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LEUKOTRIENE B_4 PRODUCTION BY STIMULATED WHOLE BLOOD: COMPARATIVE STUDIES WITH ISOLATED POLYMORPHONUCLEAR CELLS

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A new method was developed to study leukotriene B_4 (LTB_4) production by stimulated whole blood. The calcium ionophore A23187 and serum-treated zymosan induced LTB_4 production, measured by radioimmunoassay, in a dose- and time-dependent manner. The pattern of LTB_4 production by whole blood differed markedly from that observed with isolated, purified polymorphonuclear leukocytes. Higher levels of LTB_4 were reached and maintained in whole blood. The system allowed to detect drug effects on LTB_4 synthesis in vitro. This new method to study the synthesis of LTB_4 takes into account the complex interactions between different cell types which can modulate LTB_4 metabolism.

Human polymorphonuclear leukocytes (PMNs), when activated by diverse stimuli, oxydize arachidonic acid (AA) through a 5-lipoxygenase (5-LO) to form leukotriene (LT)A_4 which is in turn enzymatically transformed mainly into LTB_4 and in part into LTC_4 (1). Isolated PMNs, resuspended in buffer and stimulated, have essentially been used for studies on the synthesis and metabolism of LTB_4 and on the inhibition of its production. The use of such system may, however, have serious limitations. LT production by leukocytes may be influenced by products formed by other cells present in blood. Furthermore, the washing procedures needed to separate the leukocyte population following the administration of putative 5-LO inhibitors may partly displace the drug from its target leading to an artefactual weakening of the inhibitory effect. In addition, the use of isolated PMNs in buffer for drug testing in vitro does not reflect the in vivo situation where drug binding to proteins or other cell membranes may operate.

We have developed a method for studying LTB_4 production by whole blood stimulated in vitro with the calcium ionophore A23187 or with serum-treated zymosan (STZ). Using this method, which utilizes a radioimmunoassay (RIA) for the measurement of LTB_4, we have characterized a number of differences in the pattern of LTB_4 production from stimulated whole blood or isolated PMNs. This system was useful for testing 5-LO inhibitors in vitro.

MATERIALS AND METHODS

Blood sampling and stimulation procedures: Blood was collected in plastic tubes, containing either no anticoagulant or 0.01 vol of a 1000 U/ml solution

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of sodium heparin in saline, from drug-free healthy volunteers. One ml aliquots were rapidly dispensed into glass (non anticoagulated blood) or plastic (heparinized blood) tubes, 75x10 mm, containing known concentrations of inducers of LTB_4 synthesis or their vehicle, briefly mixed by vortexing and then placed in a water bath at 37°C. After a fixed interval the tubes were centrifuged (2,000 x g for 15 min for serum; 12,000 x g for 2 min for plasma), the serum or plasma were separated and stored frozen at -80°C until further processing. The isolation of PMNs from heparinized human blood was carried out using dextran sedimentation and Hypaque/Ficoll gradients according to Böyum. Contaminating erythrocytes were removed by hypotonic lysis. PMNs were suspended in Hanks' balanced salt solution (HBSS), pH 7.35, without Ca^{++} and Mg^{++} at a concentration of 10^7 cells/ml. The cells in these preparations were 98.8 ± 0.5% PMNs (n=5) (89.5 ± 1.5% neutrophils, 8.1 ± 1% eosinophils, 1.2 ± 0.4% basophils) and 1.2 ± 0.5% lympho-monocytes. The average platelet to leukocyte ratio was 0.66 ± 0.11 (n=15). Vitality, measured by trypan blue, was always above 95%. Ca^{++} and Mg^{++} (0.6 and 0.8 mM, respectively, final concentrations) were added to the cell suspensions immediately before starting the incubations. 0.5 ml aliquots of PMN suspensions in plastic tubes were placed in a water bath for 10 min at 37°C for various intervals. The reaction was stopped by centrifugation at 12,000 x g for 2 min and the cell free supernatant was stored at -80°C until assayed by RIA without further extraction. A stock solution of A23187 was made up in DMSO and further dilutions were prepared using autologous plasma or serum. This procedure was adopted to avoid the red cell lysis sometimes observed when using the A23187 solution in DMSO. Final concentration of DMSO never exceeded 0.5%. Zymosan was boiled for 30 min in PBS, then it was incubated with normal human serum (20 mg/ml) for 30 min at 37°C and subsequently washed twice with Ca^{++} and Mg^{++} -free HBSS and finally resuspended in HBSS containing 0.6 mM Ca^{++} and 0.8 mM Mg^{++}.

Extraction of the samples and radioimmunoassay of LTB_4: Measurement of immunoreactive-LTB_4 (i-LTB_4) was carried out by RIA on extracted serum or plasma using an anti-LTB_4 antibody purchased from Amersham Int. (Amersham, England). Four different extraction procedures were compared: triple extraction with 2.5 vol ethylacetate; protein precipitation with 9 vol methanol; protein precipitation with 9 vol aceton; extraction (0.1 ml samples, pH=3) on Sep-Pak C_{18} cartridges (Waters Associates, Milford, Mass., USA). Lipid-containing fractions were dried under vacuum in a Speed Vac Concentrator (Savant Instruments Inc., Hicksville, NY, USA) and the dry residues were resuspended in 600 µl RIA buffer (composition: 50 mM Tris:HCl containing 0.1% gelatine, pH 8.6). Aliquots of plasma or serum were spiked with labeled LTB_4 and extracted following the above procedures. Recoveries were 61.6 ± 0.7 (n=3), 79.3 ± 1.1 (n=8), 78.6 ± 0.9 (n=8), and 73.6 ± 4.6 (n=3) with the ethylacetate, methanol, aceton and Sep-Pak C_{18} methods, respectively. The aceton extraction method was adopted, for practical reasons, and used throughout the study. The recovery of cold standard LTB_4 added either to plasma and serum or to whole blood reflected that observed with the radiolabeled material. RIA was carried out according to the manufacturer's instructions. Briefly, antiserum against LTB_4 was diluted in RIA buffer and 100 µl aliquots were transferred to tubes containing 100 µl of the sample and 100 µl of ^3H-LTB_4 (approximately 2700 cpm); 100 µl RIA buffer was added and the mixture was incubated overnight at 4°C. 200 µl dextran-coated charcoal were added and the samples were mixed by vortexing, centrifuged (2 min at 12,000 x g) and the radioactivity in the supernatant counted in a liquid scintillation counter. Fifty percent displacement for LTB_4 was at 53 pg/100 µl; the least detectable amount was 12.5 pg in 100 µl buffer. The cross-reactivities (provided by the manufacturer) were: LTB_4 = 100%; diastereoisomers of 5,12-dihydroxy-6,8,10-trans-14-cis-ETE = 3.3%; 12-HETE = 2%; diastereoisomers of 5,6-diHETE = 1.6%; 5(s),12(s)-diHETE = 0.14%; LTC_4, LTD_4, 5-HETE, 11-HETE, 15-HETE, PGE_2 and other prostaglandins, arachidonic acid: all equal or < 0.04%.

Identification of immunoreactive material released by stimulated whole blood: Radiolabeled LTB_4 was added to heparinized plasma and the sample was

processed as described. In addition, 10 ml aliquots of heparinized whole blood, stimulated for 80 min either with A23187 25 µM, or STZ 2 mg/ml or not stimulated, were processed as described. The extracted samples, evaporated to dryness, were dissolved in 300 µl methanol, filtered through a millipore 0.45 µm filter (Millipore, Yonezawa, Japan) and injected for resolution in RP-HPLC. RP-HPLC was carried out on a Varian 5000 liquid chromatograph using a Nucleosil RP C_{18} column (250 x 4.6 mm, Alltech Associates), at a flow rate of 1 ml/min in an isocratic solvent of MeOH/H_2O/HOAc, 78:22:0.01, pH 4.9. 0.5 ml fractions were collected and the eluate was evaporated to dryness in a Speed Vac Concentrator. The residues were dissolved in RIA buffer and assayed by RIA as described. Standards were applied to the same column and LTB_4 was monitored at A_{270}, 15-HETE, 12-HETE and 5-HETE at A_{235}.

Materials: LTB_4, 12-HETE, 15-HETE and 5-HETE were given by Dr. J. Rokach, Merck Frosst, Canada; BW755c by Dr. G.A. Higgs, Wellcome Res. Laboratories (Beckenham, England); Nafazatrom by Dr. E. Philipp, Bayer AG (Wuppertal, West-Germany); Zymosan A and nordihydroguaiaretic acid (NDGA) were from Sigma (St. Louis, Mo, USA); A23187 from Calbiochem (La Jolla, Ca, USA); Ficoll/-Hypaque and macromolecular dextran from Pharmacia (Uppsala, Sweden); HBSS from Gibco (Paisley, Scotland); Indomethacin, from Merck Sharp & Dohme (Rahaway, NJ, USA); Acetylsalicylic acid, lysine salt from Synthélabo (Brussels, Belgium); ^3H-LTB_4 from Amersham Int.; Heparin, sodium salt, from Hoffmann-La Roche (Basle, Switzerland).

All data are expressed as mean ± SEM.

RESULTS

LTB_4 production by stimulated whole blood: Both A23187 and STZ stimulated the synthesis of i-LTB_4 by whole blood in a dose-dependent manner (EC_{50} in anticoagulated blood was around 27.5 µM and 0.5 mg/ml, respectively) (Fig. 1). i-LTB_4 production was a time dependent process when using either inducer, reaching a maximum between 10 and 15 min for A23187 in heparinized blood, and

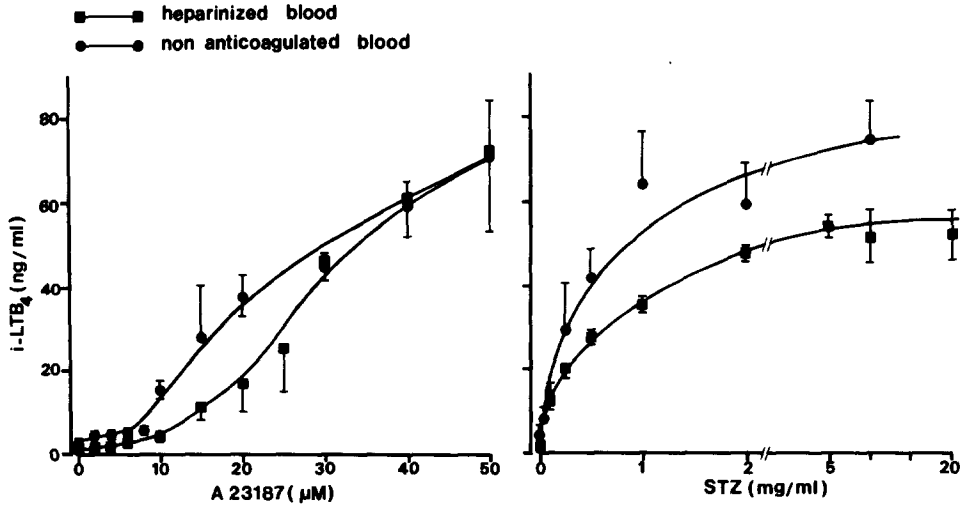

Fig. 1: Dose-response curve for A23187- (left) and STZ- (right) induced i-LTB_4 production by human heparinized (■--■) and non anticoagulated (●--●) whole blood. Each point is the mean of at least 3 experiments. The incubation time was 80 min. EC_{50} was defined as the concentration giving 50% of maximal stimulation.

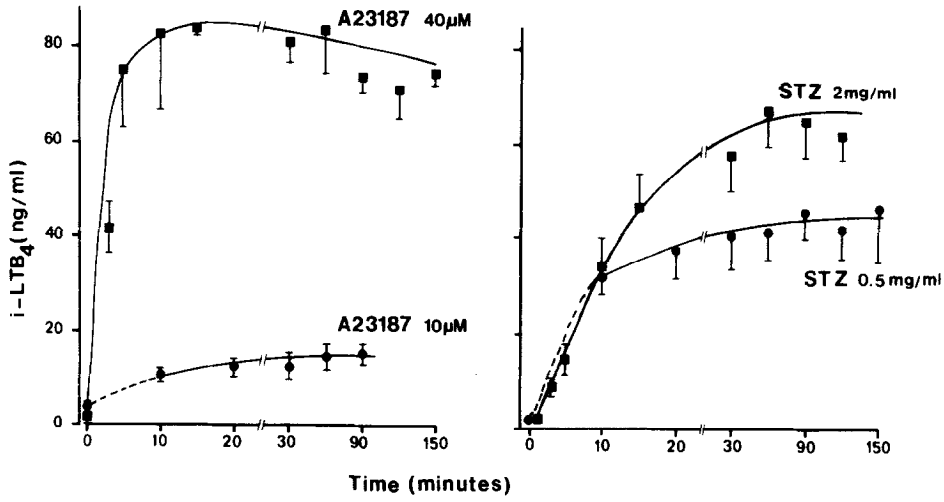

Fig. 2: Time course of the effect of A23187 (left) and STZ (right) on i-LTB_4 production by human heparinized (■--■) or non anticoagulated (●--●) whole blood. Each point is the mean of at least 3 experiments.

between 30 and 60 min for STZ and for A23187 in non anticoagulated blood; the amounts of i-LTB_4 then tended to remain constant up to 150 minutes (Fig. 2). Mean i-LTB_4 levels in maximally stimulated anticoagulated whole blood were 28.8 ± 2.7 ng/10^6 PMNs (n=34) when using A23187 and 18.9 ± 2.2 ng/10^6 PMNs (n=20) with STZ. Also in suspensions of isolated human PMNs A23187 and STZ induced a time and concentration dependent production of i-LTB_4. However, less A23187 and more STZ were required to get an optimal stimulation (EC_{50} around 2.1 µM and 8.5 mg/ml, respectively) (Fig. 3). Peak levels were reached at 5 min with a subsequent drastic drop in the following period down to levels less than 10% of the peak for A23187-induced i-LTB_4 production at 90 min (Fig. 3). Maximum levels of i-LTB_4 attained were 6.03 ± 0.7 ng/10^6 PMNs (n=8) for A23187- and 1.5 ± 0.4 ng/10^6 PMNs (n=3) for STZ-induced stimulation.

Identification of the LTB_4-immunoreactive material produced by stimulated human whole blood: ^{3}H-LTB_4 added to heparinized plasma was recovered after extraction and RP-HPLC as a single peak with an elution time similar to that of authentic unlabeled LTB_4. Recovery of radiolabeled material after extraction and HPLC was 73.1%. RIA of the fractions obtained from RP-HPLC of STZ- and A23187-stimulated anticoagulated whole blood samples showed that most of the immunoreactive material (81.7%) coeluted with LTB_4-standard (Fig. 4). Minor peaks of cross-reactivity were observed in the fractions eluting at 3 to 4.5 min (12.2% of total) and at 7.5 to 8.5 min (4.6% of total) (Fig. 4). Non stimulated whole blood samples gave two very small peaks of immunoreactive material with retention times of 4 and 11 min, respectively (data not shown).

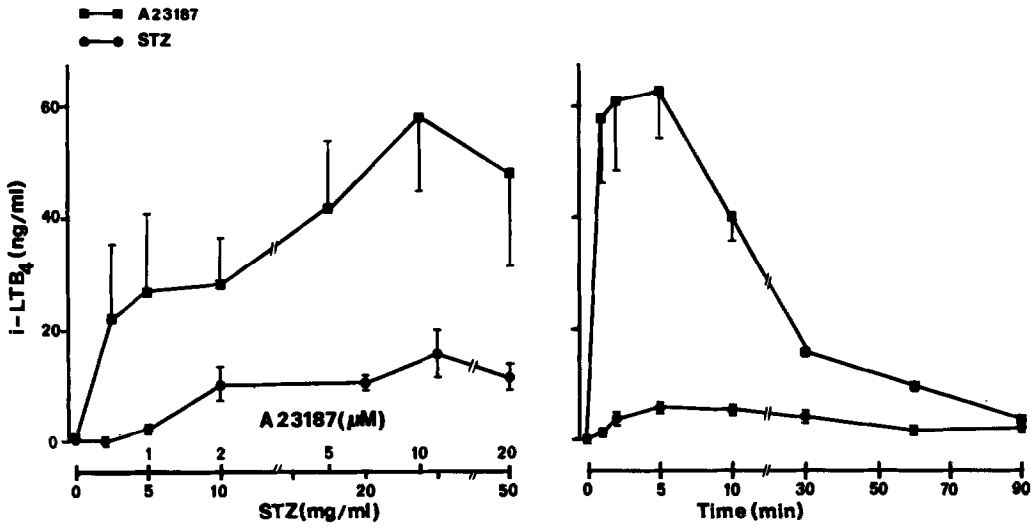

Fig. 3: Dose-response curve (5 min incubation) (left) and time course (10 μM A23187 and 20 mg/ml STZ) (right) of i-LTB_4 production by isolated human PMNs stimulated with A23187 (■—■) or STZ (●—●). Each point is the mean of at least 3 experiments.

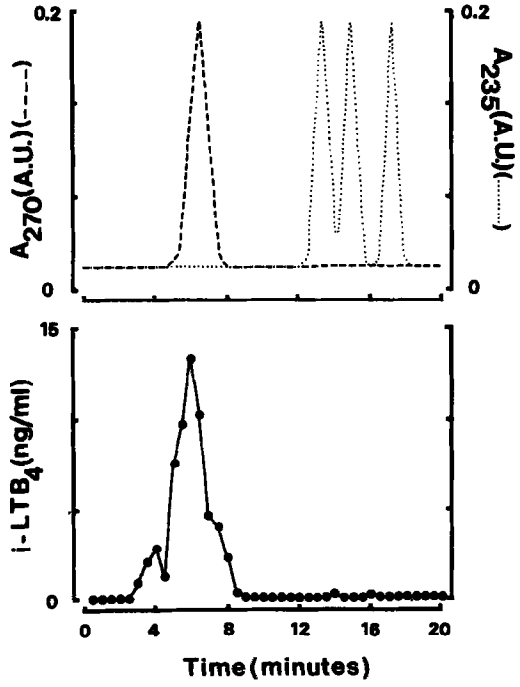

Fig. 4: RP-HPLC of the supernatant of whole blood stimulated with 2 mg/ml STZ (see methods). i-LTB_4 (lower panel) was measured on 0.5 ml fractions. In the upper panel are shown the elution times of authentic LTB_4 (first peak), 15-HETE (second peak), 12-HETE (third peak) and 5-HETE (fourth peak) measured by UV absorbance.

Table I: Reproducibility of LTB_4 RIA in extracted plasma and serum samples

	LTB_4 level (ng/ml)	Coeff. of variation	
		Intraassay (n)	Interassay (n)
SERUM	2.37 ± 0.3 (10)	12.7% (10)	-----
	8.27 ± 2.9 (15)	16.3% (10)	15.2% (5)
	70.2 ± 6.2 (7)	7.9% (4)	11.2% (3)
PLASMA	1.21 ± 0.2 (15)	4.3% (10)	17.3% (5)
	2.73 ± 0.6 (15)	9.9% (10)	23.7% (5)
	70.1 ± 14.4 (12)	5.3% (7)	29.8% (5)

Reproducibility of the measurement of LTB_4 in stimulated whole blood: The reproducibility of the assay was measured by performing repeated analyses, either on the same day or on different days, on 3 plasmas and 3 seras (1 unstimulated and 2 stimulated) containing different amounts of i-LTB_4 (Table I). Moreover, 1 volunteer was bled 10 times over a 10 days period and his blood (non anticoagulated) was stimulated with STZ 2 mg/ml: the coefficient of variation was 19.5%.

Inhibition of LTB_4 production: A series of cyclooxygenase, lipoxygenase and double cyclo/lipoxygenase inhibitors were tested for their activity on the production of i-LTB_4 by human whole blood stimulated with different concentrations of STZ or A23187 (Table II). While nafazatrom, NDGA and the dual cyclo/lipoxygenase inhibitor BW755c inhibited dose dependently i-LTB_4 production, the cyclooxygenase inhibitors aspirin and indomethacin, as expected, did not (Table II). In some cases, after cyclooxygenase inhibition an increased production of i-LTB_4 was observed in stimulated whole blood, especially when A23187 was used as an inducer (data not shown).

DISCUSSION

Our results show that human whole blood produces LTB_4 upon stimulation with A23187 or with STZ. When comparing LTB_4 formation by stimulated whole

Table II: Estimated IC_{50} (μM) of some drugs on i-LTB_4 production by A23187- and STZ-stimulated human anticoagulated blood

COMPOUND	A23187 (μM)			STZ (mg/ml)	
	10	20	30	1	2
NDGA	0.4	0.1	2.4	2	13
BW755c	4.7	---	---	---	30
NAFAZATROM	0.5	---	27	---	6.5
ASPIRIN	>500	>500	---	>500	---
INDOMETHACIN	>100	>100	---	>100	>100

blood with that by stimulated isolated PMNs some differences appear. Firstly, while much more A23187 is required in whole blood to get an optimal leukocyte stimulation, most probably due to binding of this lipophilic compound to cell membranes and/or plasma proteins (2), much less of the more physiologic stimulus STZ is required in whole blood. This phenomenon might be due to further opsonization of the zymosan when mixed with whole blood. Indeed, in one series of experiments we compared the effects of 3 different concentrations (0.25, 2 and 20 mg/ml) of opsonized or non-opsonized zymosan on LTB_4 production by isolated PMNs and by whole blood. The unopsonized zymosan was completely inactive on isolated PMNs while it was as active as the opsonized one in whole blood (data not shown). Alternative explanations for the greater effectiveness of zymosan in whole blood could be the generation of C5a (3) which might activate LTB_4 formation as other chemotactic factors do (4,5), or the liberation of AA by other cells (6,7) during the whole blood stimulation procedure, which can greatly enhance STZ-induced LTB_4 production by PMNs (4).

Secondly, the temporal pattern of LTB_4 production is rather different in whole blood and in isolated leukocytes. The drastic decrease in isolated cells after 5-10 min has already been observed and it is the expression of rapid metabolism of LTB_4 to 20-OH-LTB_4 and 20-COOH-LTB_4 (8). The addition of exogenous AA to stimulated leukocytes could maintain elevated levels of LTB_4 for a longer period of time indicating that the decreased production of LTB_4 probably reflects limited substrate availability (8). The lack of a late fall in LTB_4 levels in stimulated whole blood reflects either continued LTB_4 production, possibly favoured by an exchange of substrate between other activated cells and PMNs, or a slowing down of LTB_4 catabolism.

Thirdly, the amounts of LTB_4 produced by whole blood are higher than those produced by isolated leukocytes when using both A23187 and STZ. Besides the mentioned possible delivery of AA by platelets and monocytes (6,7) and the reduced catabolism of LTB_4 in whole blood, platelet-derived 12-HPETE could promote activation of leukocyte 5-LO (9); other cell-cell signals may exist (10). Finally, monocytes and erythrocytes by themselves can synthesize LTB_4 in response to A23187 or to LTA_4 (1,7,11,12).

The nature of the i-LTB_4 produced by stimulated whole blood was confirmed by its chromatographic behavior. The two small fractions of cross-reacting material observed were not identified, but they are likely to represent isomers of LTB_4 (13). With the antibody used a background level of i-LTB_4 was measured in unstimulated whole blood: 1.3 ± 0.4 ng/ml (n=24) for plasma and 2.55 ± 0.45 ng/ml (n=24) for serum. This material to a large extent is not an AA metabolite formed in vitro because NDGA or aspirin cause only a marginal reduction of it. The different extraction procedures tested did not affect the levels of this i-LTB_4 background measured in unstimulated samples.

Although a considerable donor to donor variability is encountered using this procedure, the coefficients of variation seem to be contained within acceptable limits so to allow repeated studies in the same subjects for the detection of drug effects. The relative easiness of the method which allows processing of large number of samples in short times, the possibility of working with minimal amounts of blood, the fact that the synthesis of LTB_4 by PMNs is studied in a more physiologic milieu, represent important advantages of this procedure. Using this method we have already tested several pharmacological products potentially affecting LT synthesis. For most of these the results obtained were essentially similar to those obtained in isolated PMNs by us or by others (5,14); however, for one experimental compound the 5-LO inhibitory activity disappeared in whole blood, due to extensive protein binding (to be published elsewhere). Using the whole blood stimulation system one can simultaneously measure the effects of drugs on LTB_4 and on TXB_2 production, thus exploring both the lipo- and the cyclo-oxygenase pathways. In our in vitro experiments BW755c inhibited TXB_2 production besides LTB_4 formation, while nafazatrom only affected LTB_4 synthesis (data not shown). Preliminary reports on the use of stimulated whole blood for testing drug effects on LTB_4 production have appeared (15,16).

In conclusion, the study of LTB_4 production by stimulated whole blood may allow to better characterize the physiology of LTB_4 metabolism by PMNs in their natural milieu. This technique may provide a relatively simple, fast and reproducible method for studying drug effects on 5-LO in vitro and ex vivo. This method might also be helpful to study LTB_4 production in conditions in which a derangement of the lipoxygenase metabolic pathway is suspected.

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