Leukotriene-induced neutrophil aggregation in vitro

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1. INTRODUCTION

Neutrophilic polymorphonuclear (PMN) leukocytes react with increased adherence, oxidative metabolism, chemotaxis, degranulation and bacterial killing after contact with substances generated in an inflammatory or infected site, e.g., the C5a-fragment and bacterial products, such as fMLP. One of the earliest recognized manifestations of an acute inflammatory response is granulocyte adhesion to the vascular endothelium, adjacent to the inflammatory focus. This increased adhesiveness may also lead to an increment in granulocyte aggregation, which can be measured in vitro in an aggregometer.

A novel group of mediators of inflammation are called leukotrienes [1]. These are formed within PMNs after stimulation with fMLP or the calcium ionophore A23187 by a lipoxygenase-dependent oxygenation of arachidonic acid. The generated epoxide leukotriene A_4 (LTA_4) is unstable and converted to leukotriene B_4 (LTB_4) by enzymatic hydrolysis. Two other 5,12-dihydroxy isomers (compounds I,II) can also be formed by a non-enzymatic hydrolysis of LTA_4.

LTB_4 can be further metabolized to a ω-hydroxylated compound (20-OH-LTB_4) and the corresponding dicarboxylic acid, (20-COOH-LTB_4) [2].

Abbreviations: LTB_4, leukotriene B_4 (5(*S*), 12(*R*)-dihydroxy-6,8,10,14-eicosatetraenoic acid); fMLP, formyl-methionyl–leucyl–phenylalanine; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; HPLC, high performance liquid chromatography

LTB_4 stimulates neutrophil migration (in vitro and in vivo), aggregation and degranulation [3–6]. Compounds I and II also induce migration and adherence but are less potent than LTB_4 and cause only a marginal degranulation [7,8]. 20-OH-LTB_4 and 20-COOH-LTB_4 also stimulate migration but to a lesser degree than LTB_4 [9]. This report concerns effects on granulocyte aggregation by several lipoxygenase-dependent oxygenation products of arachidonic acid.

2. METHODS

2.1. *Preparations of leukotrienes and related compounds*

All compounds tested in this study were obtained from incubations of human leukocytes. The leukocytes were prepared from white-blood-cell concentrates (buffy coat), kindly supplied by the Blood Central at the Karolinska Hospital. For preparation of cells, see [10]. In short, the procedure involves dextran sedimentation, ammonium-chloride-induced lysis of red cells and centrifugation. For incubation cells were suspended in Dulbecco's PBS, or in HBSS buffered with HEPES. These cells were incubated with arachidonic acid with or without ionophore A23187. To obtain LTB_4 [11] cells were incubated with arachidonic acid plus the ionophore A23187. These incubations also produced the ω-oxidation products (i.e., 20-OH and the 20-COOH derivatives) of LTB_4 [2,12]. The 20-COOH derivative of LTB_4 was also obtained in incubations of leukocytes with the tripeptide fMLP [13]. Compounds

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thus obtained were purified by acidic ether extraction, column chromatography on silica gel and HPLC. For separation of LTB_4 straight phase HPLC (SP-HPLC) was performed. The compounds were converted to the methyl esters before SP-HPLC. An additional purification on reverse phase HPLC (RP-HPLC) was performed. Hereafter, the free acid forms were regenerated by saponification and again purified on RP-HPLC.

Quantitation of thus prepared leukotrienes was based on UV-spectroscopy. The absorption maxima around 270 nm were used, and an extinction coefficient of 40 000 has been assumed. In later determinations of LTB_4, however, the extinction coefficient 50 000 was used [14].

2.2. *Neutrophil isolation*

Purified human PMN leukocytes (of >95% purity) were isolated from heparinized (10 IU/ml-without preservative) blood. The blood was layered on a discontinous Percoll gradient (Pharmacia Fine Chemicals, Uppsala). The separation was performed by a modification of the method in [15]. To the stock iso-osmotic Percoll was added a solution consisting of 1 part of HBSS/1 part 0.075 M Tris–HCl to prepare the desired densities, calculated to be 1.0990 and 1.0776 g/ml, respectively. The osmolarity was found to be 280–310 mOs/kg H_2O and pH 7.5–7.6. The gradient was centrifuged at $400 \times g$ for 5 min at 20°C. The platelet-rich plasma supernatant was then removed and centrifugation was continued for another 20 min at $800 \times g$. The granulocyte band was carefully removed and washed twice in heparinized 0.9% saline before lysis with ammonium chloride (0.155 M NH_4Cl) for 5 min to free the PMNs of contaminating erythrocytes. Purified PMN leukocytes were suspended in Hank's balanced salt solution with 1% human serum albumin (HBSS-A) at 1.15×10^7 cells/ml.

2.3. *Neutrophil aggregation*

PMN aggregometry was performed as in [16]. A standard platelet aggregometer (Model 300 BD, Payton Assoc., Buffalo NY) was used. To a siliconized cuvette, containing a stir bar revolving at 900 rev./min was added 0.45 ml PMN suspension (1.15×10^7 cells/ml). After 2 min delay to allow warming of cells to 37°C, 50 μl of the aggregant fMLP or lipoxygenase products were added, dissolved in 0.1% ethanol in HBSS. The resulting changes in light transmission were recorded as ΔT. To provide the necessary amplification for a well-defined aggregation wave the aggregometer/record system was calibrated with a PMN suspension diluted 50% (v/v) with HBSS. The tested aggregants were suspended in HBSS in serial dilutions ranging from 10^{-5}–10^{-11} M.

3. RESULTS

The maximum change in light transmission (ΔT), after stimulation with different lipoxygenase products or fMLP, is given in fig.1. LTB_4 induces aggregation to a greater degree than any of the other lipoxygenase products tested here. However, fMLP causes a more pronounced maximal light transmission than the leukotrienes. The peak response occurs for LTB_4 at 10^{-7} M and for fMLP at 10^{-6} M, but, while the lowest concentration of fMLP that induces aggregation is 10^{-9} M, LTB_4 has some effect even at 10^{-10} M. Of the other compounds tested here 20-OH-LTB_4 was more active than 20-COOH-LTB_4, and almost as active as LTB_4, but did not induce any aggregation at 10^{-10} M. Compounds I and II also had weak aggregating activity.

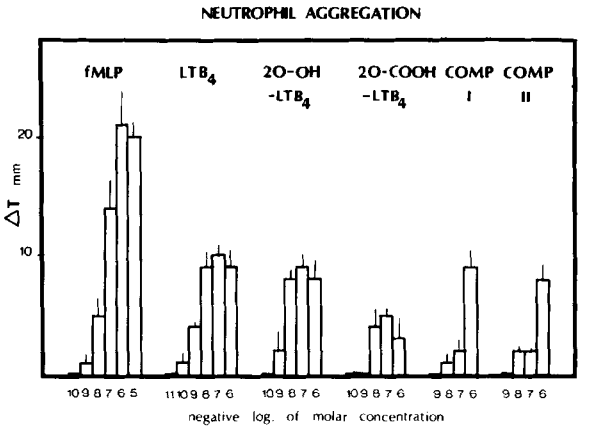

Fig.1. PMN aggregation after stimulation with fMLP and the lipoxygenase products. The results are expressed as means and SE values of the maximum change in light transmission (ΔT). PMNs were obtained from at least 3 different healthy subjects. When HBSS without fMLP or leukotrienes was added no aggregation was induced.

4. DISCUSSION

Aggregometry is a sensitive in vitro method for detecting inflammatory mediators such as fMLP, C5a and leukotrienes [17,18]. According to [19] it might be the most sensitive technique known to determine C5a and, hence, complement activation. In vivo PMN aggregation is believed to be a mechanism in the adult repiratory distress syndrome, a pulmonary dysfunction occurring in patients subjected to hemodialysis or cardiopulmonary bypass, and, possibly also, myocardial infarction [20].

Here we show that LTB_4 induces aggregation to a greater degree and at lower concentrations than any of the other lipoxygenase products tested. This is in accordance with the findings that LTB_4 is also the most potent inducer of adherence, chemotaxis and enzyme release of the hitherto tested lipoxygenase metabolites of arachidonic acid. Whereas compounds I and II do have some effect on adherence and chemotaxis they also induce aggregation but only in high concentrations. Interestingly, the order of potency of LTB_4, 20-OH-LTB_4 and 20-COOH-LTB_4 as inducers of aggregation appears to parallel their activities as chemoattractants [9].

Lipoxygenase products, particulary LTB_4 but also other leukotriene compounds, are potent stimulators of many neutrophil activities including PMN aggregation. These endogenous mediators could be the second messengers by which other inflammatory mediators, such as fMLP and C5a, exert their stimulatory effects on neutrophil functions. This hypothesis is supported by the finding that fMLP and serum-coated zymosan induce the synthesis of leukotrienes [13,21].

The various leukotriene compounds may regulate the in vivo PMN response to other inflammatory mediators; this mechanism could enhance the tissue damage that ensues when complement activation induces intravascular neutrophil aggregation.

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