Role of the 5-Lipoxygenase–activating Protein (FLAP) in Murine Acute Inflammatory Responses

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Summary

Leukotrienes are potent inflammatory mediators synthesized from arachidonic acid (AA) predominately by cells of myeloid origin. The synthesis of these lipids is believed to be dependent not only on the expression of the enzyme 5-lipoxygenase (5-LO), which catalyzes the first steps in the synthesis of leukotrienes, but also on expression of a nuclear membrane protein termed the 5-LO–activating protein (FLAP). To study the relationship of these two proteins in mediating the production of leukotrienes in vivo and to determine whether the membrane protein FLAP has additional functions in various inflammatory processes, we have generated a mouse line deficient in this protein. FLAP-deficient mice develop normally and are healthy. However, an array of assays comparing inflammatory reactions in FLAP-deficient mice and in normal controls revealed that FLAP plays a role in a subset of these reactions. Although examination of DTH and IgE-mediated passive anaphylaxis showed no difference between wild-type and FLAP-deficient animals, mice without FLAP possessed a blunted inflammatory response to topical AA and had increased resistance to platelet-activating factor–induced shock compared to controls. Also, edema associated with Zymosan A–induced peritonitis was markedly reduced in animals lacking FLAP. To determine whether these differences relate solely to a deficit in leukotriene production, or whether they reflect an additional role for FLAP in inflammation, we compared the FLAP-deficient mice to 5-LO–deficient animals. Evaluation of mice lacking FLAP and 5-LO indicated that production of leukotrienes during inflammatory responses is dependent upon the availability of FLAP and did not support additional functions for FLAP beyond its role in leukotriene production.

Leukotrienes are potent lipid mediators of inflammatory responses and have been implicated in the pathophysiology of both acute and chronic inflammatory diseases including asthma, arthritis, psoriasis, and inflammatory bowel disease (1–6). The first committed step in the production of all leukotrienes from arachidonic acid (AA)[1] is dependent on the expression of arachidonate 5-lipoxygenase (5-LO). AA is released from endogenous phospholipids in intact cells, presumably by the action of cytosolic AA-specific phospholipase A_2 (7–9). 5-LO then catalyses a two-step process involving oxygenation of AA to produce the intermediate 5-hydroperoxyeicosatetraenoic acid, followed by a dehydration step to produce the epoxide intermediate leukotriene A_4 (LTA_4). LTA_4 is either converted to leukotriene B_4 by the enzyme LTA_4 hydrolase, or is conjugated with glutathione to form the cysteinyl leukotriene compound LTC_4 (10, 11). LTB_4, synthesized predominantly by neutrophils, interacts with high-affinity G protein–coupled receptors which mediate a number of leukocyte functions. In particular, LTB_4 has been shown to be a potent chemotactic factor for neutrophils, to stimulate adhesion of leukocytes to vascular endothelia for extravasation into adjacent tissue, and to initiate neutrophil degranulation (12, 13). The peptidyl leukotrienes include LTC_4 and its metabolites LTD_4, and LTE_4, which are also known as the slow-reacting substance of anaphylaxis. These leukotrienes are secreted by eosinophils, mast cells, and macrophages, and potently stimulate bronchoconstriction and mucous secretion and cause vasodilation with increases in postcapillary venule permeability (1, 14).

[1]*Abbreviations used in this paper:* AA, arachidonic acid; DTH, delayed-type hypersensitivity; EIA, enzyme immunoassay; ES, embryonic stem; FLAP, 5-lipoxygenase–activating protein; HETE, hydroxyeicosatetraenoic acid; HSA, human serum albumin; LO, lipoxygenase; LT, leukotriene; MPO, myeloperoxidase; *neo*, neomysin resistance gene; PAF, platelet-activating factor; PG, prostaglandin; TX, thromboxane.

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In resting myeloid cells, 5-LO is found in the cytoplasm and the nucleus. When cells are stimulated to produce leukotrienes, 5-LO becomes associated with the nuclear envelope (15, 16). This translocation is believed to be related to the association of the enzyme with a protein termed the 5-lipoxygenase–activating protein (FLAP), located in the nuclear membrane. The gene encoding this 18-kD protein has been cloned (17). Based on its deduced amino acid sequence, FLAP has been hypothesized to consist of three transmembrane spanning regions with COOH and NH_2 termini on opposite sides of the membrane (18), and shows homology (31% amino acid identity, 53% similarity) with LTC_4 synthase, the enzyme responsible for peptidyl leukotriene production (19). Recent pharmacological studies identifying a new 30-kD protein with properties similar to FLAP confirm the existence of a FLAP/LTC_4 synthase gene family (20). The significance of this homology between the two proteins, however, is unknown. In contrast to the 5-LO enzyme, FLAP has been found to be associated with the membrane fraction in both resting and activated cells, and the protein has been localized to the cisternum of the nuclear envelope and to the associated endoplasmic reticulum (21). After activation, phospholipase A_2 appears to translocate to the nuclear fraction along with cellular 5-LO (15). Several lines of evidence have advanced a model for the initiation of leukotriene synthesis in which FLAP plays a critical, and perhaps essential, role in leukotriene production. This evidence includes studies of leukotriene synthesis in cells transfected with transgenes encoding 5-LO and FLAP (17), as well as studies of leukotriene inhibitors such as MK-886, which binds to FLAP and prevents 5-LO from metabolizing AA (22). These inhibitors can block leukotriene synthesis in intact cells but do not affect the catalytic activities of purified 5-LO. Current models suggest that FLAP is not involved directly in the recruitment of 5-LO to the nuclear envelope. Instead, by binding both AA and 5-LO, FLAP produces, in effect, a high local concentration of the substrate on which the enzyme can act (23).

Leukotrienes are synthesized primarily by cells of myeloid lineage. As predicted by the unique cooperation required between FLAP and 5-LO in leukotriene production, *Flap* expression has been detected in all cells where *5-lo* mRNA is present and leukotrienes are made. *Flap* expression is highest in neutrophils, but is also present in significant quantities in mast cells, eosinophils, and monocyte/macrophages (15, 22, 24–26). Quantitative comparison of *Flap* mRNA in these cell types is complicated by the fact that each has been determined in different experiments by different investigators. *Flap* and *5-lo* messages have also been detected at lower levels in B cell lines (24, 27–29) and in extrahematopoietic cells types including keratinocytes (30, 31), colonic epithelia (32), and pancreas (33). The cloning of the *Flap* and *5-lo* genes, however, has shown that their promoters do not appear to share similar control elements, which is surprising considering the unique relationship of the two proteins (34, 35). The independent regulation of these genes is further supported by the different expression patterns of *5-lo* and *Flap* in a subset of cell types and under different stimuli (27, 35). *Flap* expression in the absence of *5-lo* has been observed in several T cells lines (8, 28, 29), in the Mono Mac 6 cell line, and in U-937 cells (29). This fact, along with its unusual cellular location and high levels of expression, has led to speculation that FLAP may have other activities in addition to leukotriene synthesis. To examine this possibility and to define the role that FLAP plays in the control of leukotriene synthesis during inflammatory responses in vivo, we generated FLAP-deficient mice, and we compared the phenotype of these mice with that of mice lacking the 5-LO enzyme.

Materials and Methods

Isolation of the Murine Flap Gene. A 680-bp fragment of rat *Flap* cDNA (17) was used as a probe to screen a genomic DNA library in the Lambda FIX II vector generated from the 129 mouse strain (Stratagene Corp., La Jolla, CA). Five positive clones were obtained. One of these genomic clones, *Flap* 221, was isolated and purified to homogeneity and the insert was subcloned into the NotI site of pBluescript II SK+ (Stratagene Corp.). Regions of the insert containing coding sequence were identified and sequenced by the dideoxy chain termination method (U.S. Biochemical, Cleveland, OH) to verify the identity of the clone and to further localize regions containing coding sequence.

Generation of FLAP-deficient Mice. A targeting vector for the *Flap* gene was constructed from the murine genomic clone. Fragments identical to their corresponding regions in the gene were inserted 5′ and 3′ of the neomycin resistance gene (*neo*) of pJNS2 (36) such that coding material would be replaced by *neo* after homologous recombination of the plasmid with the endogenous locus. This construct was electroporated into E14TG2a embryonic stem (ES) cells established from the 129/Ola mouse line (37), and colonies resistant to neomycin and ganciclovir were identified as described previously (36). Genomic DNA was isolated from doubly resistant ES cell lines, digested with EcoRV and analyzed by Southern blot using a 1.2-kb BamHI/NotI fragment downstream of the targeting construct as a probe (Fig. 1 *A*). Verified recombinant ES cell lines were microinjected into mouse blastocysts, and chimeric mice were generated. Chimeras were bred to either B6D2 or 129/SvEv mice, and offspring were genotyped by Southern blot analysis of tail DNA digested with EcoRV using the 1.2-kb probe described above. All animals used in our studies of inflammation, with the exception of those assayed for platelet-activating factor (PAF) survival, were offspring of chimeras crossed with 129/SvEv mice. Likewise, the 5-LO–deficient mice used as controls for these experiments were on the 129 genetic background.

Measurement of Eicosanoid Production from Peritoneal Macrophages. Macrophages were obtained by peritoneal lavage with 10 ml of DME supplemented with 20 mM Hepes buffer 3 d after mice received an intraperitoneal injection of 1 ml of 5% thioglycollate medium. Cells were cultured in 1.5 ml DME for 30 min. Supernatant was collected and replaced by medium containing A23187 Ca^{2+} ionophore at 5 μg/ml for an additional 30 min. A23187 has been shown to stimulate the release of eicosanoids from macrophages (38). Supernatant was collected, and the number of cells was verified by using a hexosaminidase assay, as is previously described (39). Concentrations of LTC_4, prostaglandin E_2 (PGE_2) and thromboxane B_2 (TXB_2) in the pre- and poststimulation supernatants were determined using enzyme immunoassay (EIA; Cayman Chemical Co. Inc., Ann Arbor, MI).

Flap mRNA Analysis. Macrophages from mice of the indicated genotypes were isolated by peritoneal lavage as described above. Total RNA was extracted from the cells using RNAzol B (Tel-Test, Inc., Friendswood, TX) according to the protocol supplied by the manufacturer and separated by gel electrophoresis (15–20 μg/lane). Northern blot analysis was carried out according to standard techniques using the 680-bp rat *Flap* cDNA (17) as a probe to detect expression of *Flap* mRNA. A probe for rat β-*actin* was used as a positive control. Densitometry was performed to detect any changes in *Flap* expression (ImageQuaNT v4.1 software; Molecular Dynamics, Sunnyvale, CA).

Inflammatory Responses Induced by AA. 129 strain *Flap* (−/−) and (+/+) animals were injected intravenously with 0.5% Evans blue dye (10 ml of dye solution/kg of body weight) dissolved in PBS (2.7 mM KCl, 1.2 mM KH_2PO_4, 138 mM NaCl, 8.1 mM Na_2HPO_4, pH 7.5) and filtered through a 0.2 μm nitrocellulose membrane. The inside of the left ear was painted with 20 μl of AA (100 mg/ml in acetone; Sigma Chemical Co., St. Louis, MO) to induce an inflammatory response, while the right ear received acetone alone on its inner surface. After a time period of 1–2.5 h for edema assays or 7 h for myeloperoxidase (MPO) analysis, mice were killed and an 8-mm-diam disc of tissue was obtained from the center of each ear for analysis. For comparison, experiments were carried out in parallel with 129 *5-lo* (−/−) mice.

Quantification of Edema and Vascular Permeability. The wet weight of each ear biopsy was determined to assess edema formation. Each ear disc was then incubated in 1 ml of formamide at 55°C for 24 h. Extravasation of Evans blue dye was quantified by spectrophotometric analysis of the formamide extracts at 610 nm (40).

MPO Assay. 8-mm-diam ear punches were homogenized and analyzed according to a protocol modified from Bradley et al. (41) which has been previously described (42). Lysate MPO was quantified by comparison to a standard curve derived from serial dilution of MPO standard (Calbiochem Corp., San Diego, CA).

Zymosan A–induced Peritoneal Inflammation. To induce peritoneal inflammation in *Flap* (−/−), *Flap* (+/+), and *5-lo* (−/−) animals, we used a protocol modified from Rao et al. (43). Zymosan A (Sigma Chemical Co.) was dissolved in warm (56°C) PBS (pH 7.5) to a final concentration of 1 mg/ml. The Zymosan A solution was mixed until it passed freely through the 30-gauge delivery needle. Each mouse received 1 ml Zymosan A solution by intraperitoneal injection. After 3 h, mice were euthanized and a peritoneal lavage was performed with 4 ml of ice-cold PBS. The lavage fluid was collected, transferred to microcentrifuge tubes, and centrifuged at 40,000 *g* for 10 min. Supernatants were collected and LTC_4 levels were determined by EIA (Cayman Chemical Co.). In related experiments, mice received 0.5% Evans blue dye (10 ml of dye solution/kg of body weight) in PBS intravenously immediately before injection of Zymosan A solution. Mice were euthanized after 10, 20, or 30 min and underwent peritoneal lavage with 4 ml of cold PBS as described above. Evans blue dye extravasation was assessed by light spectrophotometry at 610 nm.

Detection of 12–hydroxyeicosatetraenoic Acid Levels. Peritoneal inflammation was induced in FLAP-deficient and wild-type animals as described above. Lavage fluid was collected 30 min after Zymosan A delivery and was centrifuged. 12-hydroxyeicosatetraenoic acid (HETE) levels in the supernatants were determined by EIA (PerSeptive Diag., Framingham, MA).

Delayed-type Hypersensitivity Assay. A method adapted from that of Furue and Tamaki (44) was used to analyze the effect of leukotriene deficiency on the development of a delayed-type hypersensitivity (DTH) response. In brief, 129 strain *Flap* (−/−), *5-lo* (−/−), and (+/+) mice homozygous for the *p* and c^{ch} alleles of the 129/Ola line were sensitized by application of a 100 μl epicutaneous dose of 0.5% FITC (Sigma Chemical Co.) in 1:1 acetone/dibutyl phthalate to the abdomen. After 6 d, the ears of each animal were measured using calipers before eliciting a contact hypersensitivity reaction by topical application of 20 μl of 0.5% FITC to the left ear. The right ear of each mouse received vehicle alone. 24 h later, the ear thickness was again measured with calipers. Mice were then killed, and ears were collected and weighed as described above. Caliper measurements, as well as the differences in wet weight between experimental (left) and control (right) ears, were compared in mice of each genotype.

Passive Systemic IgE-induced Anaphylaxis. Passive systemic anaphylaxis was induced as previously described (36). Animals received 20 μg of a monoclonal mouse anti–mouse DNP IgE in 200 μl of PBS intravenously. After 24 h, baseline body temperatures were determined using a rectal probe, and mice received an intravenous injection of 1 mg DNP–human serum albumin (HSA) and 0.5% Evans blue in 200 μl PBS to induce anaphylaxis. 30 min after this injection, temperature changes were measured. Mice were then killed, and edema was quantitated in 8-mm-diam ear punches as described above.

PAF-induced Anaphylaxis. Mice received intravenous injections of 50 μg of PAF per kilogram of body weight in BSA at 2.5 mg/ml and propranolol at 0.05 mg/ml in saline. Survival was assessed for 24 h after the injection of PAF.

Results

Generation of FLAP-deficient Mice. The *Flap* targeting vector was constructed using a clone isolated from a library constructed with DNA from the 129 mouse strain (Fig. 1 *A*). Fragments derived from the mouse *Flap* clone were used to prepare a plasmid that, upon homologous recombination with the endogenous locus, would result in the disruption of the gene for this protein. The 800-bp XhoI/NheI fragment, which is replaced by the neomycin resistance cassette after homologous recombination (see Fig. 1 *A*), was subcloned and sequenced. The sequence analysis showed that a 130-bp exon which encodes amino acids 108–151 is eliminated in recombinant clones. These amino acids have been shown to be highly conserved between several species (86% identity with the human amino acid sequence), and their removal would result in the loss of the third putative transmembrane domain of the protein (18). Thus, even if splicing around the missing exon were to take place, a large and conserved portion of the transcript would be absent and an inactive protein would likely result.

The JNS2/*Flap*1 plasmid was electroporated into E14TG2a ES cells, and potential targeted clones were selected by growth in the presence of G418 and ganciclovir. Resistant colonies were picked, expanded, and screened for homologous recombination by Southern blot analysis of EcoRV digested genomic DNA using a 1.2-kb BamHI/NotI probe downstream of the targeting construct (Fig. 1 *A*). This probe hybridizes to a fragment of >16 kb from the endogenous *Flap* locus, while introduction of a new EcoRV site into the targeted locus results in a hybridizing fragment of 8 kb (Fig. 1 *B*). A targeting frequency of 34% was ob-

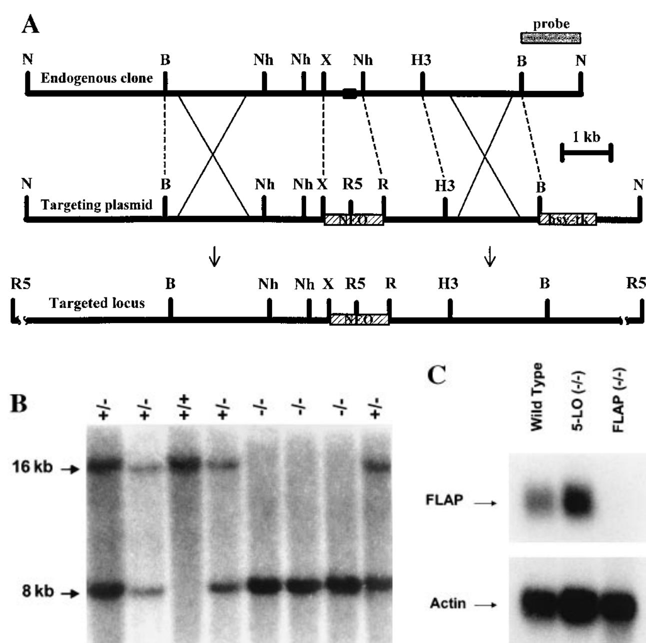

Figure 1. Targeting of the *Flap* gene. (*A*) Partial restriction enzyme maps of the targeting construct, the endogenous genomic clone, and the recombinant locus. Coding sequences replaced by homologous recombination are indicated by the filled black box. The hsv-tk and NEO selection cassettes are indicated as hatched boxes (*hsv*, herpes simplex virus; *tk*, thymidine kinase). Restriction enzyme sites are abbreviated: *N*, NotI; *B*, BamHI; *Nh*, NheI; *X*, XhoI; *R5*, EcoRV; *R*, RmaI; *H*, HindIII. The probe used to screen for homologous recombination by Southern blot analysis is indicated by the filled gray box. (*B*) Southern blot analysis of EcoRV-digested tail DNA from *Flap* (+/+), *Flap* (+/−), and *Flap* (−/−) mice hybridized with a 1.2-kb BamHI/NotI fragment downstream of the targeting construct's region of homology. (*C*) Northern blot analysis of total RNA isolated from peritoneal macrophages of *Flap* (+/+), *Flap* (−/−), and *5-lo* (−/−) mice hybridized with the rat FLAP cDNA. A rat β-actin cDNA probe was used as a positive control.

served with the JNS2/*Flap*1 construct. Microinjection of two verified recombinant ES cell lines, 160-9 and 160-37, into mouse blastocysts yielded chimeric animals which could transmit the recombinant *Flap* allele to their progeny. Each of these cell lines gave rise to mice homozygous for the *Flap* mutation in the F2 generation. *Flap* (−/−) mice were present in the litters in expected numbers based on Mendelian ratios for a heterozygous mating. This indicates that disruption of the *Flap* gene had no effect on the fetal survival or development of the animals. By simple observation, the FLAP-deficient mice were indistinguishable from their *Flap* (+/−) and *Flap* (+/+) littermates.

To verify that the predicted coding sequence had been deleted from the targeted *Flap* locus in these transgenic mice, DNA samples from tail biopsies of *Flap* (−/−), *Flap* (+/−), and *Flap* (+/+) mice were analyzed by Southern blot, using the 800-bp XhoI/NheI fragment replaced by *neo* in the recombinant locus. This probe hybridized to *Flap* (+/+) and *Flap* (+/−) DNA, but showed no hybridization to DNA from a *Flap* (−/−) animal, verifying the deletion of this critical coding region (data not shown).

Total RNA was isolated from peritoneal macrophages of *Flap* (−/−), *Flap* (+/+), and *5-lo* (−/−) mice. A Northern blot probed with the rat *Flap* cDNA (Fig. 1 *C*) shows that the murine *Flap* mRNA is present in the *Flap* (+/+) and *5-lo* (−/−) lanes, but there is no hybridization in the *Flap* (−/−) lanes. Because the full-length cDNA probe contains sequences in addition to those eliminated by targeting, these results support the interpretation that a critical region of the *Flap* gene has been deleted through recombination and that there is no stable message being synthesized.

Densitometry was performed on the autoradiograph in Fig. 1 *C*. Comparison of the integrated areas of the *Flap* mRNA bands relative to the levels of the β-*actin* mRNA indicated only a slight elevation in *Flap* expression in macrophages from *5-lo* (−/−) mice. In the (+/+) sample, *Flap* mRNA was 53.53% of β-*actin* mRNA levels which are an indicator of the amount of RNA loaded in a given lane. In the *5-lo* (−/−) lane, *Flap* mRNA was 59.87% of β-*actin* mRNA levels, or 1.12-fold of that measured in the wild type. Thus, the absence of 5-LO results in little difference in *Flap* expression.

Flap (−/−) Mice Do Not Produce Detectable Leukotrienes. Peritoneal macrophages collected from four *Flap* (−/−) mice and from three (+/+) littermates were stimulated with calcium ionophore to determine the ability of these cells to release prostaglandins, thromboxanes, and leukotrienes. After A23187 activation, a >200-fold increase in LTC_4 was detectable in supernatant from macrophages of wild-type mice, while no LTC_4 was detected in cells from *Flap* (−/−) animals (Fig. 2 *A*). The ability of the FLAP-deficient mice to produce prostaglandins and thromboxanes by the cyclooxygenase-dependent pathway remained intact (Fig. 2, *B* and *C*). Similar to results obtained with 5-LO–deficient mice (42), levels of PGE_2 and TXB_2 that were released from the *Flap* (−/−) macrophages after stimulation were greater than those released from their (+/+) counterparts. While both PGE_2 and TXB_2 production by FLAP-deficient mice was increased compared to controls, only the difference in prostaglandin production reached statistical significance ($P = 0.028$ and 0.078, respectively).

To determine whether a similar defect in leukotriene synthesis occurred in vivo, an experimental peritonitis was induced by the injection of Zymosan A into the peritoneal cavity of *Flap* (−/−), *5-lo* (−/−), and (+/+) mice. In wild-type mice, Zymosan A induced a significant stimulation of LTC_4 (1,580 ± 22 pg/ml). Consistent with the in vitro data (Fig. 2 *A*), however, no LTC_4 could be detected in either *Flap* (−/−) or *5-lo* (−/−) mice after Zymosan A injection.

This question was further addressed in the accompanying manuscript by performance of HPLC analysis of peritoneal lavage fluids after Zymosan A stimulation of *Flap* (−/−) and (+/+) animals on the DBA/1 lacJ genetic background. The chromatography verified that no leukotrienes are being synthesized by FLAP-deficient animals. Furthermore, these profiles failed to detect an increase in other AA metabolites of the 5-LO pathway (for example, 5-HETE and the nonenzymatic hydrolysis products of LTA_4) in the

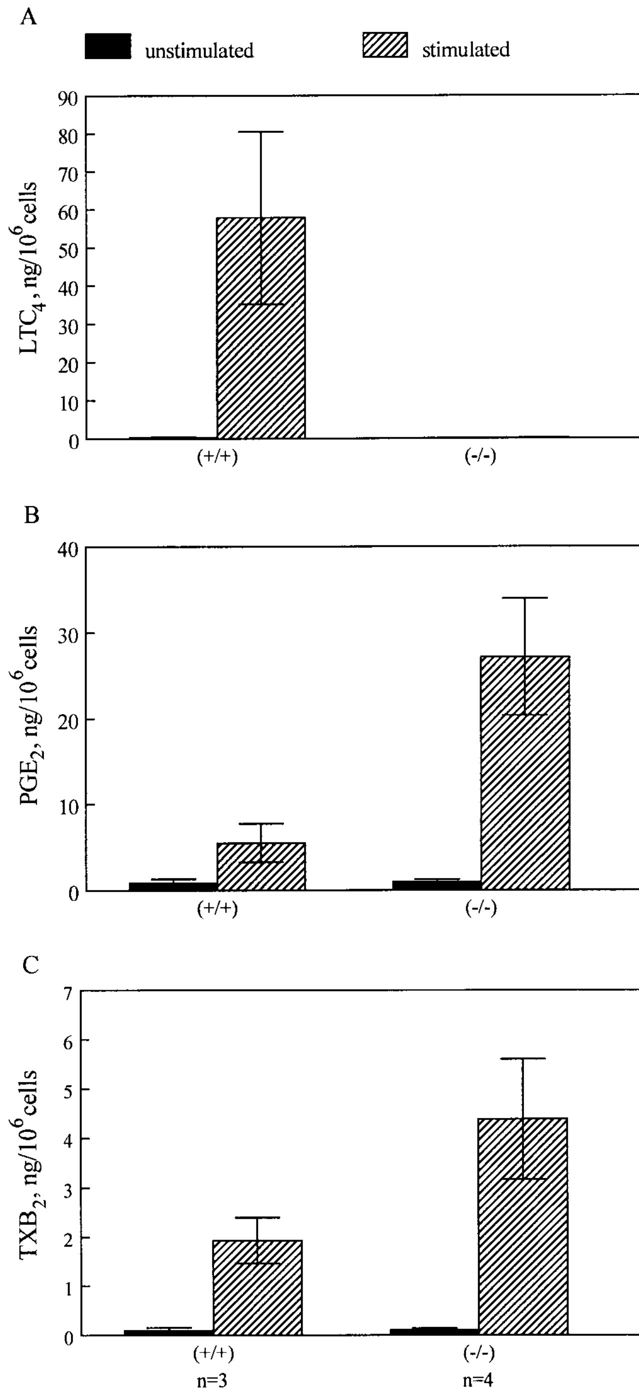

Figure 2. Ca^{2+} ionophore–induced eicosanoid production in *Flap* (+/+) and *Flap* (−/−) mice. Peritoneal macrophages from three *Flap* (+/+) and four *Flap* (−/−) mice were cultured in 1.5 ml tissue culture medium for 30 min. Medium was collected and replaced with medium containing Ca^{2+} ionophore A23187 (5 μg/ml). Supernatant was again collected 30 min after stimulation. LTC_4 (*A*), PGE_2 (*B*) and TXB_2 (*C*) levels in the prestimulation (*solid bars*) and poststimulation (*hatched bars*) supernatants were determined by EIA. After ionophore stimulation and supernatant collection, the number of adherent macrophages was determined by using a hexosaminidase assay. Eicosanoid levels were then normalized to reflect 10^6 macrophages. Error bars indicate SEM.

absence of FLAP. Surprisingly, a significant decrease in 12-HETE, a product of the 12-LO pathway, was observed in the DBA/1 lacJ *Flap* (−/−) mice. An ~54% reduction in 12-HETE compared to wild-type levels was verified in these mutant mice by EIA. We have seen a similar trend in an identical EIA for 12-HETE in 4 ml of stimulated peritoneal lavage fluid from four *Flap* (−/−) and three (+/+) mice on the 129 genetic background (50.25 ng/ml for FLAP-deficient mice versus 82.16 ng/ml in their normal controls). This reduction in 12-HETE levels, however, did not achieve statistical significance.

Flap (−/−) Mice Exhibit Decreased Vascular and Cellular Inflammatory Responses to AA. AA, when topically applied to the skin of the murine ear, causes an intense acute inflammatory reaction with both vascular and cellular components. Several lines of evidence support a role for eicosanoids in this inflammation, including the recent demonstration that this response is attenuated in 5-LO–deficient and prostaglandin H_2 synthase-1–deficient animals (42, 45, 46). To examine the role of FLAP in the edema and neutrophil recruitment characteristic of this response, the ears of *Flap* (−/−) and control mice were treated with 2 μg of AA. The ears were later biopsied, and edema was measured by determining the wet weight of each biopsy. In conjunction with the measurement of edema, alteration in plasma exudation was assessed using Evans blue dye. Since Evans blue binds to serum proteins (40), quantification of this dye in each ear biopsy gives a reliable indication of changes in vascular permeability. Edema and vascular permeability in the *Flap* (−/−) mice were compared to wild-type littermates.

As can be seen in Fig. 3 *A*, the increase in ear weight after AA treatment was significantly reduced in *Flap* (−/−) mice compared to controls ($P = 8.9 \times 10^{-9}$). To determine whether the changes seen in the FLAP-negative animals were due solely to leukotriene deficiency, *5-lo* (−/−) mice were examined in the same way. As reported, the AA-induced edema was significantly reduced in *5-lo* (−/−) animals compared to control mice ($P = 5.9 \times 10^{-16}$). Surprisingly, however, the reduction in edema in the *5-lo* (−/−) was greater than that of the *Flap* (−/−) animals ($P = 0.001$). The differences between these groups of mice were replicated in at least two experiments performed on independent days, with a total of 17 *5-lo* (−/−), 17 *Flap* (−/−), and 38 (+/+) animals examined.

Changes in vascular permeability and extravasation of serum proteins in response to topical AA followed a similar pattern for mice of these three genotypes (Fig. 3 *B*). Ears of wild-type mice displayed a deep blue color after topical AA treatment, reflecting leakage of serum proteins into the interstitial space, while *Flap* (−/−) mice and *5-lo* (−/−) mice showed a lighter blue color by observation and after quantitation ($P = 1.7 \times 10^{-7}$ and 3.5×10^{-12}, respectively). Again, the loss of *5-lo* gene function had a greater impact on edema formation than did the disruption of the gene for FLAP, and this relationship was consistent in at least two experiments performed on separate days with a total of 16 *5-lo* (−/−), 17 *Flap* (−/−), and 38 (+/+) ani-

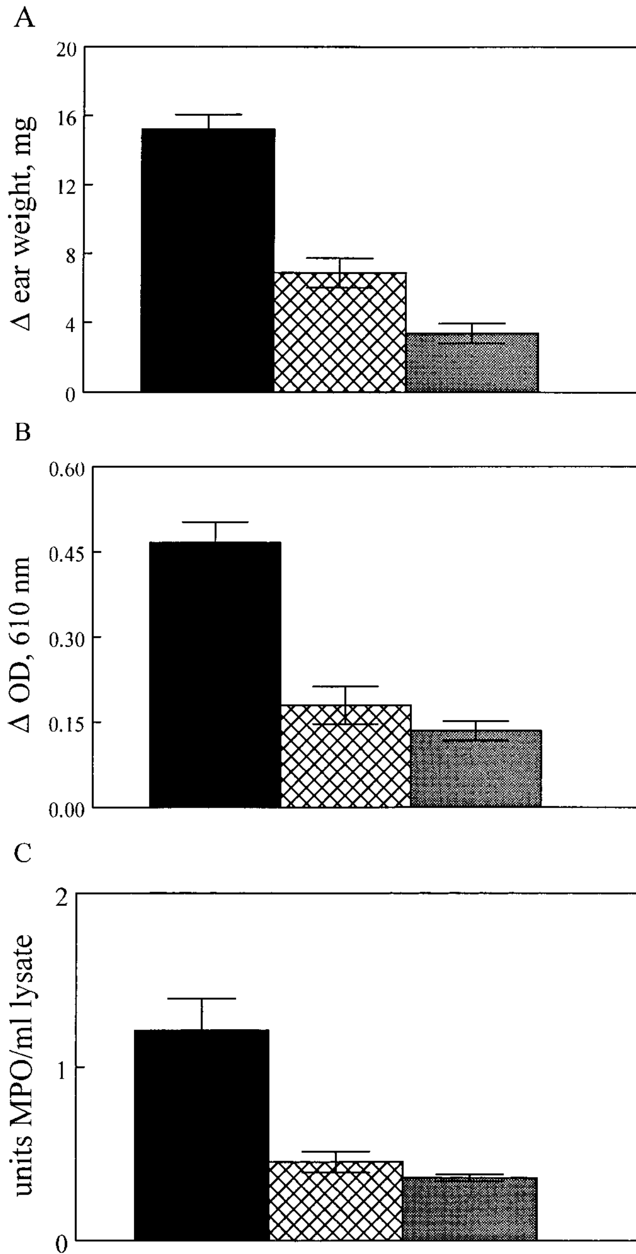

Figure 3. Acute inflammatory response to topical arachidonic acid in *Flap* (−/−), *5-lo* (−/−), and (+/+) animals. Arachidonic acid (2 mg) in acetone was applied to the left ear and acetone alone to the right ear of experimental mice. *Flap* (−/−) animals are indicated by hatched shading, *5-lo* (−/−) by grey shading, and (+/+) by solid shading. Mice analyzed for edema and plasma protein extravasation received 0.5% Evans blue dye intravenously and were killed after 1 h, whereas mice tested for neutrophil infiltration were killed after 7 h. 8-mm-diam discs were cut from each ear for analysis. (*A*) The wet weight difference between the treated and untreated ears of each animal was recorded as an indicator of tissue edema. $n = 17$ *Flap* (−/−), 17 *5-lo* (−/−), and 38 (+/+) mice. (*B*) Extravasation of plasma proteins into tissue was quantitated by extraction of dye with formamide and spectrophotometric analysis of extracts at 610 nm. The difference between the A_{610} for the left and right ears of each mouse was recorded. $n = 17$ *Flap* (−/−), 16 *5-lo* (−/−), and 38 (+/+) mice. (*C*) Neutrophil infiltration was assessed by measuring differences in MPO activity in lysates of experimental and control ear biopsies. MPO activity was determined by comparison with a standard curve of quantified MPO in an enzymatic assay. $n = 6$ *Flap* (−/−), 5 *5-lo* (−/−), and 12 (+/+) mice. Error bars indicate SEM.

mals. The difference between the two experimental groups was not as pronounced, however, as the difference in their ear weight described above ($P = 0.0445$).

In addition to their roles in edema formation and vascular changes, the leukotrienes are important in the establishment and maintenance of the cellular response to acute injury. LTB_4 exerts a potent chemotactic effect upon neutrophils, causing the adhesion of PMN to vascular endothelia and extravasation into the tissues in the acute inflammatory response. We therefore examined the ability of FLAP to affect the cellular infiltration elicited by topical AA. Levels of MPO, an enzyme specific for neutrophils, can be used as an indicator of the number of PMN present in inflamed tissue (38). The MPO level for experimental and control ears of mice of each genotype was determined by enzymatic assay 7 h after the topical AA treatment. The MPO content of unstimulated ears was uniformly undetectable by our assay for all mice tested. After AA stimulation, ear biopsies exhibited MPO activity, reflecting the recruitment of neutrophils to the treated tissue. Mean MPO activity for twelve 129 strain (+/+), six *Flap* (−/−), and five *5-lo* (−/−) mice is shown in Fig. 3 *C*. Neutrophil infiltrate was significantly decreased in the *Flap* (−/−) and *5-lo* (−/−) animals compared to the wild-type controls ($P = 10^{-5}$ and 7×10^{-6} respectively). These results were obtained in two separate experiments. In contrast to the assays of edema and vascular leakage, we observed no difference in MPO levels between FLAP– and 5-LO–deficient mice. Thus, in the *Flap* (−/−) animals we attribute reduction in recruitment of PMN to the inability of these mice to produce leukotrienes.

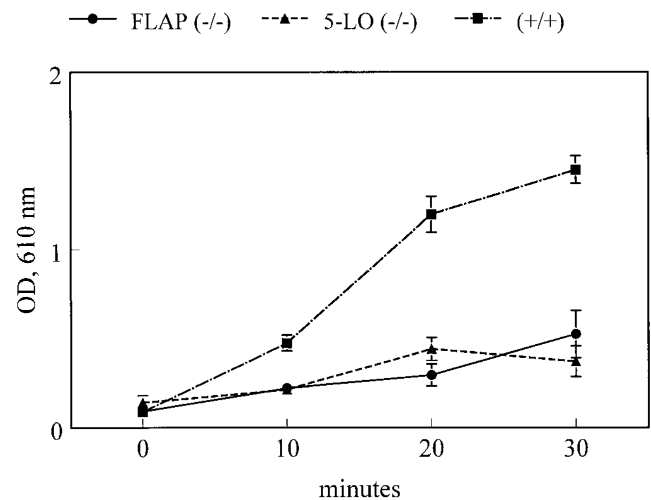

Figure 4. Leukotriene production and acute inflammation in experimental peritonitis. FLAP-deficient (*circles*), 5-LO–deficient (*triangles*), and wild-type (*squares*) mice received an intraperitoneal injection of 1 ml Zymosan A in PBS (1 mg/ml) to elicit peritoneal inflammation. To measure plasma protein influx, mice received 0.5% Evans blue dye intravenously before Zymosan A treatment. Mice were killed at time points of 10, 20, and 30 min, and peritoneal lavage was performed with 4 ml PBS. Cells were removed by centrifugation, and wash fluid was analyzed for Evans blue dye extravasation by light spectrophotometry at 610 nm. $n = 3$–8 mice at 0 min, 4–11 mice at 10 min, 5–10 mice at 20 min, and 4–11 mice at 30 min. Error bars indicate SEM.

Leukotriene-dependent Edema of Induced Peritonitis is Absent in Flap (−/−) Mice. To further explore the roles of FLAP, 5-LO, and leukotrienes in inflammatory edema, we evaluated plasma protein extravasation in response to another injurious stimulus, Zymosan A treatment. Peritoneal lavage was performed at 0, 10, 20, and 30 min time points, and the wash fluid was analyzed for Evans blue. As shown in Fig. 4, the absence of FLAP and 5-LO had similar effects on Evans blue extravasation after Zymosan A exposure. In two independent experiments, no difference was observed in the degree of inhibition conferred by FLAP or 5-LO deficiency.

Flap (−/−) Mice Exhibit a Normal DTH Response. Contact hypersensitivity to FITC is a form of DTH induced by topical application of antigen. It is initiated by T cells and can be quantitated by challenging the ears of sensitized animals and then measuring the swelling of the tissue after 24 h. Previous reports have suggested that leukotrienes may affect T cell cytokine production in vivo (47, 48). Production of leukotrienes by myeloid cells activated in this inflammatory response are likely to contribute further to the pathological changes of the DTH response. To examine a possible role for FLAP and/or leukotrienes in the DTH response, the ability of five *Flap* (−/−) and five *5-lo* (−/−) mice to mount a DTH response to cutaneous FITC in acetone/dibutyl phthalate was compared to that of nine wild-type animals. In wild-type mice, ear weight increased (14.12 ± 2.8 mg) after FITC challenge. Both FLAP– and 5-LO–deficient mice tended to exhibit a smaller increase in ear edema after similar treatment (Fig. 5). The differences between the three groups of mice, however, do not achieve statistical significance. FLAP-deficient animals showed a mean Δ ear weight of 10.42 ± 2.16 mg after treatment, whereas 5-LO–deficient mice had a Δ ear weight of 10.62 ± 2.93 mg (P = 0.16 and 0.23, respectively).

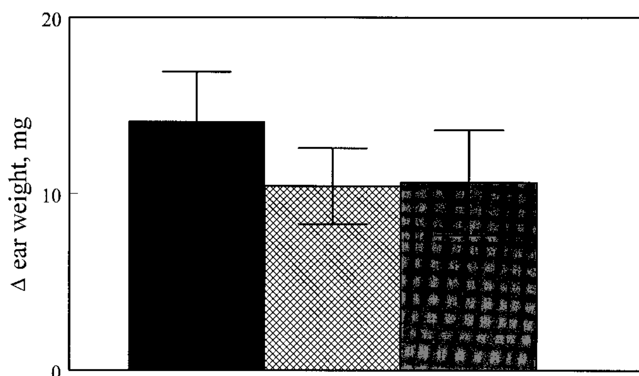

Figure 5. DTH reaction in *Flap* (−/−), *5-lo* (−/−), and (+/+) mice. Animals were sensitized with a 100 μl epicutaneous dose of 0.5% FITC in 1:1 acetone/dibutyl phthalate to the shaved abdomen. After 6 d, a hypersensitivity response was elicited by topical application of 20 μl of an identical FITC solution to the left ear. The right ear of each mouse received vehicle alone. 24 h later mice were killed, and 8-mm-diam discs were isolated from each ear. Wet weight differences between the left and right ears of each mouse were recorded as indicators of a DTH response. *Flap* (−/−) animals are indicated by hatched shading, *5-lo* (−/−) by grey shading, and (+/+) by solid shading. n = five *Flap* (−/−), five *5-lo* (−/−), and nine (+/+) mice. Error bars indicate SEM.

Flap (−/−) Mice Show Edema Formation and Temperature Change in a Passive Systemic IgE-dependent Anaphylaxis. Systemic IgE anaphylaxis in the mouse is characterized by bronchconstriction, plasma extravasation, hypotension with resultant tachycardia, and drop in body temperature. In the passive IgE-induced anaphylaxis model, these changes are dependent on binding of IgE to the high affinity IgE receptor, followed by mast cell degranulation (36). Leukotrienes, which are not stored in granules but are synthesized when the cells are activated, are among several proinflammatory mediators released by stimulated mast cells that can contrib-

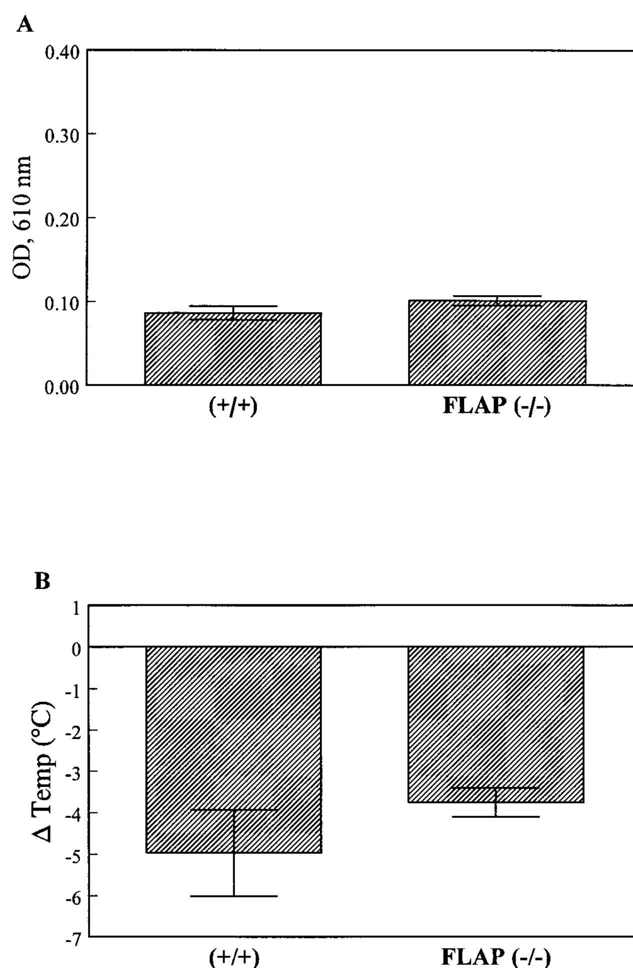

Figure 6. Passive systemic IgE-induced anaphylaxis. *Flap* (−/−) and (+/+) mice received 20 μg of a monoclonal mouse anti–mouse DNP IgE in 200 μl PBS intravenously. 24 h later basal body temperatures were recorded, and mice received an intravenous injection of 1 mg DNP-HSA and 0.5% Evans blue dye in 200 μl PBS to induce mast cell degranulation and anaphylaxis. Basal body temperature was again recorded 30 min after this injection. (*A*) Animals of the indicated genotypes were killed and 8-mm-diam ear discs were isolated. Extravasation of plasma proteins into tissue was quantitated by extraction of dye with formamide and spectrophotometric analysis of extracts at 610 nm. Dye leakage into both the left and right ears was recorded in this systemic response. (*B*) Change in basal body temperature for *Flap* (−/−) and (+/+) mice 30 min after treatment was recorded as another measure of systemic anaphylaxis. n = five *Flap* (−/−) and four (+/+) mice. Error bars indicate SEM.

ute to these physiological effects. To test the role of FLAP in the anaphylactic response, IgE-dependent passive systemic anaphylaxis was induced in five FLAP-deficient and four control mice. Mast cells of the animals were loaded with a monoclonal mouse anti–mouse DNP IgE. 24 h later DNP-HSA and Evans blue were administered. Body temperature was taken using a rectal probe before and 30 min after the DNP-HSA treatment. Finally, mice were killed, and edema was quantitated by measuring the amount of Evans blue dye in 8-mm-diam ear biopsies. *Flap* (−/−) mice averaged a 3.76 ± 0.35°C drop in body temperature after the induction of passive anaphylaxis that was less extreme than the 4.98 ± 1.04°C drop seen in the (+/+) mice (Fig. 6 *A*). This reduced response, however, was not statistically significant ($P = 0.174$). Likewise, the A_{610} of ear extracts from each group showed similar levels of Evans blue dye extravasation (Fig. 6 *B*). The mean A_{610} of extracts from the ears of FLAP-deficient mice was 0.1006 ± 0.0057. Wild-type mice edema levels reached $A_{610} = 0.0859 \pm 0.0082$, which again indicates no significant difference between *Flap* (−/−) and (+/+) animals ($P = 0.075$). Analogous results have been seen on examination of the 5-LO–deficient mice (data not shown).

Flap (−/−) Mice Are Resistant to PAF-induced Anaphylaxis. PAF is a potent endogenous mediator of inflammatory systemic anaphylaxis and shock, causing microvascular leakage, vasodilation, contraction of smooth muscle, endothelial adhesion, and activation of neutrophils, macrophages, and eosinophils. It is thought that many of the physiological changes that result from intravenous doses of PAF could be brought about by the PAF-mediated release of leukotrienes (42, 45, 49–52). Vulnerability of FLAP-deficient mice and their wild-type littermates to PAF-induced anaphalaxis was determined by treating 12 *Flap* (−/−) and 8 (+/+) animals with intravenous PAF. 75% (9 of 12) of *Flap* (−/−) animals survived this treatment, compared to only 12.5% (1 of 8) of the control group. Analysis of these results using the χ^2 test indicates a difference in susceptibility to PAF-induced anaphylaxis between the *Flap* (−/−) and (+/+) groups with a significance level of 0.0062. This difference is similar to that seen between the 5-LO–deficient and wild-type mice, as reported previously (42, 45). The protection from anaphylactic shock conferred by FLAP or 5-LO deficiency suggests a crucial role for leukotrienes in this process. While other inflammatory mediators are involved in the systemic anaphylactic response, none are able to compensate for the absence of the leukotrienes.

Discussion

FLAP was discovered by the identification of targets for agents that block leukotriene synthesis but have no effect on 5-LO enzyme activity (22). The protein has been shown to possess significant structural homology with LTC_4 synthase, another enzyme involved in the leukotriene synthetic pathway. In particular, the region of FLAP that is critical for the action of the inhibitory drug MK-886 is conserved in LTC_4 synthase, suggesting that the two proteins may also share some functional homology (19). Here we characterized the role of FLAP by examining the inflammatory responses of mice that are homozygous for a mutant allele of the *Flap* gene. To determine whether alterations in these responses are due solely to FLAP's facilitation of leukotriene synthesis, parallel experiments were performed with mice carrying a mutation in the *5-lo* gene. These mice, like the FLAP-deficient animals, were generated by introducing mutations into the genome of embryonic stem cells by homologous recombination (42).

No difference was observed in the ability of mice lacking FLAP to survive, nor was any alteration in the development of the various populations of myeloid and lymphoid cells apparent. An essential role for FLAP in the production of leukotrienes was suggested by in vitro studies and by work using drugs that were shown to bind specifically to FLAP (17, 22, 23). Here we show that leukotriene synthesis is undetectable in cells or whole animals that fail to express FLAP. This confirms the requirement of functional FLAP for normal production of leukotrienes. Also, the lack of leukotriene production in these mice indicates that the newly isolated 30-kD FLAP-2 (20), which shows homology to both FLAP and LTC_4 synthase, is not a functional complement of FLAP in AA metabolism. In addition, FLAP-deficient mice showed slight decreases in 12-HETE production in response to Zymosan A stimulus. Although we did not see a statistically significant difference in our experiments, examination of a larger group of mice in the accompanying paper confirmed this observation. It is possible, therefore, that whereas not absolutely required, FLAP facilitates the production of 12-HETE in the 12-LO pathway.

To further define the role of leukotrienes in acute inflammatory processes and to continue our exploration of potential additional functions for FLAP, we analyzed FLAP– and 5-LO–deficient mice in a number of experimental models of inflammation. We initially focused on vascular and cellular changes after topical AA administration. Attenuation of this response has been reported previously in mice deficient in the 5-LO protein (42, 45). In our own early studies, we found that edema was decreased in 5-LO–deficient mice, but the difference was not statistically significant. Much of the difficulty in achieving statistically significant differences between the two groups of animals stemmed from the considerable variability in the responses of both wild-type and 5-LO–deficient mice to AA. We reasoned that the inflammatory responses in these mice, which were 129 and B6D2 F2 offspring, were influenced by genetic factors in addition to the *5-lo* gene. To examine the roles of FLAP and 5-LO independent of these factors, the in vivo experiments described in this report (with the exception of PAF-induced anaphylaxis) were carried out on mice in which the mutated *5-lo* and *Flap* genes were maintained on the 129 background by breeding the chimeric animals (generated from 129/Ola ES cells) to 129/SvEv mice. When background variability was eliminated, a statistically significant reduction in mean edema was achieved between the 5-LO–deficient and control groups.

The edema and increased vasopermeability characteristic of AA-induced ear inflammation were reduced in the FLAP-deficient mice relative to control animals, but each remained significantly higher than that of the 5-LO–deficient animals. This was, notably, the only assay in which a reproducible and consistent difference was seen between the 5-LO– and FLAP–deficient mice. Even in this inflammatory response, significant differences were seen between these two groups only when inflammation was assessed by measuring changes in the weight of a constant area of tissue. Smaller changes were observed on evaluation of vasopermeability and cellular inflammation. One possible explanation for the difference in response between mice lacking 5-LO and FLAP in this particular assay may be that, in some circumstances of extremely high AA substrate, formation of leukotrienes is possible without the association of the enzyme with FLAP (17). This would be compatible with the finding that in vitro formation of leukotrienes from AA is possible in the absence of FLAP. Experiments that have examined the dependence of leukotriene synthesis on FLAP have shown that leukotriene production is stimulated by the treatment of cells with ionophore. Consistent with the results obtained here by stimulating FLAP-deficient peritoneal macrophages, leukotriene synthesis was found to be inhibited by drugs that interfere with the function of FLAP (23, 53). These results and our finding that FLAP-deficient mice show some response to topical AA support the theory that, under unique conditions of high substrate availability, production of leukotrienes is possible in the absence of FLAP. It is not clear why the positive response of the FLAP-deficient mice to AA was most apparent in measurements of ear weight edema and (to a lesser extent) vascular permeability, whereas levels of cellular infiltration in similarly treated ears were equivalent in the FLAP– and 5-LO–deficient mice. One possible explanation for these results is that the enzymes that carry out subsequent metabolism of LTA_4 differ depending on the intracellular localization of the 5-LO enzyme. Metabolism of AA in the absence of FLAP may occur at intracellular sites other than the nuclear envelope, where 5-LO and FLAP normally interact. The production of LTA_4 at a different intracellular location could alter its availability to LTA_4 hydrolase and LTC_4 synthase and, in turn, affect the ratio of LTB_4 to LTC_4 production in FLAP-deficient cells. Because LTB_4 and LTC_4 differ in their contributions to vascular and cellular events during inflammation, changes in the production of these two leukotrienes in the FLAP-deficient mice could explain the results we see in the edema/plasma extravasation and neutrophil infiltration assays.

Edema formation is also characteristic of the acute inflammatory response seen in IgE-mediated anaphylaxis. The absence of observable differences in edema or vasopermeability between FLAP-deficient mice, 5-LO–deficient mice, and normal controls in these experiments is not surprising given the complexity of this response. In contrast with the relatively straightforward stimulation of a single metabolic pathway by topical AA, a wide variety of mediators that can lead to inflammatory changes similar to those induced by leukotrienes are produced in IgE-mediated anaphylaxis. It is likely that the release of other mediators such as histamine, a potent vasoactive substance, plays a key role. Although no differences were observed between FLAP-deficient and control mice here, under physiological conditions in which IgE receptors are not completely occupied or antigen is limited such that few mast cells are activated, the absence of leukotrienes may possibly have an impact on the extent of the edema formation.

The lack of FLAP and leukotrienes also failed to result in detectable attenuation of the contact hypersensitivity induced by FITC. Past reports suggested that leukotrienes may influence T cell–mediated responses at a number of levels. LTB_4 has been shown to alter IFN-γ, IL-2, and IL-1 production by T cells and monocytes (47, 48, 54). In addition, leukotrienes may contribute to this inflammatory process indirectly since they are likely to be produced by the myeloid cells recruited to sites of inflammation. Our failure to see a difference in this response may again reflect the complexity of the process. Numerous pathways may exist for eliciting the physiological changes that have been observed, and, whereas leukotrienes may contribute to such changes, other mediators may mask their deficiency. It could be that lower levels of stimulation would allow us to observe a more subtle change in this response. Such a situation has been described in an analysis of trinitrochlorobenzene-mediated contact hypersensitivity in IL-1–deficient mice (55). In this case, response variation in mutant mice was apparent only at low antigen concentrations.

In both the 5-LO– and the FLAP–deficient mice, striking alterations were seen in the kinetics of peritoneal edema formation after the injection of Zymosan A. Zymosan A can induce an inflammatory response through a number of pathways including activation of alternate pathway of complement, the generation of anaphylatoxins, and the activation of mast cells and basophils. The response is also characterized by the biosynthesis of arachidonic acid metabolites and by neutrophil and macrophage phagocytosis (43). Examination of the edema formation in the 5-LO– and FLAP–deficient mice shows that leukotrienes are essential mediators of the early reaction to this stimulus. Again, the similar responses seen in the FLAP– and the 5-LO–deficient mice suggest that FLAP has no additional role in inflammation beyond its absolute requirement for leukotriene synthesis.

In conclusion, we have used a genetic approach to show that mice made deficient in FLAP survive and breed normally but are generally unable to metabolize AA to leukotriene products. Further, we show that the absence of FLAP reduces the inflammatory response in a subset of model systems. The mice lacking FLAP, along with a growing number of other animals deficient in immunologically important genes, should eventually allow specific cellular and molecular dissection of the biochemical pathways leading to inflammatory pathologies.

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