Lipoxygenase products in inflammatory synovial fluids and other exudates

Patrick B Costello,* Alan N Baer, Floyd A Green

Abstract

Forty six synovial fluid samples from 42 patients with inflammatory joint disease were analysed by reversed phase high performance liquid chromatography to determine 5-lipoxygenase products, specifically dihydroxy-eicosatetraenoic acids (diHETEs). Twenty eight per cent of the fluids which were assayed had one or more products of 5-lipoxygenase activation. Seven fluids contained leukotriene B₄ (0.1–28.1 ng/ml); three fluids had low concentrations of 20 carboxy/hydroxy-leukotriene B₄ (0.01–0.05 ng/ml); three samples had leukotriene B₄ isomers (1.5–2.4 ng/ml); and four fluids contained 5,15-diHETE (2.3–16.4 ng/ml). There was a poor correlation between synovial fluid white blood cell counts and evidence of 5-lipoxygenase activation. Several fluids contained unidentified compounds with spectra similar in shape to that of trienes, but the $\lambda_{\max}$ values of these unidentified compounds were different from those of known leukotrienes. A septic peritoneal exudate and a septic pleural fluid had concentrations of leukotriene B₄ and leukotriene B₄ isomers and metabolites in a range similar to those found in synovial fluids.

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Leukotrienes are 5-lipoxygenase products of arachidonic acid which are thought to be important mediators of the human inflammatory process.^{1,2} The studies reported here were undertaken to measure the type and concentrations of 5-lipoxygenase products and metabolites in joint effusions from patients with a variety of inflammatory joint diseases. Joint fluids were processed to optimise the recovery of total in vivo 5-lipoxygenase products present at the time of aspiration so that correlation with other parameters of inflammation could be made. In vivo leukotriene production in synovial fluids was compared with that in a limited number of exudates from the peritoneal and pleural cavities.

Materials and methods

Synovial fluid aspirates were obtained for routine diagnostic tests from 42 patients with inflammatory joint disease (rheumatoid arthritis, 16 patients; gout, 11 patients; pseudogout, two patients, septic arthritis, six patients; undiagnosed synovitis, seven patients) and the excess was flash frozen within 30 minutes and then lyophilised. In handling the peritoneal and pleural fluids, the cellular content was separated from the effusion before freezing. This separation was not performed for the synovial fluids,

but the total intra- and extracellular dihydroxy-eicosatetraenoic acid (diHETE) concentration was measured. Lyophilised samples or cells were then extracted with 25–50 ml of 75% ethanol and vortex mixed for five minutes. Prostaglandin B₂ (Sigma Chemical, St Louis, MO, USA) was added as an internal standard (five nanomoles in a volume of 100 μ l). In some instances the samples were acidified with formate buffer in a volume equal to 10% of the aqueous phase. After centrifugation at 1500 g for 10 minutes, the supernatants were rotary evaporated and the residue was transferred in methanol and ultimately reconstituted in the mobile phase for reversed phase high performance liquid chromatography (HPLC). The mean (SEM) recovery of the internal standard with this extraction technique was 58(4)%.

The mobile phase for determination of leukotriene B₄ consisted of water/acetonitrile/methanol/acetic acid, 45:37:18:1 (pH 5.6 with sodium hydroxide) and that for the determination of 20-hydroxy and 20-carboxy-leukotriene B₄ consisted of methanol/water/acetic acid, 50:50:0.1. The reversed phase HPLC was performed isocratically using a Hewlett-Packard 1090 liquid chromatograph (Hewlett-Packard, Palo Alto, CA, USA), Hewlett-Packard ODS-Hypersil columns (20 cm $\times$ 4.6 mm) and a flow rate of 0.4 ml/min. This liquid chromatograph is equipped with a diode array spectrophotometer and a computer for on-line display and storage of ultraviolet spectra, and thus an analysis of the purity of each peak and precise measurement of the $\lambda_{\max}$ of each peak can be performed after each chromatographic run. Authentic standards for leukotriene B₄, 20-hydroxy-leukotriene B₄, and 20-carboxy-leukotriene B₄ were obtained from BioMol Research Labs (Plymouth Meeting, PA, USA). The retention times of these standards were determined at the start of each HPLC run and were highly reproducible within and between HPLC assay sessions. The HPLC analyses were routinely monitored at wavelengths of 270 nm (for the detection of leukotriene B₄ and its metabolites) and 245 nm (for the detection of diHETEs). In some instances HPLC in a second system was also performed. Each 5-lipoxygenase product was identified on the basis of its characteristic ultraviolet spectrum and retention time (compared with authentic standards) on one or more HPLC systems. This instrument is capable of detecting and defining the ultraviolet spectrum of leukotriene B₄ in amounts of 10 ng or higher. The detection range of this instrument (0–3 absorbance units) is linear. In the figures the ordinate is expressed in milliabsorption units (mAU). Software programs

Division of Rheumatology, Department of Medicine, State University of New York at Buffalo and the Veterans Administration Medical Center, Buffalo, NY 14215, USA
P B Costello
A N Baer
F A Green

Correspondence to: Dr Alan N Baer, Department of Medicine, School of Medicine, Faculty of Health Sciences, Veterans Administration Medical Center, 3495 Bailey Avenue, Buffalo, New York, 14215, USA.

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*Deceased.

Table 1 Lipoxygenase products in synovial effusions

Patient No	Diagnosis*	Synovial fluid		Leukotriene (ng/ml)			5,15-diHETE‡ (ng/ml)
		Volume (ml)	Cell count ($\times 10^9/l$)	LTB ₄ †	20-hydroxy-/carboxy-LTB ₄ †	LTB ₄ isomer†	
1	Gout	25	13.30			1.8	
2	Gout	30	NA		0.05	2.4	
3	Seronegative RA	27	48.25				2.3
4	Undiagnosed	30	0.350				10.0
5	Pseudogout	12	112	1.3			
6	Gout	15	13.65	1.6			
7	Gout	3	NA	9.0			
8	Seronegative RA	20	0.425	8.8	0.2		
9	Seronegative RA	50	14.00	2.0	0.01		
10	Seropositive RA	25	11.50	28.1			
11	Sepsis	30	32.00	0.12			
12	Pseudogout	35	11.30			1.5	6.3
13	Undiagnosed	20	NA				16.4

*NA=not available; RA=rheumatoid arthritis.

†LTB₄=leukotriene B₄.

‡diHETE=dihydroxyeicosatetraenoic acid.

were used to calculate the amount of lipoxygenase products with conjugated diene and triene structures by measuring the area beneath the peak height at a given $\lambda_{\max}$. An absorption coefficient of $51\,000\text{ mol}^{-1}\text{ l cm}^{-1}$ (at 270 nm)

Figure 1 Reversed phase high performance liquid chromatograph of the extract of synovial fluid from patient 8 with seronegative rheumatoid arthritis. The HPLC run was monitored at a wavelength of 270 nm. The peak at 35.5 minutes represents leukotriene B₄. The inset shows the ultraviolet spectrum for this peak with a $\lambda_{\max}$ of 270 nm.

Figure 2 Reversed phase high performance liquid chromatogram of synovial fluid from patient 12 with pseudogout. The HPLC run was monitored at a wavelength of 245 nm. The peak at 31.4 minutes has a retention time and ultraviolet spectrum (inset) consistent with 5,15-dihydroxyeicosatetraenoic acid.

was used for leukotriene B₄, 20-hydroxy-leukotriene B₄, and 20-carboxy-leukotriene B₄ in these calculations.³ For approximate measurement of the unknown triene, the absorption coefficient and molecular mass of leukotriene B₄ were used with the assumption that these properties of the unknown triene compound were not considerably different from those of leukotriene B₄. The amount of product was corrected for the loss which occurred during extraction, as measured by the recovery of the internal standard. Cell counts were measured using a ZBI Coulter counter.

Results

Forty six synovial fluids were assayed for their total (intracellular and extracellular) concentration of specific diHETE lipoxygenase products. The system which was used allowed identification of leukotriene B₄, leukotriene B₄ isomers (6-*trans* and 6-*trans*, 12-*epi*-leukotriene B₄), and the primary metabolites of leukotriene B₄ (20-carboxy-leukotriene B₄ and 20-hydroxy-leukotriene B₄). 5,15-Dihydroxyeicosatetraenoic acid was also recognisable by its retention time and $\lambda_{\max}$ at 243 nm.

Thirteen of 46 fluids (28%) from 42 patients had one or more of these 5-lipoxygenase products (table 1) at the time of sampling. Leukotriene B₄ was present in seven synovial fluids in the concentration range 0.1–28.1 ng/ml. Figure 1 shows the chromatogram of a joint fluid from a patient with a seronegative inflammatory disease in which leukotriene B₄ elutes at 35.5 minutes. Low concentrations of 20-hydroxy-leukotriene B₄ or 20-carboxy-leukotriene B₄ metabolites were present in three samples, two of which also contained leukotriene B₄. Three synovial fluid samples contained trienes which comigrated with 6-*trans* or 6-*trans*, 12-*epi*-leukotriene B₄. None of these three contained leukotriene B₄. Four additional fluids were found to contain 5,15-diHETE in concentrations ranging from 2.3 to 16.4 ng/ml. Figure 2 is a chromatogram of an effusion from a patient with pseudogout. In this sample, a peak at 31.4 minutes had an ultraviolet spectrum with a $\lambda_{\max}$ at 243 nm, which was consistent with 5,15-diHETE.

The presence or absence of these 5-lipoxygenase products was not considerably different

Table 2 Lipoxygenase products in septic peritoneal and pleural fluids

Exudate	Volume (ml)	LTB ₄ *	20-carboxy-LTB ₄ * (ng/ml)	6-trans-LTB ₄ * (ng/ml)	6-trans-12-epi-LTB ₄ * (ng/ml)
Peritoneal Cells	5	5.2	9.8	ND	ND
Supernatant	100	0.33	7.0	0.11	1.1
Pleural Cells	7.5	1.4	Trace	1.0	1.1
Supernatant	20	0.54	0.19	0.53	0.94

ND=not detected.
*LTB₄=leukotriene B₄.

in the various inflammatory joint diseases. There was no correlation between the total white blood cell counts and 5-lipoxygenase activation. For example, one septic/gouty effusion (white blood cell count about 200×10⁹/l) was serially assayed for 5-lipoxygenase products and these were not found.

Several synovial fluid samples were noted to have spectra which were similar in shape to those of leukotriene B₄ but which did not have

elution times or a λ_{max} of known triene compounds. These ‘unknown trienes’ were present in five fluids in which they had retention times of 11–18 minutes and a λ_{max} of 280–284 nm; in two fluids they had a retention time of 11–12 minutes and a λ_{max} of 287–288 nm; and in 10 fluids they had a retention time of 34–42 minutes and a λ_{max} of 265 nm. The latter unknown triene was present in relatively large concentrations in some samples (up to 29 ng/ml).

5-Lipoxygenase products were also measured in several peritoneal cavity exudates. In four samples from chronic peritoneal dialysates complicated by staphylococcal peritonitis, no 5-lipoxygenase products were found. In one patient with septic peritonitis secondary to a ruptured bowel 100 ml of purulent (>100×10⁹/l white blood cells) peritoneal fluid was studied. A 35 ml sample of septic pleural fluid was also studied. In these samples the cells and supernatant were assessed separately (table 2). These infected fluids contained leukotrienes in a concentration range similar to that seen in the joint fluid samples. Figure 3 shows a chromatogram from the supernatant of the peritoneal fluid with 6-trans-leukotriene B₄, 6-trans, 12-epi-leukotriene B₄, and leukotriene B₄ eluting at 31, 33, and 35.5 minutes, respectively. Figure 4 shows the rechromatogram, using a different mobile phase, of the seven to 10 minutes fraction collected from the run shown in figure 3, in which the 20-carboxy-leukotriene B₄ metabolite elutes at 15 minutes.

Figure 3 Reversed phase high performance liquid chromatogram of septic peritoneal fluid. The HPLC run was monitored at a wavelength of 270 nm. The peaks at 31, 33, and 35.5 minutes comigrated with 6-trans-leukotriene B₄, 6-trans, 12-epi-leukotriene B₄, and leukotriene B₄ respectively. The ultraviolet spectrum of the peak at 35.5 minutes (inset) is consistent with leukotriene B₄.

Figure 4 Rechromatograph, using a different mobile phase (see under Materials and Methods) of the seven to 10 minutes fraction collected during the chromatographic run shown in fig 3. The HPLC run was monitored at a wavelength of 270 nm. In this mobile phase the authentic 20-carboxy-leukotriene B₄ standard elutes at 15 minutes. The ultraviolet spectrum of the peak at 15 minutes (inset) has a triene morphology and a λ_{max} of 270 nm, consistent with 20-carboxy-leukotriene B₄.

Discussion

The relative role of lipids with respect to other mediators in the inflammation of synovial and serous cavities is not known. It is possible that there may be differences in this role with respect to aetiology, time course, and even genetic factors. The first step might be to examine different inflammatory fluids for the presence of these mediators or their metabolic products in concentrations which would be compatible with a pathological role. We have therefore studied 5-lipoxygenase product generation in a variety of inflammatory joint disorders. Leukotriene B₄ or its isomers or metabolites or 5,15-diHETE (or both) was present in 28% of the samples tested. 5,15-Dihydroxyecosatetraenoic acid has not previously been reported in inflamed joint tissue. The cellular source of the 5,15-diHETE is not known. Transcellular metabolism could be part of the process.⁴

In our studies almost 50% of the fluids from patients with crystal induced arthritis contained 5-lipoxygenase products. Rae *et al* first reported

increased levels of leukotriene B₄ in gouty synovial fluids measured by a bioassay.⁵ It has also been shown that peripheral neutrophils can be stimulated to produce leukotrienes by monosodium urate in vitro. Eicosanoids other than leukotriene B₄ were thought to be important inflammatory mediators in urate induced arthritis in dogs.⁶

We assayed 12 synovial fluids from patients with seropositive rheumatoid arthritis. Only one sample contained measurable concentrations of leukotriene B₄. Other workers have reported the presence of leukotriene B₄ in rheumatoid effusions, with concentrations varying greatly depending on the technique used.⁷⁻⁸ Klickstein *et al* reported levels of 5,12-diHETE (leukotriene B₄) in inflammatory and non-inflammatory joint fluid supernatants in the range 20–500 ng/ml.⁷ In their studies leukotriene B₄ was identified only by HPLC retention times.⁷ Davidson *et al* studied rheumatoid effusions by HPLC and a bioassay technique and detected leukotriene B₄ in a concentration range similar to that seen in this analysis.⁸ Biologically active leukotriene B₄ has also been measured in low nanogram concentrations in knee fluids from dogs with carageenan induced arthritis.⁹

The finding of these eicosanoids in inflamed tissue may depend on processes which are difficult to control experimentally. For example, it has been shown that leukotriene B₄ production in antigen induced arthritis in rabbits is significantly increased only in the early phase of the lesion.¹⁰ If leukotrienes are not uniformly produced during the course of human disease, then determining them in samples taken at a single time point may be misleading. The rate of metabolism of synthetic leukotriene B₄ was higher in synovial fluids from patients with rheumatoid arthritis compared with osteoarthritic controls.¹¹ If leukotriene B₄ was rapidly metabolised to metabolites which had lost the triene chromophore, these would not be detectable by the HPLC technique used in these studies. In the patient with sepsis, the question could be raised as to whether the fact that leukotriene chemotactic factors were not formed and released early in the disease course could in some patients militates against successful phagocytosis and killing of the organism. The importance of 5-lipoxygenase products as chemotactic factors responsible for leucocyte attraction in bacterial infection has not been carefully studied in human disease or mammalian models. In a frog model of septic peritonitis in vivo 5-lipoxygenase products were routinely found early after an injection of the organism.¹² The pattern of eicosanoids found was related to

the species of the frog and not to the species of organism.

In the limited studies performed on septic fluids outside the joint the extracellular and intracellular concentrations (but not total amounts) of eicosanoids were similar. This indicates that the inflammatory cells had lost their barrier function for these products. As the inflammatory fluids by definition contain a high albumin concentration it is possible that the eicosanoids released were largely protein bound. In vivo animal studies, however, have shown some potent biological effects of these agents—that is, chemotaxis in the presence of normal serum proteins. The metabolism of leukotriene B₄ to 20-carboxy-leukotriene B₄ almost certainly took place in the neutrophils. This implies that the production of leukotriene B₄ took place not as an agonal event in the life of the neutrophil,¹³ but that the mitochondrial enzymes were intact at the time of leukotriene synthesis. This may suggest a pathophysiological role. The poor correlation between numbers of inflammatory cells and concentrations of leukotrienes suggests that other specific or selective factors which have yet to be identified may be responsible for their presence.

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