Collagen-induced Arthritis Is Reduced in 5-Lipoxygenase-activating Protein-deficient Mice

By Richard J. Griffiths,* MaryAlice Smith,* Marsha L. Roach,‡ Jeffrey L. Stock,‡ Ethan J. Stam,* A.J. Milici,* Deborah N. Scampoli,* James D. Eskra,* Robert S. Byrum,§ Beverly H. Koller,§ and John D. McNeish‡

*From the *Department of *Cancer, Immunology, and Infectious Diseases, ‡Department of Molecular Sciences, Central Research Division, Pfizer, Inc., Groton, Connecticut 06340; and §Curriculum in Genetics and Molecular Biology and Department of Medicine, University of North Carolina, Chapel Hill, North Carolina 27599-7248*

Summary

Collagen-induced arthritis in the DBA/1 mouse is an experimental model of human rheumatoid arthritis. To examine the role of leukotrienes in the pathogenesis of this disease, we have developed embryonic stem (ES) cells from this mouse strain. Here, we report that DBA/1 mice made deficient in 5-lipoxygenase-activating protein (FLAP) by gene targeting in ES cells develop and grow normally. Zymosan-stimulated leukotriene production in the peritoneal cavity of these mice is undetectable, whereas they produce substantial amounts of prostaglandins. The inflammatory response to zymosan is reduced in FLAP-deficient mice. The severity of collagen-induced arthritis in the FLAP-deficient mice was substantially reduced when compared with wild-type or heterozygous animals. This was not due to an immunosuppressive effect, because anti-collagen antibody levels were similar in wild-type and FLAP-deficient mice. These data demonstrate that leukotrienes play an essential role in both the acute and chronic inflammatory response in mice.

Leukotrienes are a class of biologically active lipids that have a variety of proinflammatory effects (1, 2). Leukotriene B_4 (LTB_4)[1] is a chemotactic and activating factor for leukocytes (3), whereas the peptido-leukotrienes, LTC_4 and LTD_4, are potent constrictors of airway smooth muscle and increase vascular permeability (4). The synthetic pathway for leukotrienes is initiated by the release of arachidonic acid from the cell membrane by phospholipase A_2, followed by its conversion to LTA_4 by the enzyme 5-lipoxygenase (5-LO) (5). In resting cells, 5-LO is found in the cytosol, but upon activation it translocates predominately to the nuclear membrane (6, 7). A second protein, 5-lipoxygenase-activating protein (FLAP) is an integral membrane protein that is required for leukotriene synthesis (8). This conclusion is based on both pharmacological and molecular biological evidence. The pharmacological evidence was provided by the discovery of a class of leukotriene synthesis inhibitors that do not inhibit 5-LO but instead bind to FLAP (9). The molecular biology evidence is based on experiments involving transfection of the genes for 5-LO and FLAP into an osteosarcoma cell line that normally express neither of the genes. Only cells transfected with both genes synthesize leukotrienes (10). It was originally believed that FLAP served as a docking protein for 5-LO but no direct interaction between the two proteins has been demonstrated (11). FLAP has sequence homology to LTC_4 synthase, suggesting that these proteins may be part of a larger gene family (12).

Leukotrienes play an important role in the allergic and inflammatory response in both animals and humans (2). Recently, using mice made genetically deficient in 5-LO by homologous recombination in embryonic stem (ES) cells, it has been demonstrated that 5-LO is not required for normal physiological functions, but is required for the full expression of certain acute inflammatory responses, e.g., platelet-activating factor (PAF) lethality, arachidonic acid–induced edema, and neutrophil infiltration in response to arachidonic acid and immune complexes (13, 14). Mice made deficient in FLAP also show reduced acute inflammatory responses to these same stimuli, as described in the accompanying report.

Collagen-induced arthritis is an experimental model of human rheumatoid arthritis that is dependent on both humoral and cellular immunity to the immunizing antigen (15). We have previously shown that LTB_4 is required for the development of collagen-induced arthritis in the mouse

[1] *Abbreviations used in this paper:* 5-LO, 5-lipoxygenase; ES, embryonic stem; FLAP, 5-lipoxygenase-activating protein; HETE, hydroxyeicosatetraenoic acid; LTB_4, leukotriene B_4.

by utilizing a potent and selective receptor antagonist (16). This conclusion has recently been questioned by a report that a FLAP antagonist, which inhibits LTB_4 production, does not inhibit this disease unless coadministered with a inhibitor of prostaglandin synthesis, suggesting that combined inhibition of both leukotriene and prostaglandin production is required to affect disease progression (17). We now show that FLAP-deficient mice have a markedly reduced severity of disease in this model despite making high levels of prostaglandins.

Materials and Methods

Reagents. Zymosan A and IFA were obtained from Sigma (St. Louis, MO). Chick type II collagen, and the anti-collagen monoclonal antibody A-2, were provided with the assistance of Dr. M. Griffiths (University of Utah). Mycobacterium tuberculosis H37RA was from Difco Laboratories, Inc. (Detroit, MI). Tetramethyl benzidine Microwell peroxidase substrate was from Kirkgaard & Perry Labs, Inc. (Gaithersburg, MD). The peroxidase-conjugated rabbit antibody against mouse IgG was from Calbiochem Novabiochem (La Jolla, CA). Recombinant murine IL-1α was expressed in *Escherichia coli* at Pfizer and purified as described (18).

Generation of FLAP-deficient Mice. ES cells derived from DBA/1 inbred mice were transfected with a targeting vector designed to inactivate FLAP in 129 ES cells (see accompanying manuscript). Three different FLAP-targeted DBA 252 ES cell lines were injected into day 3.5 blastocyst stage embryos from matings of C57BL/6 mice by established procedures (19). Microinjected blastocysts were transferred to pseudopregnant CD-1 females to continue development to term. Chimeric males were mated with DBA/1lacJ females and germline transmission of ES cell genome was evident in offspring of dilute brown coat color (for thorough description of DBA252 germline transmission and coat color, see reference 20). Homozygous FLAP −/− were generated by crossing heterozygous FLAP +/− males and females and genotypes were identified by Southern blot analysis.

Zymosan-induced Peritonitis. Male DBA/1 mice were injected intravenously with 0.2 ml of a 10 mg/ml solution of Evans blue dye to label plasma proteins. They were then injected intraperitoneally with 0.5 ml of a 2 mg/ml suspension of zymosan. 30 or 180 min later the peritoneal cavity was lavaged with 3 ml of ice-cold heparinzed saline (10 U/ml). The samples were centrifuged to remove cells and debris and a 0.2-ml aliquot of the the supernatant transfered to a microtiter plate. The absorbance of each well was read at a wavelength of 650 nm on a Molecular Devices plate reader. The remainder of the supernatant was stored frozen at −20°C until assayed for leukotriene and prostaglandin content.

Eicosanoid Assays. The LTB_4, LTE_4, and 6-keto-PGF1α content of the peritoneal lavage fluid was measured using commercially available enzyme immunoassay (EIA) kits (Cayman Chemical, Ann Arbor, MI) according to the instructions of the manufacturer. The lower limit of detection at the dilutions used was 160 pg/ml for the LTE_4 and 6-keto-PGF1α assays and 40 pg/ml for the LTB_4 assay. The LTE_4 kit has a crossreactivity of 10% with LTC_4 and LTD_4. Leukotrienes and hydroxyeicosatetraenoic acids (HETEs) were extracted and analyzed by high performance liquid chromatography with UV detection using a modification of our previously published methods (21, 22). Peritoneal lavage fluid was spiked with 100 ng indomethacin in 10 μl methanol as an internal standard with or without a mixture of leukotriene and HETE standards (25 ng each; Biomol, Plymouth Meeting, PA) and proteins were precipitated by addition of 1.0 ml acetonitrile, followed by centrifugation at 8,000 *g* for 2 min. The supernatant was acidified and diluted with 4.0 ml water/acetic acid (100:0.1, vol/vol) containing 1 mM disodium EDTA (solvent A) and passed through a BondElut C18 solid phase extraction cartridge (100 mg in 1 ml column; Varian, Harbor City, CA) previously conditioned with 4.0 ml methanol, 2.0 ml acetonitrile, and 1.0 ml solvent A. The column was washed with 2.0 ml methanol/water and 0.5 ml methanol/triethylamine (100:0.1, vol/vol) followed by 0.5 ml methanol/acetic acid (100:0.1, vol/vol). Using a Perkin-Elmer ISS-100 autosampler fitted with a 2.0-ml stainless steel loop, 500 μl of the eluate was injected into a Short One C18 column (10 × 0.46 cm, 3 μm; Rainin, Woburn, MA). The mobile phase consisted of a linear gradient of methanol/water/trifluoroacetic acid (70:30:0.075 to 90:10:0.025, vol/vol/vol) over 30 min at a flow rate of 1.5 ml/min by a Perkin-Elmer Series 4 quaternary gradient pump. The column eluant was monitored by two Perkin-Elmer LC-95 UV detectors in series, one monitoring absorbance at 280 nm for detection of LTs and the other at 235 nm for HETEs. The detector outputs were quantitated using two Perkin-Elmer LCI-100 integrators by comparison to external standard areas and corrected for recovery for each eicosanoid by comparison of each sample run with and without these standards. To confirm the identity of 12-HETE found in the peritoneal lavage by HPLC followed by EIA, and to confirm further the UV–HPLC quantitation of the other eicosanoids in the samples, we used another HPLC method using a mobile phase with a basic rather than acidic pH mobile phase (23). The samples were extracted as above with the difference that the BondElut C18 cartridge was eluted with only 1.0 ml methanol/triethylamine (100:0.1, vol/vol). The entire eluate was injected into the HPLC column (a Perkin-Elmer CR-C18 cartridge column, 3 × 0.46 cm, 3 μm) using a Gilson ASPEC autosampler programmed to dilute the eluate with 4.0 ml solvent A and inject the entire 5.0-ml diluted sample into the Rheodyne injector fitted with a 5.0 ml stainless steel loop. The HPLC column was equilibrated for 10 min before sample injection of solvent A and after injection a linear gradient methanol/water/triethylamine (20:80:0.1 to 100:0:0.1, vol/vol/vol, over 20 min) at a flow rate of 1.5 ml/min. The UV absorbance of the column effluent was monitored for UV absorbance and quantitated as above. Fractions (0.2 min each) of the column eluate were collected into polypropylene microtubes. Fractions around the retention time of 12-HETE were dried in a vacuum centrifuge and redissolved in 500 μl EIA buffer. The fractions were assayed in duplicate using a 12(S)-HETE EIA (PerSeptive Diagnostics, Cambridge, MA) according to kit instructions. The kit has a crossreactivity of less than 2% with 12-(R)-HETE, 5-HETE, and 15-HETE. Immunoreactivity was found in three consecutive fractions around the retention time of 12-HETE and were summed to give the total concentration of 12-(S)HETE in the samples.

Induction of Collagen-induced Arthritis. Groups of age-matched (10–20-wk-old) male DBA/1 mice of varying genotypes were immunized at the base of the tail with 100 μg of chick type II collagen in CFA on day 0 and 21. 1 μg of murine rIL-1α, diluted in PBS containing 1 mg/ml BSA was administered subcutaneously on days 45 and 46. This protocol causes all of the wild-type animals to exhibit a severe form of the disease (24, 25). Severity of the symptoms of arthritis were assessed weekly, by an observer unaware of the genotype of the animals, by inspection of the paws (0 = normal paw, 1 = swelling and/or redness of one toe or finger joint, 2 = two or more joints involved, and 3 = severe arthritis in the entire paw; maximum score for each animal = 12).

On day 56, the mice were injected intraperitoneally with zymosan, and leukotriene production was measured as described above. Blood was collected by cardiac puncture, and serum was prepared and stored at −20 C until assayed for anti-collagen antibody levels. Knee joints were placed in formalin and processed as described below.

Histopathology. Knee joints were decalcified in Kristensen's solution, embedded in Paraplast plus, sectioned, stained, and approximately matched sections were examined by light microscopy on a Nikon FXA microscope.

Anti-collagen Antibody Levels. IgG antibody levels against the immunizing antigen were measured by standard ELISA methodology. Microtiter plates were coated with 0.5 μg/well chick collagen, blocked with normal rabbit serum, and then incubated with dilutions of the test sera. A standard curve was included on each plate using known amounts (0.01–10 μg/ml) of a monoclonal anti-chick collagen antibody (A-2). A peroxidase-conjugated rabbit anti–mouse IgG was then added, followed by substrate. The absorbance was measured at 450 nm and the amount of antibody in each sample calculated by reference to the standard curve.

Statistics. The results are presented as the mean ± SEM. Statistical analysis was done using Student's *t* test with a Bonferroni correction factor for multiple comparisons.

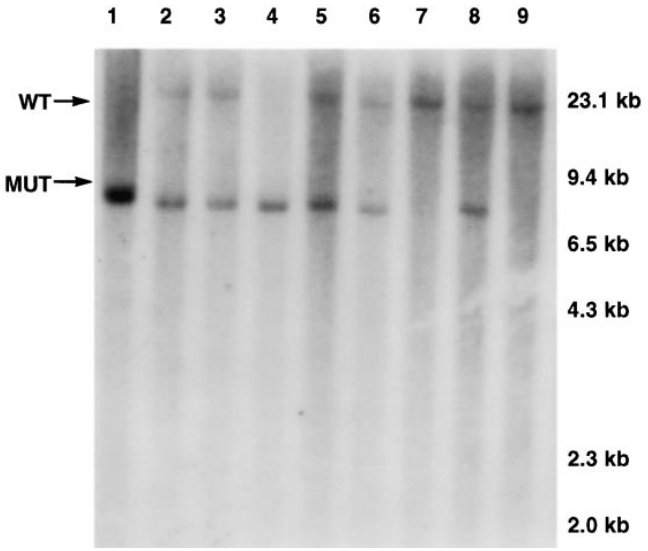

Figure 1. Southern blot analysis of DBA/1 offspring of FLAP +/− male and females. The offspring demonstrate normal Mendelian inheritance of the wild type (16 kb) and the mutated (8 kb) alleles. The genotypes are FLAP +/+, lanes *7* and *9*; FLAP +/−, lanes *2, 3, 5, 6,* and *8*; and FLAP −/−, lanes *1* and *4*.

Results

The establishment of the DBA/1 ES cell line allowed the investigation of induced FLAP mutant mice in the collagen-induced arthritis model. Previously, we reported that a targeting vector designed to inactivate FLAP in 129 ES cells undergoes homologous recombination at a similar frequency in nonisogenic DBA 252 ES cells (20). DBA/1 offspring of C57/DBA chimeric males demonstrated the mutated FLAP allele at the expected 50% ratio. Normal 1:2:1 Mendelian segregation was observed in matings between FLAP +/− males and females when identified by Southern blot analysis (Fig. 1). We observed no overt phenotypic defects in the homozygous FLAP −/− DBA/1 mutants.

To verify that, similar to the situation seen in the 129/FLAP-deficient mice, inactivation of the gene by homologous recombination leads to loss of leukotriene synthesis, zymosan was injected intraperitoneally to trigger eicosanoid synthesis associated with an increase in plasma protein extravasation. The synthesis of LTE_4 measured 30 min after injection of zymosan was abolished in the FLAP-deficient mice, whereas these mice made comparable levels of prostaglandins to wild type animals (Table 1). In addition, these samples were analyzed by reverse phase HPLC with UV detection. The major peptido-leukotriene product detected in the wild-type mice was LTE_4 with smaller amounts of LTC_4. However, no detectable peptido-leukotrienes were present in the FLAP-deficient mice. The amount of HETEs derived from the 5-, 12-, and 15-lipoxygenase pathway were also measured. Pooled samples from mice of each genotype were used. The only product detected was 12-HETE in both the wild-type and the FLAP-deficient mice. EIA of HPLC-purified fractions confirmed that this was

Table 1. *Effect of FLAP Deficiency on Zymosan-stimulated Plasma Protein Extravasation and Eicosanoid Production*

Genotype of mice	Experiment	Plasma protein extravastion (OD 650)	LTE_4 (ng/ml)	6-keto-PGF1α (ng/ml)	12(S)-HETE (ng/ml)
+/+	1	0.51 ± 0.03	95 ± 8	41 ± 3	31.8
	2	0.58 ± 0.07	87 ± 10	43 ± 6	23.9
−/−	1	0.22 ± 0.02*	<1*	40 ± 4	19.5
	2	0.31 ± 0.05*	<1*	37 ± 3	14.2

Groups of 6–9 male mice were injected intravenously with 0.2 ml of Evans blue dye to label plasma proteins. 1 mg of zymosan was then injected intraperitoneally. 30 min later the peritoneal cavity was lavaged and the amount of plasma protein quantified by measuring the absorbance of the cell-free supernatant due to Evans blue–labeled proteins. Eicosanoid levels in the same supernatants were measured either by direct EIA on the fluids (LTE4 and 6-keto-PGF1α) or EIA of HPLC-purified fractions from pooled samples (12(S)-HETE). Data are presented as mean ± SEM. *P <0.05.

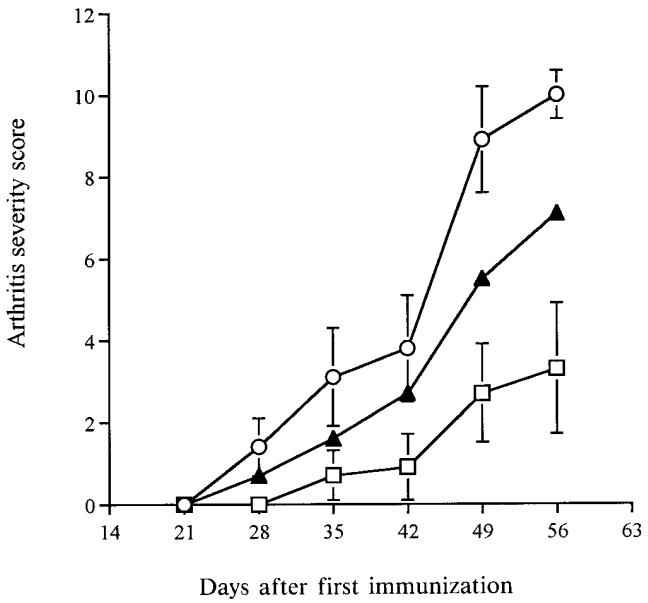

Figure 2. Collagen-induced arthritis in FLAP deficient mice. DBA/1 mice were immunized with chick type II collagen on day 0 and 21. IL-1 was administered subcutaneously on days 45 and 46 to trigger an arthritic flare. Disease severity was scored by observation of the paws for redness and swelling. Open circles, +/+ mice; closed triangles, +/− mice; open squares, −/− mice. Results are mean ± SEM, $n = 3$ experiments.

12(S)-HETE. The levels of 12(S)-HETE were slightly reduced in the FLAP-deficient mice (Table 1).

LTB_4 levels reach a maximum between 2–3 h after injection of zymosan in this model. Therefore, separate groups of mice were used to measure the levels of this eicosanoid. LTB_4 levels at 3 h after injection of zymosan were 1.52 ± 0.23 ng/ml in wild-type animals but were below the limits of detection of the assay (0.04 ng/ml) in the FLAP-deficient mice. The increase in plasma protein extravastion associated with injection of zymosan was reduced by ~50% in the FLAP-deficient mice (Table 1).

The severity of collagen-induced arthritis in the FLAP-deficient mice was reduced at all time points compared with wild-type animals (Fig. 2). The total disease score from days 21–56 (Fig. 2, area under the curve) was reduced by a mean of 73%, $P < 0.001$ (Table 2). Heterozygous animals had a smaller, but significant decrease also, mean of 37%, $P < 0.05$. The reduced severity of arthritis was maintained even after injection of IL-1, which causes all of the wild-type mice to exhibit a severe form of the disease (Fig. 2; Table 2). However, serum anti-collagen antibody levels were similar in wild-type (1050 ± 220 μg/ml) and FLAP deficient mice (1200 ± 23 μg/ml). Leukotriene levels in zymosan-injected wild-type mice were similar to nonimmunized animals (compare Table 1 with Table 2). Heterozygous animals had a slight reduction in LTE_4 levels, mean of 74% of control, and no detectable leukotriene was produced in homozygous mice.

In the FLAP-deficient mice, there generally was an absence of inflammatory cells in the joint cavity or surrounding synovial tissue (Fig. 3 *a*). The articular cartilage was intact, although there were regions of extensive depletion of proteoglycans in the calcified and noncalcified cartilage. Moreover, small pannus tongues coincided with the regions of greatest proteoglycan loss in some animals. In both the heterozygous and wild-type animals (Fig. 3, *b* and *c*), the extent of disease was much more extensive. Generally, these animals contained an extensive leukocyte infiltration of the synovial connective tissue, plasma protein extravasation into the synovial cavity, and variable amounts of articular cartilage destruction.

Table 2. *Effect of FLAP Deficiency on Collagen-induced Arthritis*

Genotype of mice	Experiment	Severity score day 56	AUC	Percentage of mice with arthritis	LTE_4 (ng/ml)
+/+	1	10.2 ± 1.6	144.9	100	84 ± 15
	2	8.8 ± 2.1	158.2	100	145 ± 19
	3	11.0 ± 0.8	163.1	100	104 ± 15
+/−	1	8.1 ± 1.6	123.6	86	71 ± 7
	2	6.4 ± 2.1	93.8	71	100 ± 23
	3	6.8 ± 1.8	79.1	74	74 ± 11
−/−	1	3.3 ± 1.4	55.7	75	<1
	2	4.8 ± 1.9	52.5	71	<1
	3	1.7 ± 0.7	18.6	75	<1

Groups of 4–9 male mice were immunized with 100 μg chick type II collagen on days 0 and 21. 1 μg of rIL-1α was injected subcutaneously on days 45 and 46. The severity of arthritis was assessed by scoring the degree of inflammation in each paw weekly. The average total severity for each group of mice was calculated as area under the curve (*AUC*) for days 21–56. On day 56, 1 mg zymosan was injected intraperitoneally. LTE_4 levels in peritoneal lavage were measured by EIA.

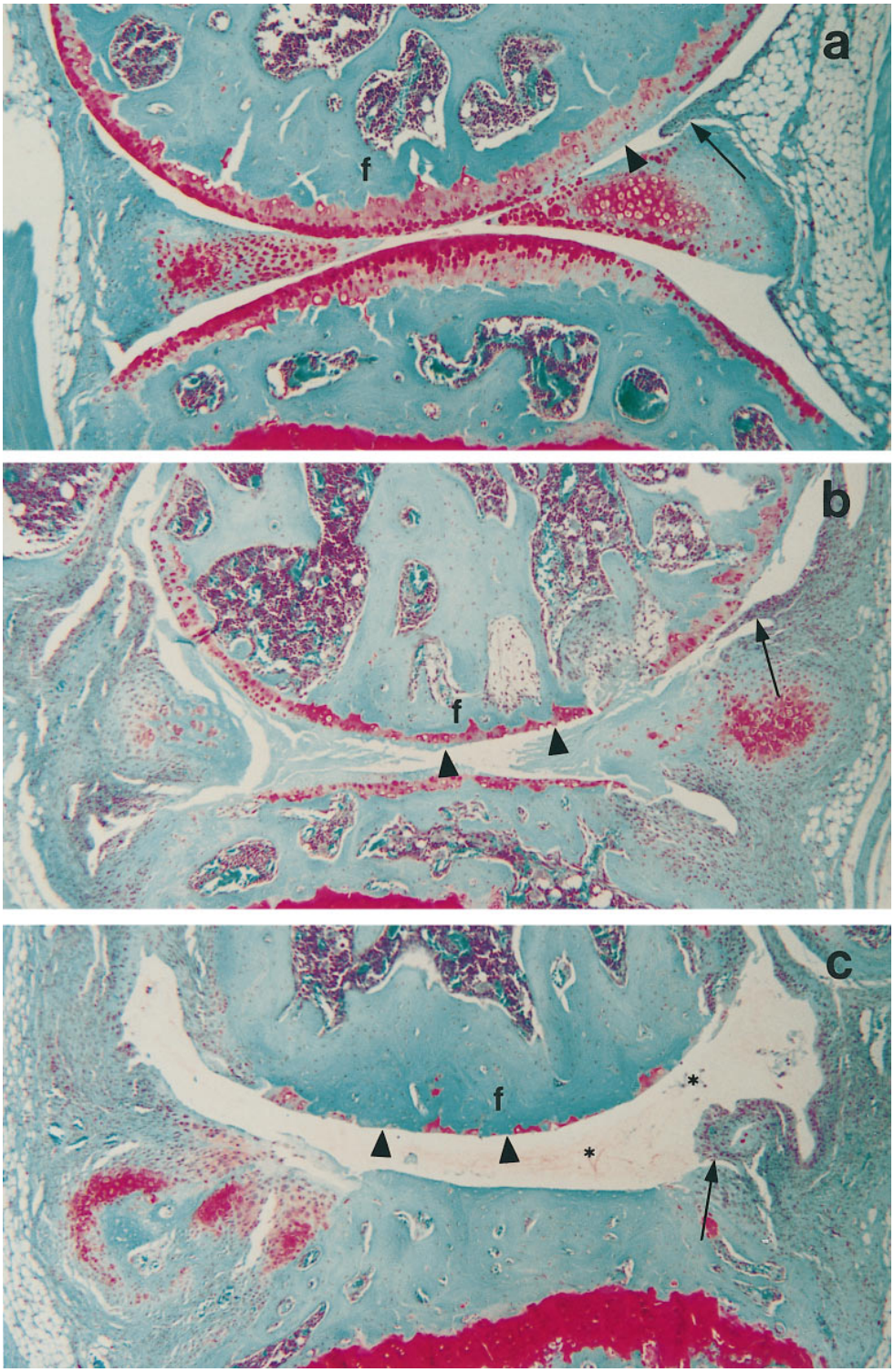

Figure 3. Light microscopy of the knee joint from FLAP −/−, +/−, and +/+ mice. In the FLAP knockout mice (*a*) there is an absence of inflammatory cells in the synovial tissue surrounding the joint cavity. An occasional pannus tongue (*arrow*) was observed and tongues generally lacked a large cellular component. The femoral condyle (*f*) articular cartilage was intact, although regions of proteoglycan depletion (*arrowhead*) were observed and these typically coincided with the pannus extensions. In the heterozygous mice (*b*), the articular cartilage was removed down to the tidemark (*arrowheads*) over large regions of the femoral condyle (*f*). Several pannus tongues (*arrow*) and an increased inflammatory cell influx into the surrounding synovial tissue were observed. In the wild-type mice (*c*), the destruction of the femoral condyle (*f*) articular cartilage (*arrowheads*) was much more extensive. In addition, some plasma proteins and cells (*) were observed within the joint cavity. The pannus tongues (*arrow*) and increased cellularity of the synovial tissue were similar to that observed in the heterozygous mice. Original magnification, ×60.

Discussion

The ability to modulate the genetic composition of mice selectively is a powerful tool to study the role of a gene product in both physiological and pathophysiological processes. Mice with a deficiency in the FLAP gene are phenotypically normal and reproduce with the expected Mendelian ratio of homozygotes to heterozygotes. Leukotriene production in vivo is absent in these mice, confirming the substantial body of in vitro evidence indicating that FLAP is a necessary component of the 5-LO pathway (8, 10). The slight reduction in 12(S)-HETE production was unanticipated and may suggest a role for FLAP in this pathway. This is a subject that requires further investigation. The

presence of high levels of prostaglandins in the FLAP-deficient mice confirms that the interaction of FLAP with the 5-LO pathway is selective.

This defect in leukotriene production results in decreased plasma protein extravasation in the peritoneal cavity in response to an injection of zymosan. This agrees with pharmacological studies with 5-LO inhibitors, which also reduce this response (26). The magnitude of the inhibiton in the DBA/1 mice is less than that observed in the 129 strain. This may be due to the contribution of mast cell amines to the response, which may vary in different strains.

Collagen-induced arthritis in the mouse is an autoimmune disease produced by immunizing mice with heterologous type II collagen. This form of collagen is found in articular cartilage and the immune response against the immunizing antigen leads to a chronic, destructive polyarthritis that has certain features in common with human rheumatoid arthritis. The disease is only produced in certain susceptible strains of mice, mainly those of the H-2^q haplotype, but multiple genes contribute to this susceptibility (15). The DBA/1 mouse has been the strain most commonly used to study this disease. The 129 ES cell lines, normally used for gene targeting, generate mice that are not susceptible to collagen-induced arthritis. Therefore, to study this model it is necessary to breed the gene-deficient mouse to the DBA/1 background. Because the disease is polygenic, it is not clear how many generations of breeding are required to ensure that any diminution in severity of disease observed is due to the lack of the targeted gene, rather than to the fact that the mice may not be congenic. Therefore, we have recently developed a ES cell line from DBA/1 mice, that has allowed us to circumvent this problem by gene targeting directly on the arthritis-susceptible background.

We have previously reported that collagen-induced arthritis can be prevented by the administration of a potent and selective LTB_4 receptor antagonist, implicating products of the 5-LO pathway as mediators of this disease (16). However, arachidonic acid metabolites derived from the monooxygenase pathway can also act through this receptor, e.g, 12(R)-HETE (27), and it has recently been reported that a FLAP inhibitor does not prevent collagen-induced arthritis alone, but only when coadministered with an inhibitor of prostaglandin synthesis (17). This latter study suggests that inhibition of the 5-LO pathway alone is not sufficient to inhibit disease. However, our experiments with FLAP-deficient mice provide very strong evidence that products of the 5-LO pathway are essential for the development of this disease. We do not believe that the reduction in 12(S)-HETE production contributes to the reduced inflammatory responses in these mice because, unlike 12(R)-HETE, this product does not bind to the LTB_4 receptor and is not chemotactic for leukocytes (25).

The animals were successfully immunized against collagen, as judged by the fact that they make normal levels of antibody, but exhibit a profound reduction in the signs of arthritis. This result is in agreement with our previous studies with an LTB_4 receptor antagonist. The observed effects are particularly impressive given the fact that we challenged the mice with IL-1, which causes control mice to develop a very severe form of the disease.

In summary, FLAP-deficient mice are phenotypically normal but exhibit a complete defect in leukotriene production. They also show reduced acute and chronic inflammatory responses, confirming the important role of leukotrienes as mediators of the inflammatory response in mice.

Address correspondence to Dr. John D. McNeish, Molecular Sciences, Central Research Division, Pfizer, Inc., Eastern Point Road, Groton, CT 06340

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