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IN VIVO METABOLISM OF [1-^{14}C]ARACHIDONIC ACID DURING DIFFERENT PHASES OF GRANULOMA DEVELOPMENT IN THE RAT

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Abstract—The metabolism of [1-^{14}C]arachidonic acid was studied *in vivo* during the development of carrageenin-induced granuloma in the rat. For this purpose a double-cannulated teflon cylinder was implanted into the dorsal subdermal tissue of rats, and the cannulae were exteriorised through the skin at the scruff of the neck. The "chamber" allowed the injection of substances and collection of exudate at different stages of granuloma development. When [1-^{14}C]arachidonate was injected into the chamber one hour before recovery of the exudate at periods up to 12 days after initiating the inflammation, hydroxy fatty acids and prostaglandin E_2 appeared to be the main products formed. Only very small amounts of thromboxane B_2 and 6-ketoprostaglandin $F_{1\alpha}$ were detectable. These *in vivo* results are in marked contradiction to observations of other workers with granuloma tissue *in vitro*. The present findings are discussed in relation to the postulated negative-feedback function of E-type prostaglandins and a possible role of hydroxy fatty acids in inflammation.

The main precursor of endogenous prostaglandins (PG's) is arachidonic acid (AA), which occurs abundantly in cell membrane lipids of virtually all mammalian tissues [1]. PG's are released upon a variety of stimuli [2] and increased amounts of PG's, particularly those of the E-type, are detectable at inflamed sites [3]. PG's exert pro-inflammatory actions, such as vasodilation [4], increased vessel permeability [5] and potentiation of similar actions of other inflammatory mediators [6]. It is now widely accepted that PGE's contribute significantly to the development of acute inflammatory conditions [7]. However, with respect to chronic inflammation the function of PG's is not clear [8]. Dietary depletion of AA in rats, resulting in a shortage of PG's at the inflamed site, leads to an enhancement of the granulomatous component of chronic inflammation [9] and this enhancement is associated with an increased synthesis of collagen [10]. While this indirectly suggested that PG's exert a suppressive action on granulomatous inflammation, direct evidence was obtained more recently. Thus it was shown that local administration of PGE_1 inhibits the formation of inflammatory granulomata in rats, provided that the administration is carried out a stage of granuloma development when tissue growth is already in progress [11]. A similar inhibitory action on granuloma formation was also demonstrated with PGE_2 [12].

Prostacyclin (PGI_2), a recently discovered product of arachidonate bioconversion, has been found to be even more potent than PGE_2 in producing vasodilation [13, 14]. However, PGI_2 fails to inhibit granuloma formation, when injected locally in the chronic phase of inflammation at a dose which is equivalent to the inhibitory dose of PGE_2 [12].

Studies involving *in vitro* incubations of AA with a fraction of the homogenate of rat granuloma tissue, identified thromboxane B_2 (TXB_2) and 6-keto$PGF_{1\alpha}$ (a metabolite of PGI_2) as the major products formed [15, 16]. Furthermore, at different stages during the development of granuloma tissue, the capacity of the particular tissue fraction used to convert AA into TXB_2 increased, whereas its capacity to form 6-keto$PGF_{1\alpha}$ decreased [17].

The present study was undertaken to investigate the metabolism of AA *in vivo* at different stages of granuloma development in the rat, using a modification of the model involving implantation of sponges with indwelling cannulae [18]. This modification allowed withdrawal of exudate from the intact rat at any desired moment during the development of inflammatory granuloma.

MATERIALS AND METHODS

Materials. [1-^{14}C]Arachidonic acid (specific radioactivity 56.2 Ci/mole) was purchased from the Radiochemical Centre, Amersham, England; Carrageenin (Viscarin) from Marine Colloids Inc. Springfield, U.S.A.; arachidonic acid, prostaglandin A_2, E_2 and $F_{2\alpha}$ from Sigma, St. Louis, U.S.A.; 12L-hydroxy-5,8,-10,14-eicosatetraenoic acid (HETE) and 12L-hydroxy-5,8,10-heptadecatrienoic acid (HHT) were gifts from Unilever, Research, Vlaardingen, The Netherlands. 6-Keto$PGE_{1\alpha}$ was a gift from Wellcome Research, Beckenham, England and PGD_2 and TXB_2 from Upjohn, Kalamazoo, U.S.A. Phosphomolybdic acid and 60 F_{254}-silicagel thin layer chromatography (t.l.c.) plates were purchased from E. Merck, Darmstadt, Germany and dioxane scintillation fluid from Packard, Brussels, Belgium. Teflon tube: 8 × 10 mm, was purchased from Eriks, Alkmaar, The Netherlands, silicone adhesive (Silastic) from Dow Corning Co., Midland, U.S.A. and nylon tube: 0.62 × 1.02 mm, from Rubber, Hilversum, The Netherlands. All other chemicals used

were of the highest degree of purity commercially available.

Animal treatment. 4 Male Wistar rats (TNO, Zeist, The Netherlands), weighing 245–260 g, were each implanted with a Teflon cylinder (length 30 mm, inner diameter 8 mm) provided with 108 holes of 1.5 mm diameter and two longitudinally indwelling nylon cannulae of 0.65 mm in diameter. The ends of the cylinder were sealed with silicone kit and the whole then implanted subcutaneously in the lumbar region, the cannulae being exteriorised at the back of the neck.

Inflammation was induced by injection through a cannula of 1 ml of a 1% (w/v) gel of carrageenin in sterile pyrogen-free saline into the implanted cylinder. The cannulae were then closed by means of tube-sealer. The injected carrageenin had access to the surrounding tissue via the holes in the implanted chamber, thus inducing granulomatous inflammation. The granuloma developed initially on the outside of the cylinder, subsequently growing in through the holes. Macroscopically, the whole granuloma had a similar appearance to that induced by carrageenin-soaked sponges [18]. A detailed description of the methodology will be published elsewhere.

Rats had free access to water and standard laboratory food (Hope Farms, Woerden, The Netherlands).

Injection and collection of radioactive material. Arachidonic acid (10 μCi) was dissolved in 800 μl ethanol after having evaporated the stock solvent, benzene, under a stream of nitrogen. Of this solution 200 μl were injected through the cannula into the teflon cylinder in each of the 4 rats on days 1, 4, 8 and 12 of the inflammatory process. After 1 hour of *in vivo* incubation with the arachidonate, exudate was collected by infusing 1 ml saline into one cannula, while fluid was drawn out of the other one into a 5-ml syringe. During handling, rats were under slight ether anaesthesia. Exudate was transferred into a calibrated glass tube and its volume recorded.

Extraction and thin layer chromatography. Extraction of lipid material was carried out twice, by mixing the exudate with 4 ml ethylacetate after acidification to a pH of 3 with 2 M HCl. Phase separation was facilitated by centrifugation at 1000 *g* for 5 min. The separate extracts of each exudate were pooled, the volumes recorded and subsequently the solvent was evaporated *in vacuo* at room temperature. Samples of 10 and 100 μl exudate and extract, respectively, were taken up into 10 ml scintillation fluid and recovery was estimated by counting radioactivity in a Packard Tricarb Model 3375 liquid scintillation counter with external standardization for quench correction. T.l.c. plates were activated at 100° for 10 min and after cooling 5 μg samples of arachidonic acid, PGA_2, D_2, E_2, $F_{2\alpha}$ and 6-keto$PGF_{1\alpha}$ were spotted at one point as internal standards. The extract residue was dissolved in 50 μl chloroform and spotted on the t.l.c. plate at the same point as the reference compounds.

T.l.c. plates were initially developed in a solvent system consisting of chloroform:methanol:acetic acid:water (90:8:1:0.8) [19] and subsequently in the organic phase of a mixture of ethylacetate:*iso*-octane:acetic acid:water (11:5:2:10) [20]. This two-dimensional chromatography procedure produced excellent separation of the metabolites under study.

Radioactive spots were visualized by scanning the t.l.c. plates in a Berthold LB 2723 thin layer scanner equipped with a dot scan unit for β-radiation. After scanning, plates were sprayed with a freshly prepared 7.5% (w/v) solution of phosphomolybdic acid in absolute ethanol followed by heating for 10 min at 100°. Blue coloured spots, and finally the uncoloured remainder, were scratched off the plates into scintillation vials, mixed with 10 ml scintillation fluid and radioactivity was estimated by liquid scintillation counting.

Calculation of the radioactivity of metabolites. Conversion of c.p.m. into d.p.m. was performed by correction for quenching via an external standard. Amounts of metabolites of arachidonic acid were expressed as percentages of total plate radioactivity. Activity representing PGE_2 was estimated by calculating the sum of radioactivities of the original spots of PGE_2 and PGA_2, because handling solutions containing PGE_2 may lead to dehydration and hence PGA_2 formation [21]. In addition, enzymatic conversion of PGE_1 into PGA_1 by a placental dehydrase has been reported [22]. In our experiments radioactivity representing PGA_2 never exceeded that for PGE_2.

RESULTS

The implantation of a porous Teflon chamber with two indwelling cannulae appeared to be a convenient model for studies of the metabolism of [1-^{14}C]arachidonic acid *in vivo* in carrageenin-induced inflammation. It enables the biochemical changes occurring within the inflamed locus to be investigated at any time during granuloma development in the same animal. Recovery of exudate is a simple atraumatic procedure, although on day 12 of inflammation accumulation of cell debris occurred within the chamber. Injection of 1 ml saline into one cannula to reduce viscosity and removal of the perfusate through the other one overcame the problem of obstruction. The injected [1-^{14}C]arachidonate was more than 99.9 per cent pure as revealed by prior thin layer chromatography and subsequent scanning of radioactive dots.

T.l.c. of the extract of the inflammatory exudate indicated that arachidonate was metabolised into hydroxy fatty acids, prostaglandins and thromboxane B_2 (Fig. 1). Maximally, 1.5 per cent of total radioactivity remained at the origin after development in both solvent systems. The amount of unconverted arachidonate, which represents the main spot in the chromatogram, showed a decrease during the development of inflammation. In contrast, the total amounts of products of cyclo-oxygenase activity, i.e. prostaglandins, thromboxane B_2 and hydroxy fatty acids, increased three-fold from day 1 to day 12 (Fig. 2). During this period radioactivity representing other lipids, running at the solvent front, increased from 8.2 per cent on day 1 to 21.8 per cent of total radioactivity on day 12 (Table 1). Elution of this fraction, followed by rechromatography, indicated that the fraction consisted mainly of triglycerides (unpublished results), presumably originating from the membranes of accumulated cells.

Our results further indicate that the major products formed by arachidonate cyclo-oxygenase activity were hydroxy fatty acids and PGE_2 (Fig. 1). The major part of the hydroxy fatty acids, produced during metabolism

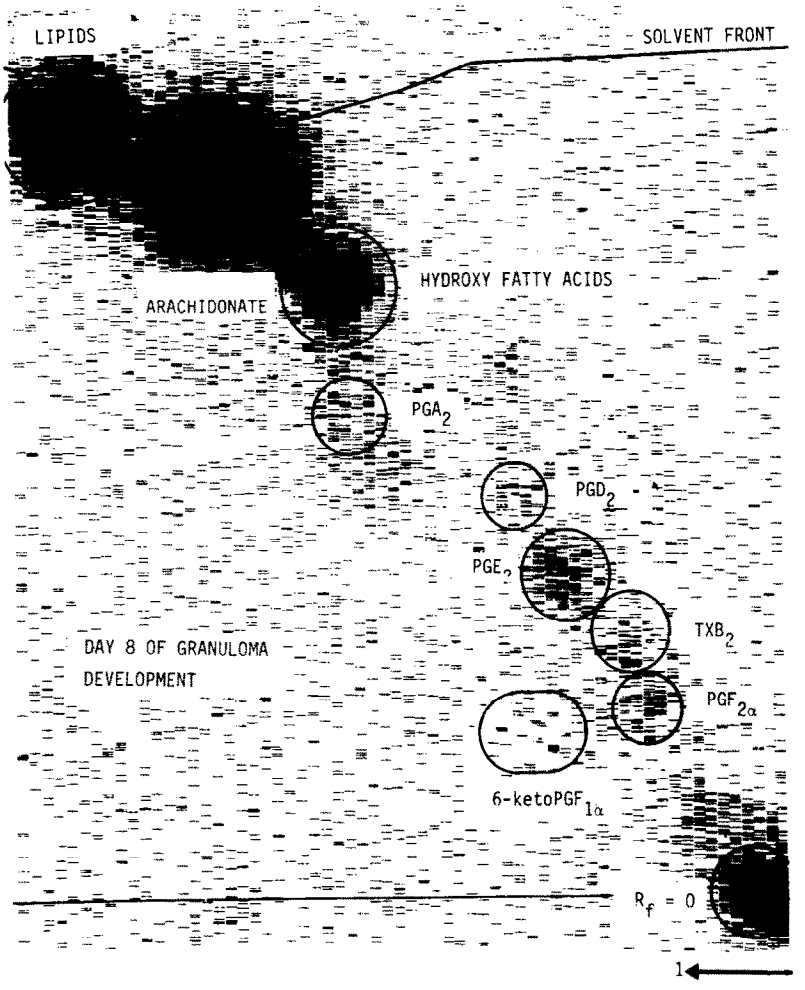

Fig. 1. Distribution of radioactivity on a t.l.c. plate after two-dimensional chromatography of the lipid material from an inflammatory exudate. Radioactive spots, accentuated by circles, were visualised by dot-scanning and the intensity of a dot is proportional to its radioactivity. $R_F = 0$ indicates the origin.

Table 1. Frequency of radioactive materials originating from locally injected arachidonate after 1 hour *in vivo* incubation

Radioactive spot	Per cent of total radioactivity*			
	Day 1	Day 4	Day 8	Day 12
PGE_2	0.23 ± 0.04	0.88 ± 0.42	0.76 ± 0.14‡	1.40 ± 0.59§
PGF_2	0.18 ± 0.04	0.27 ± 0.10	0.38 ± 0.04‡	0.58 ± 0.20§
PGD_2	0.11 ± 0.02	0.23 ± 0.07	0.24 ± 0.01**	0.35 ± 0.12§
PGA_2	0.23 ± 0.05	0.24 ± 0.03	0.29 ± 0.03	0.40 ± 0.06§
Unidentified lipids	8.21 ± 4.14	9.19 ± 2.67	19.73 ± 3.68§	21.80 ± 4.42§
Origin	0.57 ± 0.14	0.76 ± 0.15	1.11 ± 0.20§	1.35 ± 0.25§§
Ratio+ $\frac{TXB_2}{PGE_2 + PGA_2 + PGF_2}$	0.41 ± 0.13	0.11 ± 0.01§	0.17 ± 0.04	0.09 ± 0.03§

*Values represent means ± S. E. M. of 4 rats at different stages of granuloma development. The total formation of PGE_2 (not included in this table) was calculated from the sum of the individual values of PGE_2 and PGA_2 shown in the table.

+Ratios of TX/PGs were calculated for individual rats and the ratios shown represent means ± S. E. M. of 4 rats.

Metabolites of arachidonate included in the figures are not presented in this table. Significance was tested versus day 1 (one-tailed Student's *t* test, § = $P < 0.05$, ‡ = $P < 0.01$, §§ = $P < 0.025$ and ** = $P < 0.001$).

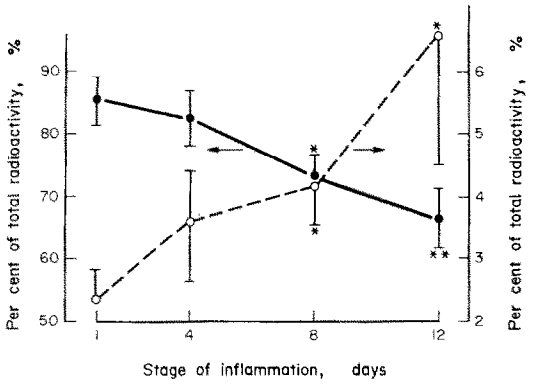

Fig. 2. Decrease in the amount of unconverted arachidonate (closed circles, unbroken lines) and increase in the total amount of prostaglandins, hydroxy fatty acids and thromboxane (open circles, broken line), during the development of granuloma in the rat. Arrows indicate the respective axes for the two curves. Each point represents the mean ± S.E.M. of 4 experiments. Significance was tested versus day 1 values (one-tailed Student's t test, * = $P < 0.05$, ** = $P < 0.01$).

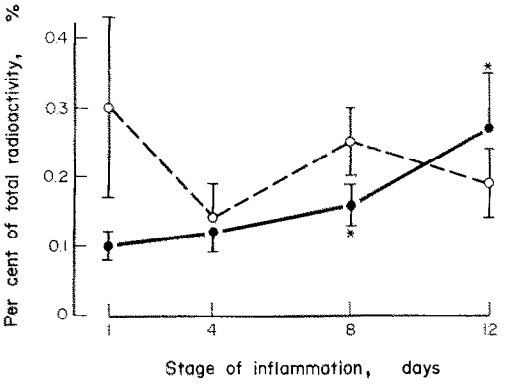

Fig. 4. Bioconversion of the injected arachidonate to 6-keto-prostaglandin $F_{1\alpha}$ (closed circles, unbroken line) and thromboxane B_2 (open circles, broken line) at different stages of granulomatous inflammation in the rat. Each point represents the mean ± S.E.M. of 4 experiments. Significance was tested versus values from day 1 of granuloma development (one-tailed Student's t test, * = $P < 0.05$).

of arachidonate *in vivo*, may consist of 12L-hydroxy-5,8,10-heptadecatrienoic acid (HHT) as deduced from the R_F value of the unlabelled standard. However, this cannot be concluded with certainty because the closely related substance, 5L-hydroxy-6,8,12,14-eicosatetraenoic acid [23], may occupy more or less the same position in the chromatogram. Moreover, the relatively small resolution capacity of the dot-scanner did not allow specification of the exact chemical nature of the fraction of hydroxy fatty acids. As our interest was primarily the formation of PGE_2, 6-ketoPGF$_{1\alpha}$ and TXB_2, we did not make an attempt to separate the fraction into HETE and HHT.

The capacity of the inflamed locus to convert arachidonate into both hydroxy fatty acids and PGE_2 showed an approximately threefold increase during granuloma development, reflecting an enhancement of cyclo-oxygenase activity in the later stages of inflammation (Fig. 3). At all time periods investigated, hydroxy fatty acids were formed in greater amounts than PGE_2. However, $PGF_{2\alpha}$ and PGD_2 were minor metabolites, which also increased approximately threefold between days 1 and 12 of the inflammatory process (Table 1). Another interesting observation was the very small capacity of the tissue for the biosynthesis of TXB_2 and 6-keto$PGF_{1\alpha}$ from arachidonate *in vivo*. From day 4 to day 12 both metabolites were synthesized in amounts even smaller than those of PGD_2 and $PGF_{2\alpha}$. The capacity for synthesis of 6-keto$PGF_{1\alpha}$ increased during granuloma development by two- to threefold (Fig. 4). TXB_2 formation did not increase in later stages when compared with day 1, but actually showed a tendency to decrease during the development of inflammation. From day 1 to day 8 TXB_2 formation exceeded that of 6-keto$PGF_{1\alpha}$, whereas on day 12 6-keto$PGF_{1\alpha}$ synthesis dominated.

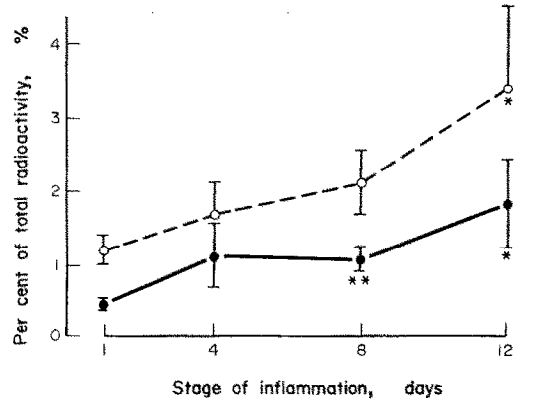

Fig. 3. Increase in hydroxy fatty acids (open circles, broken line) and prostaglandin E_2 (closed circles, unbroken lines) as a result of the *in vivo* metabolism of arachidonate by a developing granuloma in the rat. Each point represents the mean ± S.E.M. of 4 experiments. Significance was tested versus values from day 1 of inflammation (one-tailed Student's t test, * = $P < 0.05$, ** = $P < 0.005$).

The ratio $TXB_2/(PGE_2 + PGF_{2\alpha})$ showed a progressive decrease from day 1 to day 12 (Table 1). As the ethanol vehicle might possibly have influenced conversion of arachidonic acid or the ratios between the different metabolites, we carried out a separate experiment, using two rats, in which we investigated the metabolism of arachidonate, injected in a saline vehicle, on day 1 of inflammation. However, when suspending the arachidonate in saline it was found that a large percentage of the fatty acid adhered to the test tube and did not enter suspension. Nevertheless, when the small amount of arachidonate, which was suspended in saline, was injected into the cylinder, TXB_2 and 6-keto$PGF_{1\alpha}$ were again minor metabolites as compared to hydroxy fatty acids and PGE_2 while hydroxy fatty acid levels were somewhat less than those obtained using arachidonate dissolved in ethanol (data not shown). When acetone was used as a vehicle the results were similar to those obtained with the ethanol vehicle (data not shown).

DISCUSSION

The presence of prostaglandin-like activity in the exudate of a carrageenin-induced granuloma in the rat was first demonstrated by Willis in 1969 [3], using bioassay. Ever since, the role of prostaglandins as mediators of the early inflammatory response has been widely investigated [24–26]. However, it was discovered recently that PGE_1 can display an anti-inflammatory effect during the later stages of chronic inflammation [9, 11]. Possible mechanisms underlying this phenomenon have been reviewed [8].

It has also been suggested that labile intermediates in prostaglandin biosynthesis may also play an important role in inflammation [27], and granuloma tissue has been shown to convert arachidonate into TXB_2 [15] and 6-keto$PGF_{1\alpha}$ [16] *in vitro* as major metabolites.

The sodium salt and methylester of prostacyclin (PGI_2) both increased local vascular permeability when injected into an 8 day old granuloma in the rat. 6-Keto$PGF_{1\alpha}$ and TXB_2 were inactive in this respect [28]. PGE_1 and PGE_2 also increased vascular permeability, when administered locally on day 7, but failed to exhibit any effect when injected on day 1 [29].

Depriving rats of the essential fatty acid precursors for prostaglandin and thromboxane biosynthesis leads to a reduction in exudate accumulation but an increase in granuloma formation, when examined on day 8 of granuloma development, suggesting an inhibitory effect of fatty acid derivatives on the granulomatous component of inflammation [9]. Indeed, local injection of 1 μg PGE_1/day on days 4–7 of inflammation resulted in a decreased dry granuloma weight, whereas injection on day 1 enhanced granuloma formation [11]. Administration of PGE_2 showed similar effects on granuloma formation, but PGI_2 failed to reduce the weight of an 8 day old granuloma, when injected locally at 1 μg twice a day on days 4–7 of granuloma development [12]. The results reported in the present paper indicate that PGE_2 is a major metabolite formed from locally injected arachidonate by granulomatous tissue *in vivo*. During development of the granuloma an increased cyclo-oxygenase activity, measured as the sum of prostaglandins, thromboxane and hydroxy fatty acids formed from a fixed amount of AA in 1 hr, seems to parallel tissue growth. Capacity for biosynthesis of PGE_2 is enhanced in the later stage of granuloma development as compared to the initial phase, emphasising the possibility that PGE plays an important role in the chronic phase of inflammation. However, it should be noted that other authors have observed a decrease in the concentration of PGE in exudate during later phases of carrageenin-induced granuloma [30].

Our results further show the very small capacity of the granuloma tissue to convert arachidonate into TXB_2 and 6-keto$PGF_{1\alpha}$ *in vivo*. This is in strong disagreement with the observations of Chang *et al.* [31] obtained from *in vitro* experiments, showing that PGE_2 and $PGF_{2\alpha}$ were hardly formed but rather two other products, later established as being TXB_2 [15] and 6-keto$PGF_{1\alpha}$ [16], were synthetised. However, these results were obtained by the use of the 600 *g* supernatant of the homogenized granuloma tissue as an enzyme source, while membrane fractions of the homogenate were not investigated.

Moreover, in our inflammatory model, consisting of a s.c. implanted Teflon cylinder containing carrageenin, the metabolism of injected AA is not restricted to the granulomatous tissue itself. The inflammatory exudate may contribute to the metabolism of AA by supplying enzymes originating from cell breakdown. Phagocytozing polymorphonuclear leukocytes [32, 33], macrophages [34, 35], fibroblasts [36, 37], and platelets [38] accumulating in the inflamed area [39], have been shown to convert AA into prostaglandins, thromboxanes and hydroxy fatty acids.

Our results reveal the small tendency for the bioconversion of injected AA into TXB_2. This is consistent with results from the clinical experiments of Trang and his colleagues [40], who measured TXB_2 and PG levels in the synovial fluid of patients with rheumatoid arthritis. PGE_2 amply exceeded TXB_2 levels and the ratio $TXB_2/(PGF_{2\alpha} + PGE_2)$ was an average of 0.1 ± 0.05 in 5 patients. This ratio is in good agreement with the ratio in the chronic phase of experimental inflammation observed in our study (Table 1). Only at day 1 was this ratio relatively high, and this may indicate that thromboxanes play a major role in the early inflammatory stage.

The observation that hydroxy fatty acids are the major products formed from AA *in vivo*, suggests a possible role for these compounds in inflammation. It has been shown that the hydroxy fatty acids HETE and HHT exert a selective chemotactic effect on human polymorphonuclear (PMN) leukocytes but not on monocytes, and these compounds enhance random cell migration [41, 42]. In addition, increased concentrations of HETE have been reported to occur in the epidermis during psoriasis, an inflammatory skin disorder [43].

The hydroxy fatty acid HHT is formed from arachidonate by a cyclo-oxygenase, whereas HETE is formed by lipoxygenase activity, which is insensitive to inhibition by nonsteroidal anti-inflammatory drugs [38]. Both HHT and HETE can be identified and quantified by chromatography in combination with mass spectrometry, using deuterated standards [38]. Despite the availability of authentic standard compounds, we did not attempt to further specify whether HHT or HETE were present in the inflammatory exudate, because the resolution capacity of the dot-scanner was insufficient. Furthermore we had no access to mass spectrometry.

Nevertheless, our observation that hydroxy fatty acids, irrespective of their exact chemical nature, are the main metabolites formed during arachidonate bioconversion *in vivo*, combined with the chemotactic properties of these molecules, tempts speculation that HHT and HETE play an important role in inflammation.

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