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Enhanced capacity for release of leucotriene B_4 by neutrophils in rheumatoid arthritis

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SUMMARY The calcium dependent metabolism of endogenous arachidonic acid (AA) was investigated in 17 patients with rheumatoid arthritis during treatment with dextropropoxyphene alone and in 25 healthy volunteers. Incorporation of $[1-{}^{14}C]$AA into intracellular phospholipids of purified neutrophils was achieved by incubation until steady state before activation with ionophore A23187. Analysis of extracellular metabolites was performed by extraction, thin layer chromatography, autoradiography, and laser densitometry. The patients showed a twofold increase in the total capacity for oxidation of AA. Release of leucotriene B_4 (LTB_4) and its omega oxidation products, 20-OH LTB_4 and 20-COOH LTB_4, was 29%, range 11–48%, in patients compared with 8%, range 4–12%, in healthy volunteers. Total amounts of radioactivity released and the specific activity of LTB_4, as assessed by high pressure liquid chromatography, were equal in experimental and control groups. The demonstrated increased capacity for metabolism of AA to the major proinflammatory metabolite, LTB_4, via the 5-lipoxygenase pathway may contribute to perpetuation of inflammation and to tissue destruction in rheumatoid arthritis.

Key words: arachidonic acid.

Neutrophil granulocytes are present at the interphase of cartilage with pannus in rheumatoid arthritis[1] and are abundant in the cellular exudate of the rheumatoid joint.[2] Release of the potent lipoxygenase product of arachidonic acid (AA), leucotriene B_4 (LTB_4), by inflammatory leucocytes[3] may lead to further accumulation and activation. This will induce release of neutrophil derived elastase,[4] neutral protease,[5] and oxygen radicals[3] and cause local destruction of the tissue.

LTB_4 ranks with the most potent chemoattractants for leucocytes, complement 5a, and chemotactic tripeptide, f-MLP (unpublished data). This inflammatory mediator and its 20-OH and 20-COOH derivatives are present in significant amounts in rheumatoid joint fluid,[6 7] and its activity is markedly lowered for two to three weeks after a single, intra-articular injection of steroid.[7]

The aim of the present study was to assess the total enzymatic capacity of peripheral neutrophils for oxidation of endogenous arachidonic acid to the potent, proinflammatory lipoxygenase products, LTB_4 and 5-hydroxyeicosatetraenoic acid (5-HETE), in patients with rheumatoid arthritis. Calcium ionophore A23187 was chosen as the activating agent in order to bypass specific receptors with possible functional defects[8] and to secure a maximal activation of the metabolic pathways.

Patients and methods

PATIENTS

During three months, 17 consecutive outpatients with classic or definite rheumatoid arthritis according to the criteria of the American Rheumatism Association[9] were selected for the study. None of the patients had received treatment with gold compounds, D-penicillamine, or steroids within the preceding six weeks. Non-steroidal anti-inflammatory drugs were withdrawn for at least 10 days before blood sampling. During this wash out period, only dextropropoxyphene was administered.

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Table 1 *Clinical data and routine laboratory values in 17 patients with rheumatoid arthritis*

	Sedimentation reaction (mm/h)	*C reactive protein (nmol/l)*	*Joint score†*	*Morning stiffness (min)*
Rheumatoid arthritis	35 (2–110)*	70 (3–411)	8·5 (0–21)	60 (0–300)
Healthy volunteers	0–20	0–74	—	—

*Values are medians with range in parentheses.
†According to Ritchie *et al*[10]

The patients included 10 women and seven men, median age 66 years, range 20–84 years. The control population consisted of 25 healthy volunteers taking no medication and matched in respect to age and sex. Clinical data and routine laboratory values are given in Table 1.

REAGENTS AND EQUIPMENT

The following reagents were used: ethylenediaminetetra-acetate (EDTA) (Merck, Darmstadt, FRG); methylcellulose (Apodan, Copenhagen, Denmark); Lymphoprep (Nygaard, Oslo, Norway); glutamine (Gibco, Middlesex, UK); [1–^{14}C]AA (37×10^3 Bq/ml, $2\cdot2\times10^9$ Bq/mmol, Amersham International, Buckinghamshire, UK); purified human albumin (Behringswerke, Marburg, FRG); and calcium ionophore A23187 (Calbiochem, La Jolla, CA, USA).

The measurements were performed using thin layer chromatography, Kieselgel 60 F_{254} (Merck Cat No 11·798); autoradiography, Osray-C-films (Agfa Gevaert, NV, Mortsel, Belgium); laser densitometry, Ultroscan 2202 (LKB, Bromma, Sweden) with integrator, Spectra-Physics 4100 (Spectra-Physics, San José, CA, USA).

Pure standards, 5-HETE and LTB_4 (Paesel, Frankfurt, FRG), and prostaglandin E_2, 12-hydroxy-5,8,10-heptadecatrienoic acid (HHT) (Upjohn, Kalamazoo, MI, USA) were used. High pressure liquid chromatography was performed with Waters M45 pump, NovaPack column, and absorbance detector 440 (Waters, Milford, MA, USA) at 229 and 280 nm.

NEUTROPHIL FUNCTION TEST

Neutrophils were isolated from peripheral blood drawn into EDTA (10 mM) by a modification of Böyum's method,[11] including (*a*) sedimentation of erythrocytes with methylcellulose (0·8%), (*b*) gradient centrifugation of buffy coat leucocytes on Lymphoprep, and (*c*) hypotonic lysis of residual erythrocytes. The intracellular pools of phospholipids were labelled with [1–^{14}C]AA (37×10^3 Bq/ml) until steady state by incubation ($0\cdot5\times10^7$ leucocytes/ml) in RPMI 1640 (2 mM glutamine) for five hours under carbon dioxide (5%).[12] The labelled neutrophils were challenged with ionophore A23187 (10 μM, 15 min). Released radiolabelled compounds were extracted by the method of Folch *et al*,[13] separated by thin layer chromatography (TLC) (developing solvents: chloroform: methanol: acetic acid: water; 90:9:1:0·65), and measured by autoradiography and laser densitometry.[12 14]

The specific activity of LTB_4 and 5-HETE was measured by high pressure liquid chromatography (HPLC) both in samples of patients and controls.

Results

Challenge of [1–^{14}C]AA labelled neutrophils with ionophore A23187 led to mobilisation of phospholipids from intracellular pools and to oxidation, mainly via the lipoxygenase pathway.[12] This process was measured in cells obtained from patients with rheumatoid arthritis by the release of total radioactivity, ranging from 3 to 10×10^2 Bq/5×10^6 cells (Table 2). Fractionation by TLC showed a twofold increase in the capacity for oxidation of AA by cells from patients with rheumatoid arthritis (Table 3, Fig. 1) with a corresponding increase in the relative contribution of both LTB_4 (Table 3, Fig. 2) and its 20-OH and 20-COOH catabolites (Table 3). These two degradation products of LTB_4 were not separated in the TLC system used.

HPLC controls revealed identical specific activities of LTB_4 in patients and controls, ranging from 3·4 to 6·9 Bq/mmol. Only trace amounts of *6-trans-*

Table 2 *Release of total radioactivity, representing AA and its metabolites, by [1–^{14}C]AA labelled neutrophils during challenge with A23187*

Group	*Radioactivity* $\times 10^{-2}$ *(Bq/5×10⁶ leucocytes)*
Rheumatoid arthritis (n=17)	6·7 (2·6–9·9)*
Healthy volunteers (n=25)	6·4 (2·6–11·1)

*Values are medians with range in parentheses.

Table 3 *Percentage contribution of arachidonic acid (AA) and its metabolites to total release of activity by activated, [1-^{14}C] labelled neutrophils*

Group	*AA†* (%)	*LTB_4†* (%)	*20-OH LTB_4/ 20-COOH LTB_4†* (%)	*5-HETE* (%)	*HHT* (%)
Rheumatoid arthritis (n=17)	30 (22–66)*	17 (7–20)	13 (3–28)	15 (13–20)	1 (0–8)
Healthy volunteers (n=25)	66 (56–84)	5 (3–10)	3 (1–6)	15 (10–19)	2 (1–5)

*Values are medians with range in parentheses.
†$p<0.01$.
LTB_4 = leucotriene B_4; LTB_4 degradation products = 20-OH LTB_4 and 20-COOH LTB_4; 5-HETE = 5 hydroxyeicosatetraenoic acid; HHT: 12-hydroxyheptadecatrienoic acid.

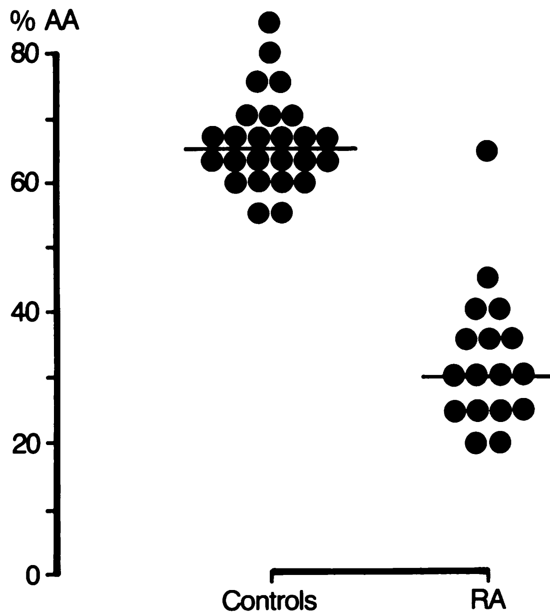

Fig. 1 *AA as percentage of total radioactivity released by [1-^{14}C]AA labelled neutrophils after challenge with A23187 in patients with rheumatoid arthritis (RA) and healthy volunteers (controls).*

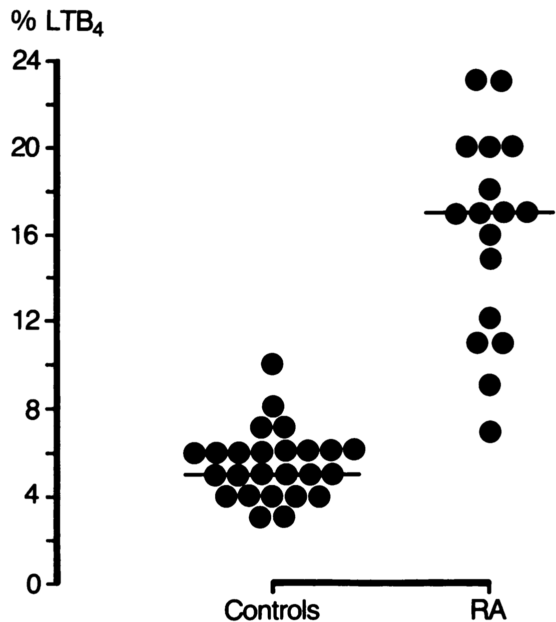

Fig. 2 *LTB_4 as percentage of total radioactivity released by [1-^{14}C]AA labelled neutrophils after challenge with A23187 in patients with rheumatoid arthritis (RA) and healthy volunteers (controls).*

LTB_4 were identified by HPLC. Little cyclo-oxygenase activity was found, either in patients or controls, and there was a minor contribution from a metabolite inhibited by indomethacin and identified as 12-hydroxyheptadecatrienoic acid (HHT) (Table 3).[12]

The main fraction of the residual activity was more lipophilic than AA, possibly indicating esterification to cholesterol or glycerol. This fraction was not calculated by laser densitometry. Fig. 3 shows a representative chromatogram of [1-^{14}C]AA metabolites released by neutrophils from a patient with rheumatoid arthritis and a healthy volunteer. None of the data correlated with the clinical score of joint involvement, with the duration of morning stiffness, or with the acute phase reactants.

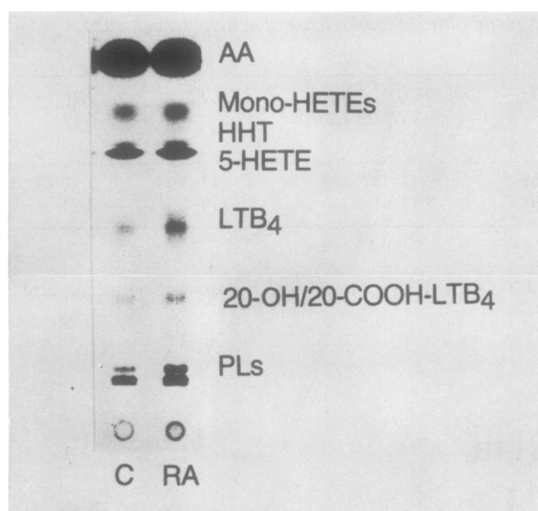

Fig. 3 *Separation of arachidonic acid and its oxidation products by thin layer chromatography in a representative sample of healthy volunteers (controls (C)) and of patients with rheumatoid arthritis (RA). PLs = phospholipids.*

Discussion

In patients with rheumatoid arthritis the present study showed an increased capacity of neutrophils to oxidise endogenous arachidonic acid to the proinflammatory metabolite, LTB_4, via the lipoxygenase pathway. The increased extracellular level of LTB_4 catabolites, 20-OH and 20-COOH LTB_4, speaks against an underlying decrease in LTB_4 degradation. The increased oxidation capacity of the neutrophils apparently is specific, since the cyclooxygenase activity, the capacity for release of endogenous arachidonate, and the oxidation to 5-HETE were within normal values. An increased LTB_4 synthetase activity may explain the enhanced LTB_4 release in rheumatoid arthritis in association with the decreased level of arachidonate.

The 5-lipoxygenase enzyme responsible for leucotriene synthesis in human neutrophils has recently been shown to exhibit an absolute requirement for calcium ions.[15] Accordingly, the calcium ionophore A23187 was chosen as the activating agent. It is assumed to produce a maximal synthesis of leucotrienes in response to calcium influx.

The nature of this cellular abnormality remains obscure. Considerable variation exists in the distribution of AA in various intracellular pools and of AA metabolising enzymes in different human cell types.[16] Even mononuclear phagocytes, i.e., the same cell type, may differ in respect to AA metabolism according to the site of origin.[17] The cellular mechanism responsible for these differences has not been elucidated.

Recently, sulphasalazine, a drug used in the treatment and prophylaxis of chronic inflammatory bowel disease, has been recommended for the treatment of rheumatoid arthritis.[18 19] The active moiety of sulphasalazine, 5-aminosalicylic acid, inhibits the 5-lipoxygenase activity of neutrophils in vitro[20] and may thus correct the abnormality shown by the present experiments, without the side effects of steroid treatment. High concentrations (mM) are needed, however, for a substantial inhibition of the synthesis of the proinflammatory mediators, LTB_4 and 5-HETE.[20] Such concentrations of 5-aminosalicylic acid are found in the distal part of the colon during treatment of chronic inflammatory bowel disease with conventional doses of sulphasalazine,[20] but these exceed the plasma level by a factor of approximately 10^3. The possibility that sulphapyridine and not 5-aminosalicylic acid is the pharmaceutically active moiety in the treatment of rheumatoid arthritis with sulphasalazine has recently been supported by clinical data.[19] The capacity of non-steroidal anti-inflammatory drugs selectively to inhibit arachidonate conversion to prostaglandins, i.e., cyclo-oxygenase activity,[21] points to the importance of other cellular elements, such as macrophages, with an approximately equal capacity for cyclo-oxygenation and lipoxygenation of endogenous AA.[22]

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