly, 50 μ l loading buffer supplemented with 5.0 mM probenecid (Sigma-Aldrich) was added to each well and incubated at 37°C for 30 min. Cytoplasmic [Ca²⁺]_i was determined at an extinction wavelength of 494 nm and an emission wavelength of 516 nm, using a Flexstation fluorescence spectrophotometer (Molecular Devices) with 20 μ l each stimulus added for a final volume of 120 μ l. Each condition was set up in triplicate and the

average of the three measurements used for analysis. ATP (300 μ M) was used as a positive control. Data analyses were performed using GraphPad Prism 6 (GraphPad Software, San Diego, CA). Additional studies were performed in the additional presence of ethylene glycol-bis(β -aminoethyl ester)-N,N,N',N'-tetraacetic acid (EGTA), 1 mM, to chelate extracellular sources of Ca²⁺.

Eosinophil progenitor activation

PBMCs were isolated through Ficoll-Hypaque (Sigma-Aldrich) density centrifugation from blood obtained from healthy volunteers. CD34⁺ cells were enriched from PBMCs using positive magnetic affinity column purification (Miltenyi Biotec), and eosinophil progenitors were derived using the technique of Hudson et al. (22) by culturing purified CD34⁺ cells in complete medium (RPMI 1640 and 10% FBS) supplemented with stem cell factor [(SCF) 25 ng/ml; BD Biosciences], thymopoietin (25 ng/ml; R&D Systems, Minneapolis, MN), Fms-like tyrosine kinase 3 ligand (25 ng/ml; BD Biosciences), IL-3 (25 ng/ml; BD Biosciences), and IL-5 (25 ng/ml; BD Biosciences) with or without IFN- γ (20 ng/ml; BD Biosciences) for 3 d and then cultured for an additional 3 wk with just IL-3 and IL-5 ($\pm$ IFN- γ). Cells were washed, and fresh media and cytokines were applied weekly. Maturation was accessed as previously described (17), and cells were activated as described above.

Mast cells. For studies involving mast cells, CD34⁺ cells isolated as described were cultured for 8 wk with SCF and IL-6 and, for the first week only, with IL-3 according to the methodology of Kirshenbaum et al. (23, 24).

Statistical analyses

Data were contrasted between unstimulated and stimulated cells by the Wilcoxon rank sum test for nonparametric data analyses or paired *t* test for parametric data. Normal and AERD cohorts were compared using unpaired *t* tests. Statistical analyses were performed using GraphPad Prism 6.

Results

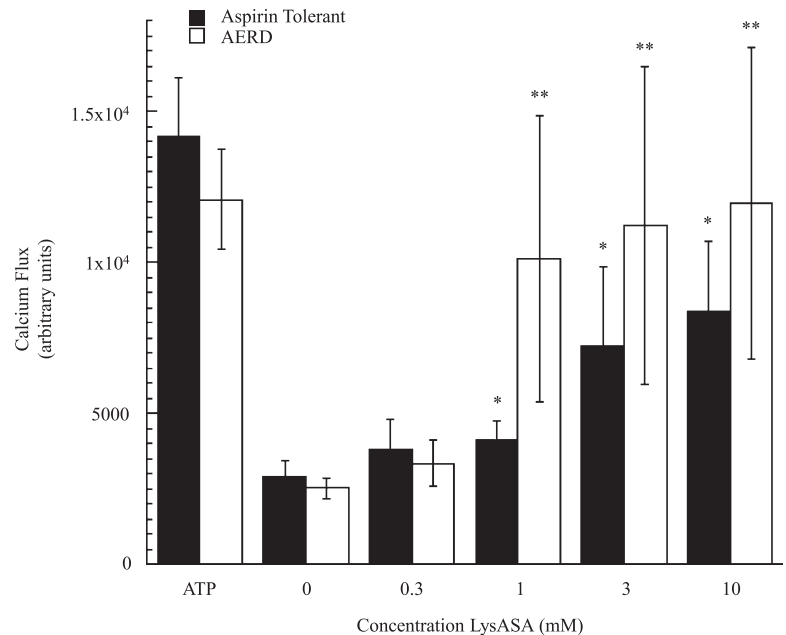
Eosinophil Ca²⁺ flux

We evaluated the capacity of aspirin and other NSAIDs to directly activate enriched peripheral blood eosinophils, initially by examining induction of calcium fluxes. For these studies, we used the aspirin-like compound LysASA, which retains the biological properties of aspirin while being water soluble. Ca²⁺ fluxes were consistently observed at physiologically relevant concentrations for eosinophils in a dose-dependent manner [*p* < 0.05; data from 12 subjects (6 each with and without AERD) are displayed in Fig. 1]. The presence of AERD made no statistically significant difference in either the extent of Ca²⁺ mobilization with eosinophils to LysASA or in sensitivity (EC₅₀ = 2.1 mM for controls and 0.8 mM for AERD). The calcium flux studies were also performed with the related anti-inflammatory compound NaSal, which retains many of the off-target anti-inflammatory activities of aspirin but does not inhibit COX, and ketorolac, which is a nonselective COX inhibitor. Unlike LysASA, both NaSal and—unexpectedly—ketorolac were unable to induce calcium flux (Fig. 2A). To determine whether the calcium mobilized in the assay was derived from intra- or extracellular stores, additional studies were performed in the presence of EGTA. Insignificant decreases in Ca²⁺ flux were observed (Fig. 2B), suggesting that the main source of calcium was being derived from intracellular stores.

Eosinophil degranulation

To investigate whether NSAID stimulation would lead to release of eosinophil-derived mediators, EDN and CysLT secretion was assessed. Incubation with LysASA in a dose-dependent fashion resulted in statistically significant increases of EDN secretion (*p* < 0.05) (Fig. 3). As with the Ca²⁺ flux, no differences were observed between AERD and control cohorts (data not shown). Further, no EDN release was observed after incubation with NaSal or ketorolac. However, incubation with celecoxib did result in release of EDN (*p* < 0.01) at the 3 and 10 mM concentrations, although not as robust a release as with LysASA (Fig. 3). In contrast to the

FIGURE 1. LyASA stimulation of eosinophils from AERD and non-AERD subjects. Calcium mobilization assays were performed on eosinophils by plating 125,000 cells per well and incubating the cells with Fluo-4 NW wash dye mix supplemented with 5 mM probenecid for 1 h. Dose–response curves were generated by incubation of the cells, increasing concentrations of LysASA (0.3–10 mM), and measuring cytoplasmic calcium flux. ATP was used as a positive control. Data are presented as the mean $\pm$ SEM of separate studies performed in triplicate (six aspirin-tolerant, six AERD subjects: * $p < 0.05$, ** $p < 0.05$ compared with unstimulated healthy control and AERD control, respectively).



EDN results, LysASA did not increase CysLT secretion from these peripheral blood-derived eosinophils, again, even when obtained from AERD subjects (data not shown).

Influence of IFN- γ on aspirin activation of eosinophils derived from CD34⁺ progenitors

Circulating eosinophils display comparatively modest expression of LTC₄S, even when derived from AERD subjects, compared with what is observed in their sinonasal tissue and lungs (8, 15; J.W. Steinke and L. Borish, unpublished observations). However, we have recently demonstrated that eosinophils matured in the additional presence of IFN- γ —as occurs locally in the sinonasal tissue

and lungs of AERD patients—display upregulated LTC₄S expression and markedly increased their release of CysLTs (and EDN) when activated with PMA/ionomycin (17). We therefore queried whether eosinophils differentiated from CD34⁺ hematopoietic progenitor cells in the presence of IFN- γ would demonstrate CysLT secretion when exposed to LysASA. As with eosinophils obtained from circulating blood, eosinophils differentiated in the absence of IFN- γ ($n = 6$) secreted EDN after exposure to LysASA (Fig. 4A), but only modest secretion of CysLTs was observed (Fig. 4B). The presence of IFN- γ during maturation significantly enhanced the capacity of in vitro differentiated eosinophils to degranulate and release EDN (Fig. 4A; $p < 0.0004$ at the 1 and 10 mM concentrations). More

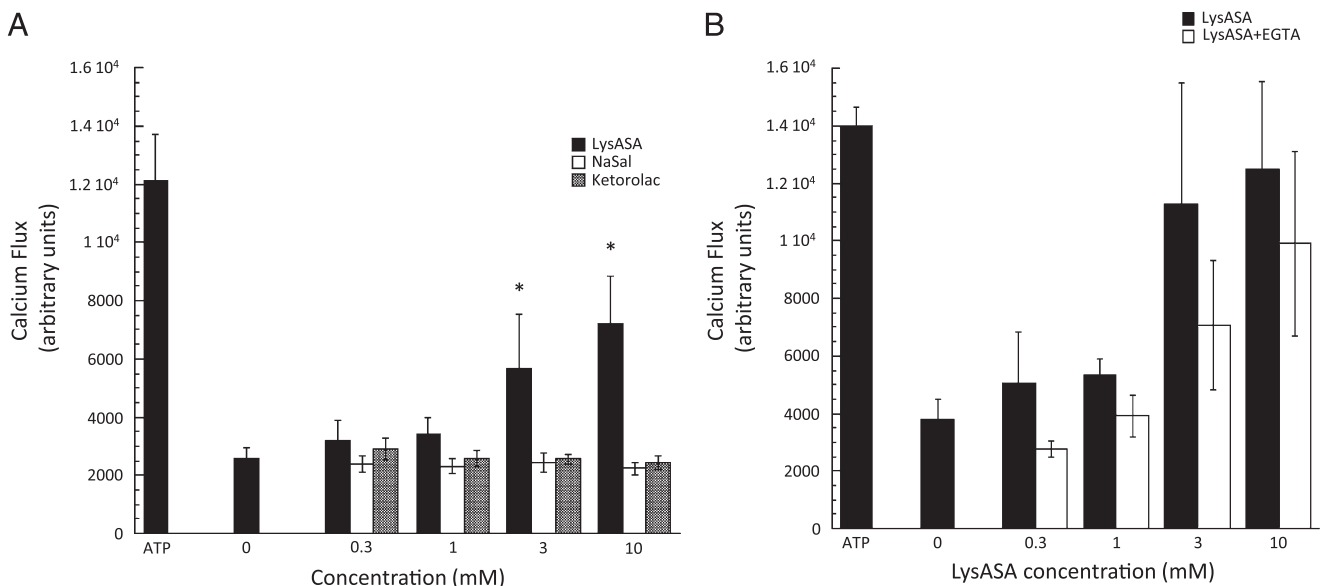
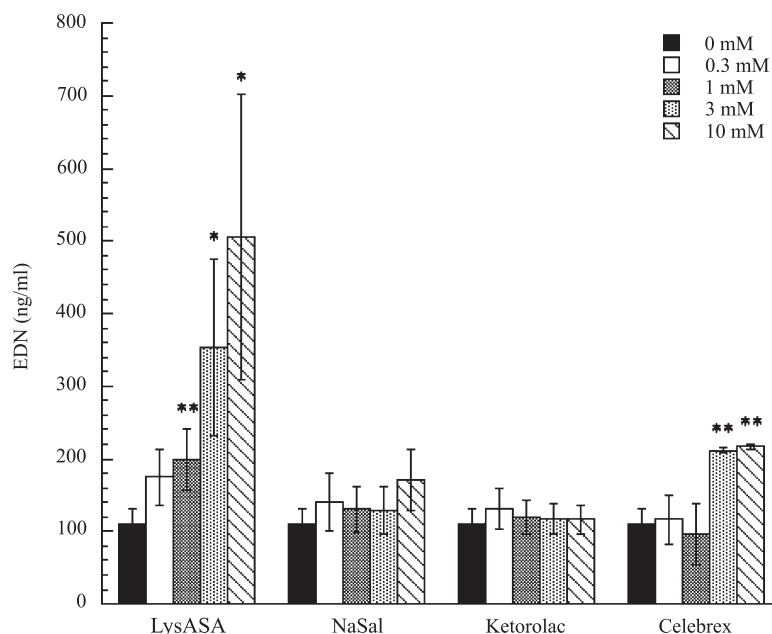


FIGURE 2. Ca²⁺ flux of NSAID-stimulated eosinophils. Calcium mobilization assays were performed on eosinophils by plating 125,000 cells per well and incubating the cells with Fluo-4 NW wash dye mix supplemented with 5 mM probenecid for 1 h. (A) Dose–response curves were generated by incubation of the cells; increasing concentrations of LysASA, NaSal, or ketorolac (0.3–10 mM); and measuring cytoplasmic calcium flux. ATP was used as a positive control. Data are presented as the mean $\pm$ SEM of separate studies performed in triplicate ($n = 9$ LysASA and ketorolac, $n = 6$ NaSal: * $p < 0.05$, compared with unstimulated control). (B) Dose–response curves were generated by incubation of the cells with increasing concentrations of LysASA (0.3–10 mM) in the presence or absence of 1 mM EGTA and measuring cytoplasmic Ca²⁺ flux.

FIGURE 3. Activation of peripheral blood eosinophils by NSAIDs. Eosinophils were purified from peripheral blood and stimulated in a dose-dependent fashion with LysASA, NaSal, ketorolac, or celecoxib (0.3–10 mM) for 30 min. EDN was measured in supernatants and measured in nanograms per milliliter. Data are presented as the mean $\pm$ SEM of 4–13 separate studies. * p < 0.05, ** p < 0.01, compared with unstimulated cells.



importantly, the concomitant presence of IFN- γ led to significant induction of the capacity of these eosinophils to secrete CysLTs (Fig. 4B; p < 0.03 at the 1 and 10 mM concentrations).

Ketorolac activation

AERD subjects react to aspirin and other nonselective NSAIDs, including ketorolac (25, 26). We therefore interrogated the ability of this compound to activate CD34⁺ progenitor-derived eosinophils and, as with LysASA, observed significant CysLT release (Fig. 4B). The activation induced by ketorolac at the 10 mM concentration was more pronounced than that observed with LysASA (p < 0.002) and, as displayed in Fig. 4A, was further enhanced when the cells were matured with IFN- γ .

Aspirin activation of CD34⁺ progenitor-derived mast cells

Having demonstrated aspirin activation of eosinophils, we wanted to address whether similar activation pathways were induced in mast cells. As with eosinophils, we initially investigated the capacity of these compounds to drive Ca²⁺ fluxes in mast cells differentiated from CD34⁺ progenitor cells. Similar to the results for eosinophils, LysASA was able to significantly (p < 0.05) stimulate Ca²⁺ flux (Fig. 5A). Although ketorolac induced modest Ca²⁺ fluxes, the results were not significantly different from those with unstimulated cells (Fig. 5A). Because aspirin/NSAID reactions in AERD are associated with PGD₂ release, PGD₂ secretion was measured after CD34⁺-derived mast cells were stimulated with LysASA. Similar to the Ca²⁺ flux data, PGD₂

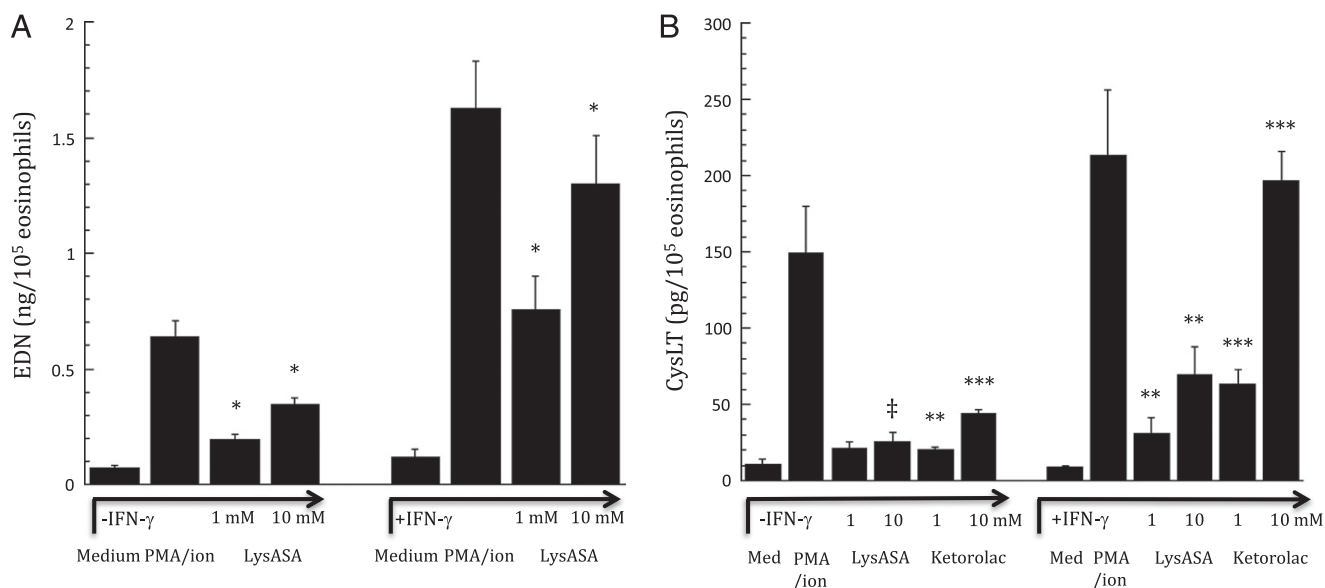


FIGURE 4. Activation of eosinophils differentiated from hematopoietic precursors in the additional presence of IFN- γ . CD34⁺-enriched hematopoietic progenitor cells were cultured for 3 d with SCF, thymopoietin, Fms-like tyrosine kinase 3 ligand, IL-3, and IL-5, after which they were cultured for 3 wk more with just IL-3 and IL-5, with or without the additional presence of IFN- γ . Eosinophils matured from CD34⁺ progenitors were activated with or without calcium ionophore/PMA, LysASA (1 and 10 mM), or ketorolac (1 and 10 mM) for 30 min. Supernatants were collected and (A) EDN and (B) CysLT levels were quantified and measured in nanograms per milliliter or picograms per milliliter, respectively. Data are presented as the mean $\pm$ SEM of a minimum of six separate studies. $^{\dagger}p$ = 0.05, * p < 0.0004, ** p < 0.03, *** p < 0.002, compared with unstimulated cells.

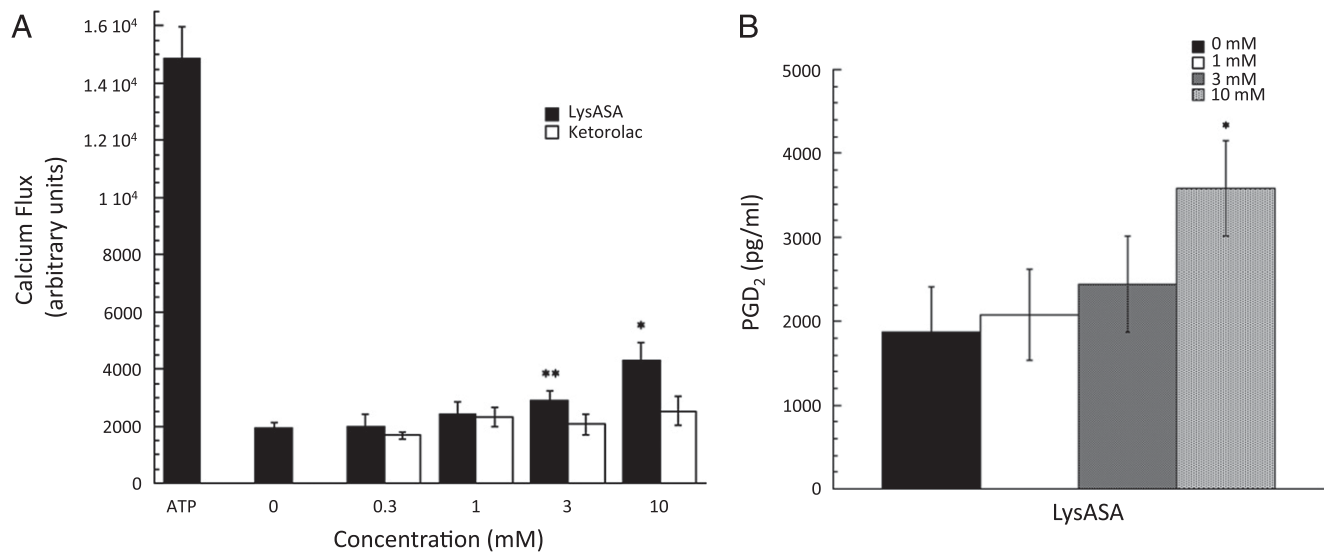


FIGURE 5. Activation of CD34-differentiated mast cells. **(A)** Calcium mobilization assays were performed on CD34-derived mast cells as described. Dose-response curves were generated by incubation of the cells and increasing concentrations of LysASA or ketorolac (0.3–10 mM). ATP was used as a positive control. Data are presented as the mean $\pm$ SEM of three separate studies performed in triplicate (* $p < 0.05$, ** $p = 0.08$). **(B)** CD34-derived mast cells were stimulated with LysASA (0.3–10 mM) for 30 min. PGD₂ release was measured in supernatants as picograms per milliliter. Data are presented as the mean $\pm$ SEM of 10 studies ($p < 0.03$).

release was observed at the highest concentration of LysASA (Fig. 5B; $p < 0.05$).

Discussion

AERD is characterized by eosinophilic infiltration into the upper (sinus and nasal polyp) and lower (bronchial) airway that is markedly greater than that observed in aspirin-tolerant asthmatics (13–15), and these eosinophils are individually characterized by much higher expression of the rate-limiting enzyme permitting CysLT synthesis, LTC₄S (8). As a result, when eosinophils are activated after ingestion of aspirin or other nonselective inhibitors of COX, a surge in CysLT secretion occurs (27, 28). CysLT secretion is further enhanced by the distinct tendency of platelets to adhere to eosinophils and neutrophils in AERD and the transcellular conversion of LTA₄ into CysLTs by the platelets (10). CysLTs underlie much of the symptomatic component of these anaphylactoid reactions, as demonstrated by the ability of leukotriene modifiers to greatly attenuate their presence and severity (15, 29, 30). Although many cell types are capable and likely contribute to the CysLT production and inflammatory response in AERD, these studies focused on the contribution of these products by eosinophils and mast cells.

The stimulus driving eosinophil and mast cell activation is not known. In part, these reactions reflect the ability of PGE₂ acting through the anti-inflammatory receptor EP2 to constrain cellular activation. Thus, coadministration of synthetic PGE₂ prevents the bronchospastic response to aspirin challenge (6). This explanation, however, is incomplete. If a low PGE₂ milieu were the only condition required for eosinophil activation to occur, then eosinophil activation would also occur in all asthmatics and even in nonasthmatics, albeit in the relative absence of CysLT generation. This observation suggests the concomitant need for a positive signal driving eosinophil activation. Aspirin and other NSAIDs have numerous off-target effects, independent of their ability to inhibit COX (20). Recently, as an example, aspirin was demonstrated to engage an L-type Ca²⁺ channel to promote cellular activation (21). We therefore posited that aspirin itself is responsible for cellular activation in AERD.

With the use of peripheral blood eosinophils, induction of Ca²⁺ flux was measured following stimulation with LysASA (Fig. 1). In a dose-dependent manner, LysASA generated a Ca²⁺ flux, with a maximal response observed at 10 mM, but significant changes also observed at pharmacologically meaningful concentrations. When aspirin-tolerant and aspirin-sensitive circulating eosinophils were compared, no difference was found in their responses to LysASA stimulation. Similar results were observed when we examined EDN secretion in response to LysASA (Fig. 3). However, in addition to observing activation with control subjects, many other features of this LysASA-induced activation of circulating eosinophils were inconsistent with what is observed in AERD subjects, including especially the absence of CysLT production, but also the failure to observe activation with ketorolac.

This induction of Ca²⁺ fluxes and EDN secretion by LysASA acting on eosinophils derived from control subjects was unexpected. Numerous potential explanations exist for the failure to observe—even modest—hypersensitivity reactions in aspirin-tolerant asthmatics and healthy controls. To some extent, this reflects the relative dearth of eosinophils in these other conditions with several studies, including ours (13, 15), demonstrating >10-fold fewer tissue eosinophils, and as such, the impact of eosinophil activation would be proportionately less. However, the more likely explanation is the relative impact of COX inhibitors in these conditions. PGE₂ acts through the anti-inflammatory EP2 receptor to mediate its protective responses (6, 31). In AERD, the PGE₂ synthetic pathway is suppressed, and both constitutive tissue PGE₂ concentrations and expression of the anti-inflammatory PGE₂ receptor EP2 are diminished (15, 32, 33). Thus, AERD patients exist on the precipice of cellular activation with their borderline expression of PGE₂ and EP2 such that even a modest decrease in PGE₂ can have the observed catastrophic effects. This model is consistent with recent observations that in a murine model of AERD, knockout of the rate-limiting enzyme responsible for PGE₂ synthesis, specifically murine PGES-1, renders these mice susceptible to anaphylactoid reactions after exposure to aspirin (34).

A further explanation for lack of reactivity in control cohorts is that a large component of the syndrome induced by aspirin is driven

by CysLTs, as shown by the ability of LT modifiers to greatly attenuate the severity of reactions (29, 35). Of interest, we did not observe CysLT secretion from eosinophils even when derived from the circulation of AERD subjects. We recently demonstrated that AERD is characterized by a high tissue IFN- γ milieu and, in contrast to the Th2 signature cytokines (IL-3, IL-4, IL-5, IL-13, and GM-CSF), IFN- γ was uniquely capable of driving the expression of LTC₄S and the secretion of CysLTs from eosinophils differentiated from CD34⁺ hematopoietic progenitor cells (17). AERD sinonasal and lung tissue is characterized by high numbers of eosinophilic hematopoietic progenitor (CD34⁺IL-5R α ⁺) cells (18, 19) that will mature in this presence of IFN- γ and acquire the ability to produce CysLTs; this observation would suggest that the robust CysLT production would be limited to airway—and not circulating—eosinophils. In the current studies, we therefore investigated whether eosinophils differentiated from progenitor cells in the presence of IFN- γ would recapitulate the sensitivity to aspirin displayed by tissue eosinophils in vivo in AERD. Not only was EDN release increased in IFN- γ -stimulated eosinophil progenitors (Fig. 4A), but consistent with their increased LTC₄S expression, CysLT secretion was thereby also detected upon LysASA activation (Fig. 4B). Importantly, activation was again also significant at lower, pharmacologically relevant, concentrations of LysASA.

Having observed activation by LysASA, we tested the ability of other NSAIDs to activate eosinophils. The absence of effects by NaSal on eosinophils on both Ca²⁺ flux (Fig. 2A) and EDN release (Fig. 3) indicates that the action by LysASA is not being mediated by the salicylate component (20), a result consistent with the ability of AERD patients to tolerate salicylates (36). In contrast, AERD patients are variably sensitive to nonselective NSAIDs capable of COX inhibition (37), including ketorolac (25, 26). In contrast to circulating eosinophils, ketorolac was able to activate newly differentiated eosinophils to stimulate CysLT release, especially in the presence of IFN- γ (Fig. 4B). Thus, IFN- γ exposure fully recapitulates the AERD phenotype by rendering these eosinophils responsive to ketorolac.

Finally, we addressed the ability of LysASA to also activate mast cells. Although less prevalent (8, 12, 13), studies examining mediator release following aspirin challenge in AERD identified histamine, tryptase, and PGD₂ generation demonstrating mast cell involvement (16, 38, 39). Using mast cells generated from CD34⁺ progenitors, we observed a dose-dependent increase in Ca²⁺ flux, similar to that observed with eosinophils, when the cells were stimulated with LysASA and a slight but nonsignificant increase with ketorolac (Fig. 5A). In addition, PGD₂ secretion was increased after stimulation with LysASA (Fig. 5B). Again, it was surprising that ketorolac failed to elicit a more robust response given its ability to cause reactions when administered to AERD subjects. This, again, may reflect the inherent variability in sensitivity to NSAIDs and that, as with eosinophils, the mast cells need to be matured in the presence of an additional agent, such as IFN- γ , to render the cells fully responsive to ketorolac.

In summary, our data demonstrate the ability of aspirin to directly activate eosinophils and mast cells, causing the release of inflammatory mediators. Further, the concomitant presence of high sinonasal and asthma tissue concentrations of IFN- γ with infiltrating eosinophil progenitors in AERD subjects, along with the current study's demonstration of the ability of IFN- γ to "sensitize" the eosinophil toward CysLT release on activation, explains part of the surge of CysLT production and release seen on exposure to aspirin. Although this activation can occur in aspirin-tolerant asthmatics and nonasthmatics, it is constrained in these subjects by their continued expression of sufficient PGE₂ to inhibit cellular

activation acting through their much higher expression of the anti-inflammatory PGE₂ receptor EP₂.

Disclosures

The authors have no financial conflicts of interest.

References

- Steinke, J.W., S.C. Payne, and L. Borish. 2012. Interleukin-4 in the generation of the AERD phenotype: implications for molecular mechanisms driving therapeutic benefit of aspirin desensitization. *J. Allergy*. DOI: 10.1155/2012/182090.
- Mascia, K., L. Borish, J. Patrie, J. Hunt, C. D. Phillips, and J. W. Steinke. 2005. Chronic hyperplastic eosinophilic sinusitis as a predictor of aspirin-exacerbated respiratory disease. *Ann. Allergy Asthma Immunol.* 94: 652–657.
- Szczeklik, A., and D. D. Stevenson. 1999. Aspirin-induced asthma: advances in pathogenesis and management. *J. Allergy Clin. Immunol.* 104: 5–13.
- Szczeklik, A., and E. Nizankowska. 2000. Clinical features and diagnosis of aspirin induced asthma. *Thorax* 55(Suppl 2): S42–S44.
- Berges-Gimeno, M. P., R. A. Simon, and D. D. Stevenson. 2002. The natural history and clinical characteristics of aspirin-exacerbated respiratory disease. *Ann. Allergy Asthma Immunol.* 89: 474–478.
- Sestini, P., L. Armetti, G. Gambaro, M. G. Pieroni, R. M. Refini, A. Sala, A. Vaghi, G. C. Polco, S. Bianco, and M. Robuschi. 1996. Inhaled PGE₂ prevents aspirin-induced bronchoconstriction and urinary LTE₄ excretion in aspirin-sensitive asthma. *Am. J. Respir. Crit. Care Med.* 153: 572–575.
- Sladek, K., and A. Szczeklik. 1993. Cysteinyl leukotrienes overproduction and mast cell activation in aspirin-provoked bronchospasm in asthma. *Eur. Respir. J.* 6: 391–399.
- Cowburn, A. S., K. Sladek, J. Soja, L. Adamek, E. Nizankowska, A. Szczeklik, B. K. Lam, J. F. Penrose, F. K. Austen, S. T. Holgate, and A. P. Sampson. 1998. Overexpression of leukotriene C₄ synthase in bronchial biopsies from patients with aspirin-intolerant asthma. *J. Clin. Invest.* 101: 834–846.
- Daffern, P. J., D. Muilenburg, T. E. Hugli, and D. D. Stevenson. 1999. Association of urinary leukotriene E₄ excretion during aspirin challenges with severity of respiratory responses. *J. Allergy Clin. Immunol.* 104: 559–564.
- Laidlaw, T. M., M. S. Kidder, N. Bhattacharyya, W. Xing, S. Shen, G. L. Milne, M. C. Castells, H. Chhay, and J. A. Boyce. 2012. Cysteinyl leukotriene overproduction in aspirin-exacerbated respiratory disease is driven by platelet-adherent leukocytes. *Blood* 119: 3790–3798.
- Dahlén, B., E. Nizankowska, A. Szczeklik, O. Zetterström, G. Bochenek, M. Kumlin, L. Mastalerz, G. Pinis, L. J. Swanson, T. I. Boodhoo, et al. 1998. Benefits from adding the 5-lipoxygenase inhibitor zileuton to conventional therapy in aspirin-intolerant asthmatics. *Am. J. Respir. Crit. Care Med.* 157: 1187–1194.
- Kamil, A., O. Ghaffar, F. Lavigne, R. Taha, P. M. Renzi, and Q. Hamid. 1998. Comparison of inflammatory cell profile and Th2 cytokine expression in the ethmoid sinuses, maxillary sinuses, and turbinates of atopic subjects with chronic sinusitis. *Otolaryngol. Head Neck Surg.* 118: 804–809.
- Payne, S. C., S. B. Early, P. Huyett, J. K. Han, L. Borish, and J. W. Steinke. 2011. Evidence for distinct histologic profile of nasal polyps with and without eosinophilia. *Laryngoscope* 121: 2262–2267.
- Bachert, C., M. Wagenmann, U. Hauser, and C. Rudack. 1997. IL-5 synthesis is upregulated in human nasal polyp tissue. *J. Allergy Clin. Immunol.* 99: 837–842.
- Pérez-Novo, C. A., J. B. Watelet, C. Claeys, P. Van Cauwenberge, and C. Bachert. 2005. Prostaglandin, leukotriene, and lipoxin balance in chronic rhinosinusitis with and without nasal polyposis. *J. Allergy Clin. Immunol.* 115: 1189–1196.
- Fischer, A. R., M. A. Rosenberg, C. M. Lilly, J. C. Callery, P. Rubin, J. Cohn, M. V. White, Y. Igarashi, M. A. Kaliner, J. M. Drazen, and E. Israel. 1994. Direct evidence for a role of the mast cell in the nasal response to aspirin in aspirin-sensitive asthma. *J. Allergy Clin. Immunol.* 94: 1046–1056.
- Steinke, J. W., L. Liu, P. Huyett, J. Negri, S. C. Payne, and L. Borish. 2013. Prominent role of interferon- γ in aspirin-exacerbated respiratory disease. *J. Allergy Clin. Immunol.* 132: 856–865.e1–3.
- Kim, Y. K., M. Uno, D. L. Hamilos, L. Beck, B. Bochner, R. P. Schleimer, and J. A. Denburg. 1999. Immunolocalization of CD34 in nasal polyposis. Effect of topical corticosteroids. *Am. J. Respir. Cell Mol. Biol.* 20: 388–397.
- Denburg, J. A. 2000. Haemopoietic mechanisms in nasal polyposis and asthma. *Thorax* 55(Suppl 2): S24–S25.
- Tegeger, I., J. Pfeilschifter, and G. Geisslinger. 2001. Cyclooxygenase-independent actions of cyclooxygenase inhibitors. *FASEB J.* 15: 2057–2072.
- Suzuki, Y., and C. Ra. 2009. Analysis of the mechanism for the development of allergic skin inflammation and the application for its treatment: aspirin modulation of IgE-dependent mast cell activation: role of aspirin-induced exacerbation of immediate allergy. *J. Pharmacol. Sci.* 110: 237–244.
- Hudson, S. A., H. Herrmann, J. Du, P. Cox, el-B. Haddad, B. Butler, P. R. Crocker, S. J. Ackerman, P. Valent, and B. S. Bochner. 2011. Developmental, malignancy-related, and cross-species analysis of eosinophil, mast cell, and basophil siglec-8 expression. *J. Clin. Immunol.* 31: 1045–1053.
- Kirshenbaum, A. S., J. P. Goff, T. Semere, B. Foster, L. M. Scott, and D. D. Metcalfe. 1999. Demonstration that human mast cells arise from a progenitor cell population that is CD34(+), c-kit(+), and expresses aminopeptidase N (CD13). *Blood* 94: 2333–2342.
- Laidlaw, T. M., J. W. Steinke, A. M. Tinana, C. Feng, W. Xing, B. K. Lam, S. Paruchuri, J. A. Boyce, and L. Borish. 2011. Characterization of a novel

- human mast cell line that responds to stem cell factor and expresses functional FcεRI. *J. Allergy Clin. Immunol.* 127: 815–822 e811–815.
25. White, A., T. Bigby, and D. Stevenson. 2006. Intranasal ketorolac challenge for the diagnosis of aspirin-exacerbated respiratory disease. *Ann. Allergy Asthma Immunol.* 97: 190–195.
 26. Lee, R. U., A. A. White, D. Ding, A. B. Dursun, K. M. Woessner, R. A. Simon, and D. D. Stevenson. 2010. Use of intranasal ketorolac and modified oral aspirin challenge for desensitization of aspirin-exacerbated respiratory disease. *Ann. Allergy Asthma Immunol.* 105: 130–135.
 27. Christie, P. E., P. Tagari, A. W. Ford-Hutchinson, S. Charlesson, P. Chee, J. P. Arm, and T. H. Lee. 1991. Urinary leukotriene E4 concentrations increase after aspirin challenge in aspirin-sensitive asthmatic subjects. *Am. Rev. Respir. Dis.* 143: 1025–1029.
 28. Szczeklik, A., K. Sladek, R. Dworski, E. Nizankowska, J. Soja, J. Sheller, and J. Oates. 1996. Bronchial aspirin challenge causes specific eicosanoid response in aspirin-sensitive asthmatics. *Am. J. Respir. Crit. Care Med.* 154: 1608–1614.
 29. Dahlén, B. 2000. Treatment of aspirin-intolerant asthma with antileukotrienes. *Am. J. Respir. Crit. Care Med.* 161: S137–S141.
 30. Steinke, J. W., D. Bradley, P. Arango, C. D. Crouse, H. Frierson, S. E. Kountakis, M. Kraft, and L. Borish. 2003. Cysteinyl leukotriene expression in chronic hyperplastic sinusitis-nasal polyposis: importance to eosinophilia and asthma. *J. Allergy Clin. Immunol.* 111: 342–349.
 31. Steinke, J. W. 2012. Editorial: yin-yang of EP receptor expression. *J. Leukoc. Biol.* 92: 1129–1131.
 32. Kowalski, M. L., R. Pawliczak, J. Wozniak, K. Siuda, M. Poniatowska, J. Iwaszkiewicz, T. Kornatowski, and M. A. Kaliner. 2000. Differential metabolism of arachidonic acid in nasal polyp epithelial cells cultured from aspirin-sensitive and aspirin-tolerant patients. *Am. J. Respir. Crit. Care Med.* 161: 391–398.
 33. Ying, S., Q. Meng, G. Scadding, A. Parikh, C. J. Corrigan, and T. H. Lee. 2006. Aspirin-sensitive rhinosinusitis is associated with reduced E-prostanoid 2 receptor expression on nasal mucosal inflammatory cells. *J. Allergy Clin. Immunol.* 117: 312–318.
 34. Liu, T., T. M. Laidlaw, H. R. Katz, and J. A. Boyce. 2013. Prostaglandin E2 deficiency causes a phenotype of aspirin sensitivity that depends on platelets and cysteinyl leukotrienes. *Proc. Natl. Acad. Sci. USA* 110: 16987–16992.
 35. Christie, P. E., C. M. Smith, and T. H. Lee. 1991. The potent and selective sulfidopeptide leukotriene antagonist, SK&F 104353, inhibits aspirin-induced asthma. *Am. Rev. Respir. Dis.* 144: 957–958.
 36. Stevenson, D. D., A. J. Hougham, P. J. Schrank, M. B. Goldlust, and R. R. Wilson. 1990. Salsalate cross-sensitivity in aspirin-sensitive patients with asthma. *J. Allergy Clin. Immunol.* 86: 749–758.
 37. Mathison, D. A., and D. D. Stevenson. 1979. Hypersensitivity to nonsteroidal antiinflammatory drugs: indications and methods for oral challenges. *J. Allergy Clin. Immunol.* 64: 669–674.
 38. Higashi, N., M. Taniguchi, H. Mita, M. Osame, and K. Akiyama. 2002. A comparative study of eicosanoid concentrations in sputum and urine in patients with aspirin-intolerant asthma. *Clin. Exp. Allergy* 32: 1484–1490.
 39. Bochenek, G., K. Nagraba, E. Nizankowska, and A. Szczeklik. 2003. A controlled study of 9α,11β-PGF2 (a prostaglandin D2 metabolite) in plasma and urine of patients with bronchial asthma and healthy controls after aspirin challenge. *J. Allergy Clin. Immunol.* 111: 743–749.