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PURIFICATION AND MASS SPECTROMETRY IDENTIFICATION OF LEUKOTRIENE D_4 SYNTHESIZED BY HUMAN ALVEOLAR MACROPHAGES

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SUMMARY : Human AM obtained by BAL from normal subjects and asthmatic patients converted [1-^{14}C]-AA into a polar labeled metabolite. The structure of this metabolite, after two successive purifications on TLC (silicagel plates then reversed phase plates) and mass spectrometric analysis was shown to be identical to an authentic sample of LTD_4. The amount of LTD_4 recovered in the culture medium of AM was attempted to be related to pathological lung profile. In our experimental conditions AM from allergic asthmatics synthetized more LTD_4 than cells from healthy subjects and from aspirin sensitive asthmatic patients.

AM have an important role in non specific and immunologic lung defenses. Human AM synthetize a variety of substances involved in these functions including metabolites of AA (1, 2, 3, 4, 5, 6). Previous works have demonstrated production by AM of normal and asthmatic human lungs, of cyclooxygenase and lipoxygenase products from AA (7, 8, 9, 10, 11). SRS-A (slow-reactive substance of anaphylaxis) which is now considered to be a mixture of leukotrienes predominantly LTC and LTD (12), has been considered to be a mediator of broncho-constriction in asthma. This belief is the result of experimental observations, such as the ability of sensitized human lung to release SRS-A when exposed to antigen (13) and the prolonged contraction of human airway preparation by SRS-A "in vitro" (14, 15). Holroyde and coll. (16) have recently demonstrated that synthetic leukotrienes C and D were able to produce moderate broncho-constriction and coughing when these substances are inhaled by human volonteers.

Abbreviations : AM, alveolar macrophage ; BAL, bronchoalveolar lavage ; AA, arachidonic acid ; LTD_4, leukotriene D_4 ; RBL, rat bosophylic leukemia cells ; BSTFA, N-O-bis trimethylsilyltrifluoroacetamide ; TMCS, trimethylchlorosilane ; TMS, trimethylsily ether derivative ; m/e, mass/charge ratio.

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In the present study we investigated the lipoxygenase pathway of AA metabolism in human AM and particularly the release of LTD_4 by these cells labeled with [1-^{14}C] AA. LTD_4 released in the culture medium of AM was purified with an easy proceeding and identified by mass spectrometry. Comparative studies were made with LTD_4 produced by RBL (17) and with synthetic LTD_4.

MATERIALS AND METHODS

. Selection of subjects : Six healthy humans (ages 20 to 35 years), ten patients with allergic bronchial asthma (ages 18 to 62 years) and five with aspirin sensitive asthma (ages 31 to 66 years), underwent bronchial lavage, none of these subjects had ever smoked and non had used anti-inflammatory drugs including aspirin for 8 days prior to the study.

BAL was performed as it was previously described (10).

. Materials : [1-^{14}C] AA (specific activity 50 mCi/mmole) was purchased from the Radiochemical Center (Amersham, England). LTD_4 standard was generously supplied by J. Rokach, Ph. D. (Merck Frosst Canada Inc.). Latex beads (1,1µ) and Ionophore A 23187 came from Sigma Chemical Co. (St-Louis, Missouri), RBL was purchased from ATCC (Rockeville, Maryland). Materials and media used for cell cultures were from Flow Laboratories (Puteaux, France).

. Culture cell and precursor incubations :

1. Macrophages culture and [1-^{14}C] AA labeling : Macrophages monolayers were processed and cultured as it was described in previous published methods (9, 10). The cells (2 x 10^6 cells for a 60 x 35 Petri dish) were incubated with 5 ml of 199 medium for 2 hours. The cultures were then washed three times and the remaining adherent cells were incubated for an additional 24 h of medium 199 and labeled with 500 000 d.p.m. of [1-^{14}C] AA. At the end of the labeling the viability of adherent cells was assessed from duplicate culture plates (35 x 15 Petri dish, 1 x 10^6 cells) by trypan blue dye exclusion test and phagocytosis of latex particules was performed during an additional 40 min. incubation in the same culture medium. Mast cells were never observed on culture plates staining with Giemsa and toluidine blue.

2. RBL culture and [1-^{14}C] AA labeling : RBL cells were grown and incubated with AA in the same conditions as those described by Samuelsson (17).

. Extraction and purification procedure :

1. Extraction and purification of labeled LTD_4 obtained in the culture media of RBL cells : the incubation and extraction were proceeded according to B. Samuelsson (17) and G.S. Blackwell (18), except that the precursor (AA) was radiolabeled, and purification according to P. Piper (19).

2. Extraction, purification and structure analysis of the polar radiolabeled AA metabolite obtained in the culture media of AM : the cell supernatant was made 80 p. 100 (vol/vol) in ethanol. After 4 hr. at 4°C, the ethanol solution was centrifuged and evaporated to dryness under reduced pressure. The extract was then analyzed by TLC on silicagel plates prewashed with methanol by ascending elution for 16 hr. TLC was run in the solvent system I ethyl acetate-1 p. 100 acetic acid. The radiolabeled products were localized by scanning on a radiochromatograph Scanner Berthold (Model LB 2760).

. Purification of the polar metabolite of AA : the area in the origin of plates was scraped off and eluted with methanol. The methanol was evaporated under a nitrogen stream and the residue was spotted on KC 18 reversed phase plates (Whatman). The chromatography was carried out in the solvent system II ehtanol-water (80-20, vol/vol). The radioactive peak with the same Rf of an authentic sample of LTD_4 was scraped off and eluted with methanol. The methanol was evaporated under nitrogen and the residue stored at -80°C for mass spectrometry analysis.

. Derivatization of the polar metabolite of AA : the purified metabolite was dissolved in 10 µl of methanol and methylated by 500 µl of ethereal diazomethane. After evaporation the residue was treated with 100 µl of a pyridine/BSTFA/TMCS (1-6-1 vol/vol/vol) mixture during 20 min. The reactants were evaporated and the sample stored in 30 µl of anhydrous hexane at -80°C until Mass Spectrometry analysis.

. Mass Spectrometric Analysis of the polar metabolite of AA by direct inlet system : Derivative study was performed on a LKB 2901 instrument. Temperature of the ion source was 240°C. The accelerating voltage and the electron beam energy current were respectively 3,5 kV and 70 eV.

An authentic sample of 2 µg of LTD_4 was treated upon the same conditions.

RESULTS AND DISCUSSION

When [1-^{14}C] AA was incubated with human AM and the radiolabeled products separated by TLC, the major radioactive metabolite detected was at the origin (Fig. 1A). The level of radioactivity in the 6 keto $PGF_1\alpha$, PGE_2, TxB_2 zones was very low, compared to the origin area. Two different purification proceedings were carried out on the polar material recovered from the TLC plate.

1) Comparison of the radiolabeled polar material with radioactive LTD_4 obtained from RBL cells. In the presence of Ionophore A 23187 and cysteine according to the conditions described by B. Samuelsson (17), RBL cells released in the culture medium the only radiolabeled metabolite detected at the origin of silicagel plate when analysed by TLC in the solvent system I (Fig. 1B). This polar product purified according to the processus described by Samuelsson (17), showed the UV spectrum characteristic of the triene chromophore (λ max 280 nm) and identical to the litterature data (19, 20). The LTD_4 was then methylated, acetylated by the soft acetylation method described by P. Piper and then silylated. The mass spectrum was shown to be a mixture of N-acetyl, OTMS, methyl ester (M^+ 638) and N-acetyl O-acetyl methyl ester (M^+ 608).

The polar metabolite provided by several experiments on human AM was purified in the same conditions and comparison with the labeled LTD_4 was greatly in favour of the same structure.

2) Comparison of the polar [1-^{14}C] AA metabolite in macrophages culture medium with an authentic non radioactive sample of LTD_4.

To prevent so a long and tedious purification, the polar metabolite of human macrophages medium, detected at the origin of TLC in the solvent system I was spotted on reversed phase KC 18 plates (RP KC 18) in the solvent system II. The radiochromatogram of figure 2 showed the apparition of two radioactive peaks A and B. An authentic sample of LTD_4, spotted on FKC 18 developped in the same solvent system, comigrated with peak B. B was scraped off, eluted with methanol. The corresponding product, derivatizated as descri-

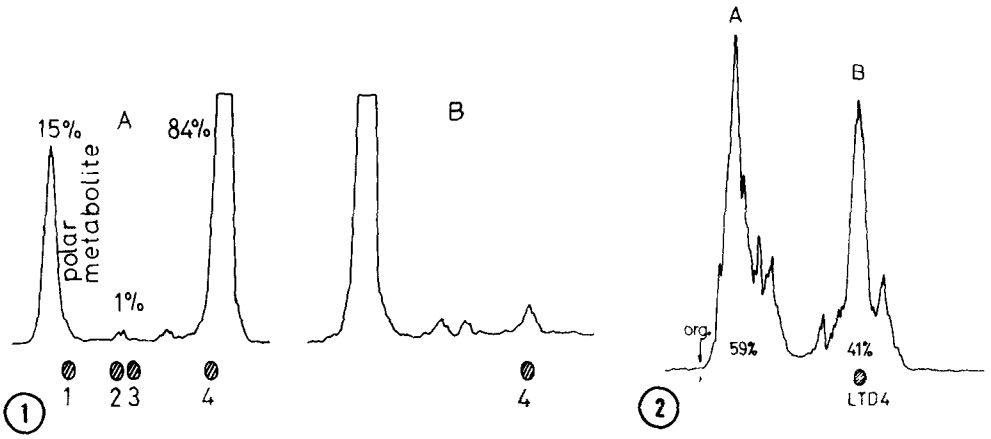

Fig. 1 : A. Chromatograms on silicagel plates of the radioactive products formed from [1-^{14}C] by human AM : 1 = 6 keto $PGF_1\alpha$; 2 = PGE_2 ; 3 = TxB_2 ; 4 = AA B. Chromatogram on silicagel plate of the labeled metabolite of the [1-^{14}C] AA released in the RBL cell medium.
(For A. and B. = solvent System I).

Fig. 2 : Chromatogram on KC 18 reversed phase TLC of the radioactive polar metabolite of [1-^{14}C] AA in the culture medium of human AM (Solvent System II)

bed in "Materials and Methods", gave the only N-TMS, O-TMS, methyl ester derivative. Simultaneous derivatization was carried out on 2 µg of non radioactive LTD_4. The same fragments (except m/e 653) were recovered for the mass spectrum obtained from the AA polar metabolite (peak B, Fig. 3). The absence of molecular ion M^+ 668 does not affect the interpretation as the structure was found by combining the informations from the main characteristic fragment ions (Table I).

The data system coupled with the LKB 2900 apparatus gave every characteristic fragment ions for several experimentations : the fraction of LTD_4 recovered after reversed phase KC 18 TLC purification of the origin area was always in the order of 40 p. 100 (Fig. 2). This result, the mass spectrometric identification of LTD_4 and the reproductibility in the existence of the polar [1-^{14}C] AA metabolite in macrophage culture media after 24 hours allowed us to extend the LTD_4 quantification to a series of three different populations of subjects submitted to BAL : healthy subjects (6), patients with allergic bronchial asthma (10) and patients with sensitive asthma (5). Figure 4 reported the percent of labeled LTD_4 recovered after reversed phase TLC purification of the polar metabolite. These data were analyzed by the Mann Whitney U test extended by Milton (21). At the level of 0,01 a significant difference was observed between the healthy subjects and the patients with allergic asthma : AM of allergic asthma patients synthetized more LTD_4 than healthy subjects. The amount of LTD_4 synthetized by aspirin sensitive asthmatics was the same as healthy subjects.

In our incubation conditions of [1-^{14}C] AA metabolization by normal and pathological human AM (i.e. without any addition of calcium Ionophore

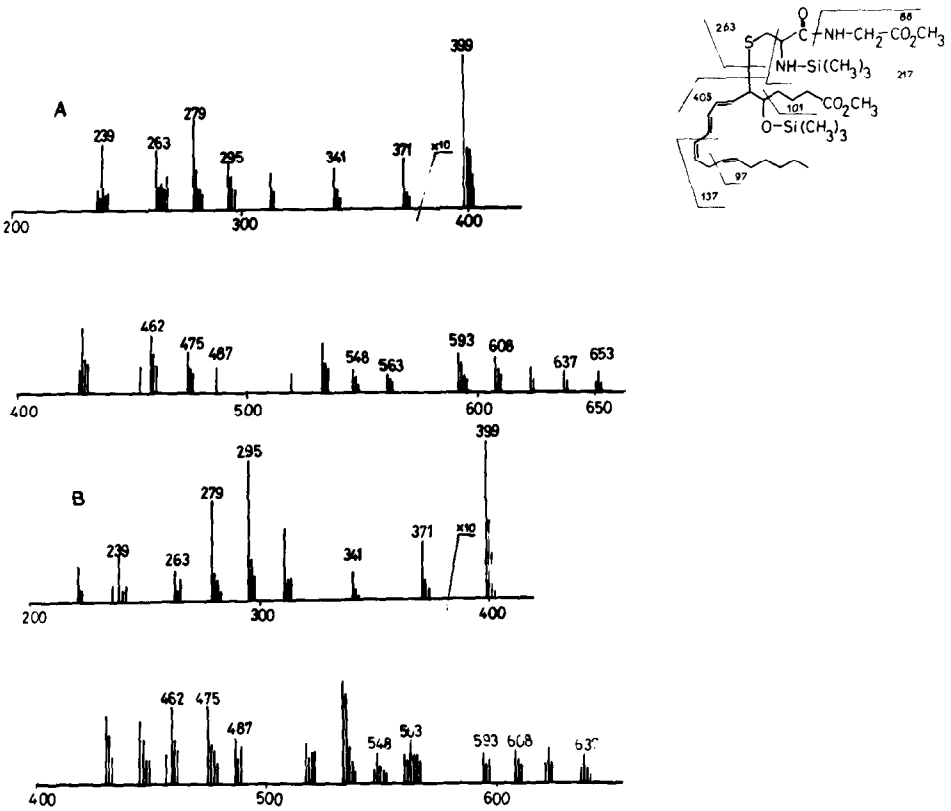

Fig. 3. Trimethyl silyl methyl ester derivative mass spectrometric fragmentation :
A : authentic sample of LTD_4
B : polar product released in A.M. culture media.

A 23187) reversed phase TLC was demonstrated to be a very easy purification proceeding and mass spectrometric analysis, a powerful tool for identifying structure of very low amounts of LTD_4.

TABLE I : Main characteristic mass spectrometry fragmentations of the N-trimethylsilyl O-trimethylsilyl methyl ester derivative of LTD_4 (263 corresponds to the non lipophilic part of the molecule). OHTMS = 90 ; NH_2TMS = 89.

m/e		m/e	
653	M^+-CH_3	399	$M^+-(90 + 89 + OCH_3 + CO_2CH_3)$
637	M^+-OCH_3	371	$M^+-(90 + 89 + OCH_3 + (CH_2)_2CO_2CH_3)$
564	$M^+-(89 + CH_3)$	341	$M^+-(90+89+CO_2CH_3+NH_2CH_2CO_2CH_3)$
563	$M^+-(90 + CH_3)$	295	$M^+-(217 + 97 + CO_2CH_3)$
549	$M^+-(89 + 2CH_3)$	279	$M^+-(203 + 89 + 97)$
548	$M^+-(90 + 2CH_3)$	263	M^+-405
463	$M^+-(89 + CH_3 + (CH_2)_3CO_2CH_3)$	239	$M^+-(203 + 89 + 137)$
462	$M^+-(90 + CH_3 + (CH_2)_3CO_2CH_3)$		

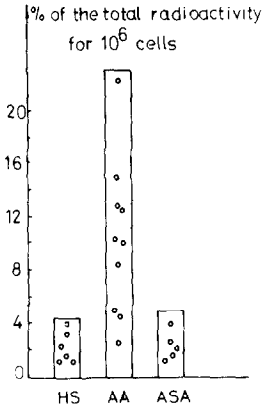

Fig. 4 : Amounts of labeled LTD_4 recovered in macrophage culture media for three different populations of subjects : HS healthy subjects. AA : allergic asthmatics. ASA : aspirin sensitive asthmatics.

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