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LEUKOTRIENE BIOSYNTHESIS AND METABOLISM DETECTED BY THE COMBINED USE OF HPLC AND RADIOIMMUNOASSAY

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A radioimmunoassay for leukotriene D_4 (LTD_4) has been developed which exhibits sufficiently high sensitivity to be useful in conjunction with RP-HPLC in the detection of LTC_4, LTD_4 and LTE_4 in physiological samples. The detection limit of the assay was approximately 240 amoles, using antiserum TG1 at a dilution of 6×10^3, with 50% displacement at 70 fmoles. Antiserum NW1, also at a dilution of 6×10^3, displayed a detection limit of 9 fmoles with 50% displacement at 100 fmoles. The two antisera have similiar crossreactivities, both manifesting useful affinities for LTE_4 and LTC_4, and low or negligible affinities for other arachidonic acid metabolites, or their derivatives. The radioimmunoassay was used to detect 1) LTC_4, LTD_4 and LTE_4 released from perfused rat lung in response to platelet-activating factor (PAF) stimulation, 2) conversion of exogenous LTD_4 to LTE_4 in human blood, and 3) endogenous leukotrienes in human blood samples.

Leukotriene D_4 (LTD_4) is a major constituent of slow reacting substance of anaphylaxis (1), a mixture of lipoxygenase products of arachidonic acid (2), possessing potent smooth muscle contracting activity (3, 4), in addition to cardiovascular and respiratory actions such as plasma exudation (5) and mucus secretion (6). The peptidoleukotrienes are produced from human and guinea pig lung in response to allergic hypersensitivity reactions (7) and stimuli such as C5a (8), and their production has been demonstrated in rat basophilic leukemia (RBL-1) cells (9), mastocytoma cells (10), macrophages (11), and eosinophils (12).

Until recently the major form of assay for LTD_4 has been the guinea pig ileum bioassay (13, 14). The drawbacks to the bioassay are a detection limit of approximately 1 pmole, and a lack of sensitivity to the related leukotrienes C_4 and E_4(15). Speculation as to a contributory role for leukotrienes in pathological conditions, such as the bronchoconstriction associated with asthma, was the driving force for the structure elucidation (1, 16, 17) and is now a major

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impetus for the development of alternative means of quantitation. In the past two years a number of immunochemical assays have been reported, exhibiting a wide range of specificities and sensitivities to LTB_4 , LTC_4, LTD_4 and LTE_4 (18-24). The specificity of the peptidoleukotriene assays has however relied only upon the low crossreactivity of the antisera to other leukotrienes. Here we report a radioimmunoassay (RIA) protocol which now allows us to specifically measure low levels of LTC_4, LTD_4, and its major metabolite, LTE_4 in clinical samples and perfusates. The methodology was tested in LT generation from rat perfused lungs stimulated by PAF, LTD_4 incubation in vitro in human blood and the measurement of endogenous LTs in human blood samples, using reverse phase high performance liquid chromatography (RP-HPLC) to separate leukotriene species, thus giving specificity to the assay (25).

MATERIALS AND METHODS

Synthetic LTD_4, C_4, and E_4 were quantitated using U.V. absorbance on a Cary 219 spectrophotometer at 280 nm, using the peptidoleukotriene extinction coefficient of 40,000.

Preparation of immunogen: Four rabbits were immunised with immunogen prepared from 300 ug thyroglobulin (TG) (Sigma, UK) and 100 ug LTD_4 with 0.05 uCi (^{3}H)LTD_4 (New England Nuclear, UK) conjugated with 0.03% gluteraldehyde at 4°C for 48 hours, and subsequently dialysed (yeild 70%). The dose of conjugated LTD_4 was 5-10 ug per rabbit. Immunogen was made up to 2.6 ml with physiological saline prior to mixture with Freund's Adjuvant (Difco Laboratories, USA). Keyhole lympet haemocyanin (KLH) (Calbiochem, USA), (20 mg) was mixed with 0.75 mg LTD_4 and 0.05 uCi (^{3}H)LTD_4 and conjugated and dialysed in the same manner as the TG conjugate and brought to a final volume of 5 ml (yield 85%). 80 ug conjugated LTD_4 was introduced per rabbit in the KLH regime.

Immunisation: Four rabbits were initially given the TG immunogen in the first two innoculations, and switched to KLH immunogen for subsequent boosts. Two rabbits were started with KLH immunogen, and were administered only one boost. The TG rabbits were given a ten week rest after the initial immunisation, and then were boosted at four week intervals until the 16th week, at which point immunisation was suspended for 3 months. The rabbits administered only KLH immunogen were given a three month rest after the first innoculation. Bleeding was conducted eight weeks after the first immunisation, and seven to nine days after each boost. Immunisations were commenced with Freund's Complete Adjuvant and subsequently alternated with Incomplete Adjuvant, in a ratio of immunogen/adjuvant of 1:3, v/v. Innoculations were introduced intradermally, in volumes of approximately 50 ul, at between 25 and 35 sites on the rabbits' backs. Blood was collected via an ear vein, allowed to clot at room temperature for 2 hours, and filtered using Millex-AA filters (Millipore Ltd., UK). Antisera were aliquoted into 1 ml fractions and stored at -20°C.

Assay: Sample or standards were introduced to a final volume of 50 ul assay buffer (phosphate buffered saline, ionic strength 0.15, 0.25% gelatine, pH 7.4). Serum was diluted to 2.5×10^3 in assay buffer, and 50 ul was added to the sample or standards, which were assayed in triplicate. After mixing, the tubes were allowed a preincubation period of 30 min at room temperature before addition of label. (^{3}H)LTD_4 (6000 dpm, 75 fmoles) was added in 50 ul assay

buffer, mixed, and the incubation allowed to continue at room temperature for 90 min.

Free label was precipitated by addition of 500 ul charcoal mixture (1g Norit charcoal (BDH Chemicals, UK), 100 mg dextran (Sigma, UK) in 250 ml assay buffer) and centrifugation at 2,000 g for 5 min. at 4°C. The supernatant, 700 ul, was added to 4.5 ml Aquassure scintillation fluid (NEN,UK) and counted on an Intertechnique scintillation counter.

Rat lungs were perfused as previously described (14,26). Indomethacin (2.8 uM) was infused 15 min prior to injection of PAF (5 ug) and perfusate was collected for 2 min after PAF stimulation.

10 ml samples of venous blood were collected from volunteers into tubes containing 1 mg AA 861 (Takeda Industries, Japan), cooled on ice and the plasma separated by centrifugation.

Sep-pak extraction, HPLC and LTD_4/blood incubation were conducted as described previously (25, 27, 28).

RESULTS AND DISCUSSION

Of the six rabbits immunised, all showed production of antibodies, the titres gradually rising to between 10^2 and 10^3 in the TG immunised animals. After the introduction of KLH and large amounts of hapten (80 ug LTD_4/rabbit) the titres increased more dramatically, and after the three month rest two bleeds displaying the high titres (both of 6 X 10^3) were used in the radioimmunoassay; these corresponded to one of the initial TG immunised rabbits (TG1) and one of the KLH-only immunised rabbits (NW1).

Antiserum TG1 showed a 50% displacement of (^{3}H)LTD_4 by LTD_4, LTC_4 and LTE_4 of 70 fmoles, 1.0 pmoles, and 410 fmoles respectively, and an LTD_4 detection limit of 240 amoles ($p<0.0025$, Student's t test) (Table 1). The Ka of this antisera was 1.1×10^{10}.

Antiserum NW1 showed a greater sensitivity to LTE_4 , with 50% displacement of 100 fmoles LTD_4 ,1.3 pmoles LTC_4, and 210 fmoles LTE_4, and a relatively higher affinity to LTA_4-methyl ester, LTB_4, and arachidonic acid (Table 1). Antisera

Table 1 CROSSREACTIVITY OF ANTISERA TG1 (NW1)

	50% DISPLACEMENT (^{3}H)LTD_4 pmoles/tube		% CROSSREACTIVI	
LTD_4	0.07	(0.10)	100	(100)
LTE_4	0.41	(0.21)	17	(48)
LTC_4	1.00	(1.30)	7	(8)
LTB_4	800	(140)	0.008	(0.05)
LTA_4 methyl ester	18 000	(190)	<0.001	(0.04)
Arachidonic acid	7 500	(750)	<0.001	(0.009)
FPL55712	>20 000	(>20 000)	<0.001	(<0.001)
Glutathione	>20 000	(>20 000)	<0.001	(<0.001)
Glutathione-S-paracetamol	>20 000	(>20 000)	<0.001	(<0.001)
PGE_2	>20 000	(>20 000)	<0.001	(<0.001)

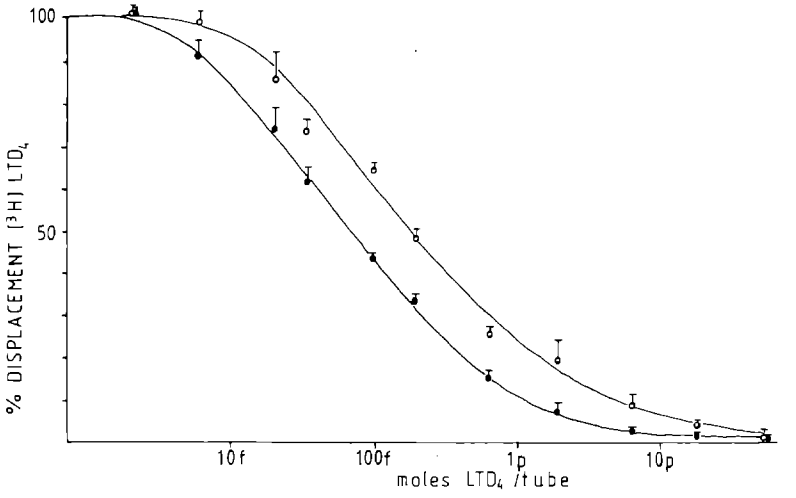

Figure 1: The effect of preincubation of standard and antibody on $(^3H)LTD_4$ binding to TG1 antiserum. Closed circles (—●—) represent experiments conducted including a 30 min preincubation with increasing amounts of LTD_4, with a 90 min subsequent incubation in the presence of 6 000 dpm $(^3H)LTD_4$. Open circles (—○—) indicate experiments where no preincubation was included, and $(^3H)LTD_4$ was added with antibody and standard for a total incubation time of 120 min. Results are means + S.D..

NW1 had a Ka of 1.0 X 10^9 with a detection limit of 9 fmoles ($p<0.05$, Student's t test).

Neither antisera showed any affinity, up to 20 nmoles, for glutathione, glutathione-S-paracetamol, or PGE_2.

FPL 55712 produced 10% displacement of $(^3H)LTD_4$ binding to antiserum TG1 and 15% displacement to antiserum NW1 at 5 nmoles (data not shown).

Figure 1 shows the increased sensitivity of the LTD_4 standard curve with antiserum TG1 when a preincubation step was introduced, the $(^3H)LTD_4$ being added 30 min after mixing standard LTD_4 and antibody. This was incorporated into the assay protocol.

The detection limits of the RIA for LTD_4 ,LTE_4 and LTC_4 were 0.24 fmoles, 1.4 fmoles and 3.4 fmoles respectively ($p<0.0025$, Student's t test). One-sixth of HPLC fractions were assayed, therefore the limit of quantitation of total individual LT levels in post-HPLC fractions was 1.4 fmoles for LTD_4 11.8 f moles for LTE_4 and 20.4 f moles for LTC_4.

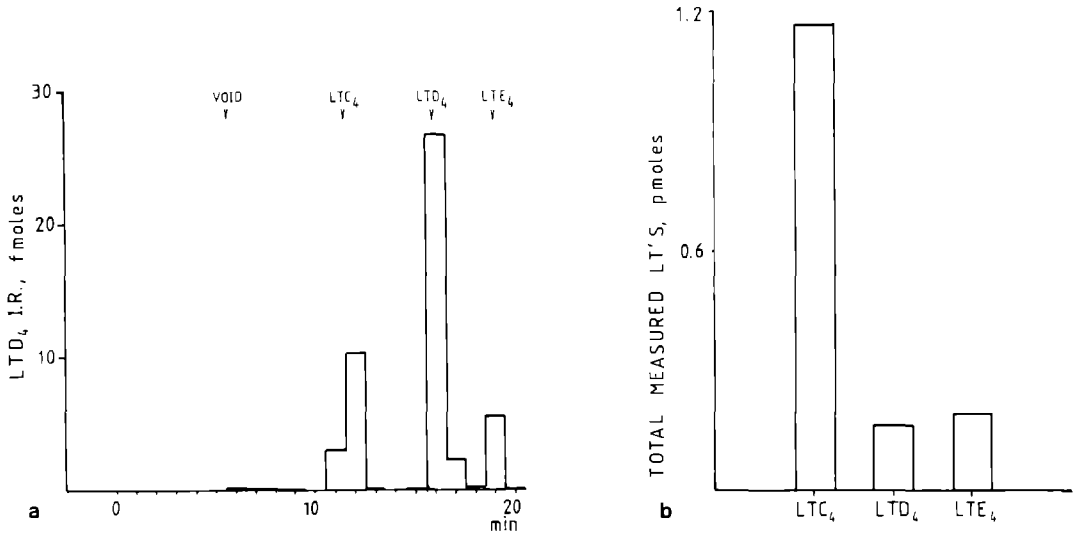

Figure 2a: The LTD_4 immunoreactivity of a typical HPLC run, assayed in 1/6th aliquots of HPLC fractions, is shown. This experiment shows the chromatography of a PAF and indomethacin-stimulated perfused rat lung extract (for HPLC conditions and RIA protocol see Materials and Methods). Elution positions of LTC_4 , LTD_4 , and LTE_4 are indicated.

Figure 2b: This figure shows LTD_4 immunoreactivity from Fig.2a, translated into quantitation of peptidoleukotriene levels. The data from Fig.2a is subjected to crossreactivity calculations for LTC_4 and LTE_4, and multiplied by six, to arrive at the post-HPLC values for the total experiment.

The RIA data from screening HPLC fractions of a rat perfused lung/PAF experiment are shown in Fig.2a, with the quantitation of total individual LT levels corrected for crossreactivities shown in Fig.2b. Importantly, these data illustrate the competence of the assay protocol to measure the individual leukotriene levels, and its suitability for use with biological samples, where background immunoreactivity may be encountered.

We have previously developed methods for extraction of leukotrienes from blood samples based on C_{18} Sep-pak adsorption and elution (27). In preliminary studies utilising the above procedure followed by HPLC and bioassay we were able to demonstrate the presence of LTD_4 in a blood sample of a drug-free asthma patient taken during the asthma crisis (27). The majority of such studies were however negative, which we concluded was due to the conversion of LTD_4 to a metabolite having a relatively poor response on the guinea pig ileum bioassay. Further work involving isolation and mass spectrometric structure

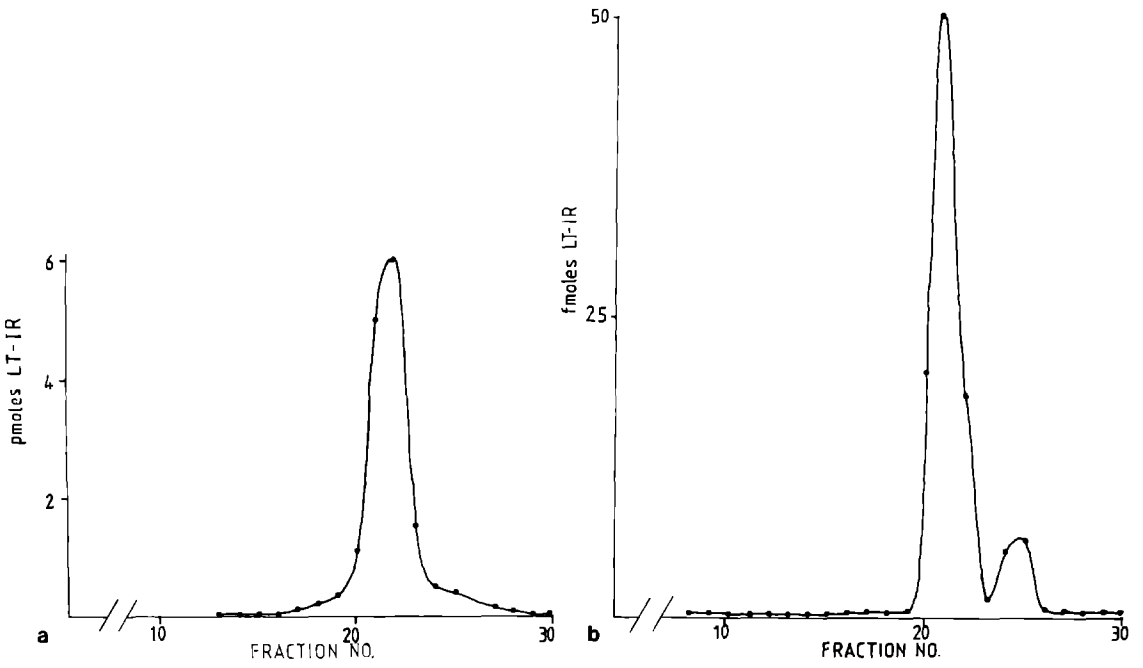

Figure 3: *in vitro* incubation of LTD_4 in blood (antiserum TG1). Fig. 3a shows LT-IR recovered from human blood after incubation at 4°C for 15 min prior to centrifugation, extraction, and HPLC separation. The assay was conducted on 0.02% of fractions. Fig. 3b shows the consequences of 37°C incubation for 15 min, with subsequent steps as Fig. 3a. In this experiment 0.8% of each fraction was used in the RIA. Incubation, extraction, and HPLC as refs. 25, 27 and 28.

determination has rigorously identified one such major metabolite as 5-hydroxy 6-cysteinyl 5, 7, 11, 14 eicosatetranoic acid, or LTE_4 (29), substantiating observations of leukotriene metabolism based on HPLC retention times (30).

An important test of the present protocol, therefore, was whether the RIA developed was capable of detecting LTE_4 in blood samples, where contaminating background activity can present problems to many assay systems. The data produced is shown in Fig. 3 for LTD_4 incubations at both 4° and 37°C for 15 min. There is little LTE_4 generation at 4°C, suggesting that the sampling and extraction protocol should not of itself lead to extensive metabolism. The 37°C data clearly show conversion to LTE_4, which, when corrected for the known crossreactivity to LTE_4 amounts to 50% of the total identified leukotriene. Significantly, the assay is not noticeably affected by background cross-reacting species when using this extraction protocol.

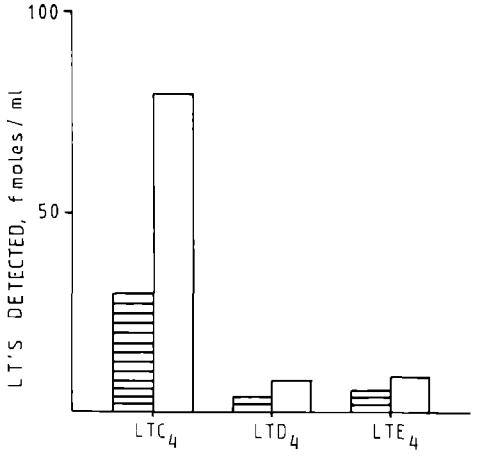

Figure 4: This figure shows leukotrienes measured in plasma from 10 ml blood from two volunteers. Volunteer 1, hatched bars and volunteer 2, open bars. Data is calculated per ml plasma.

In further experiments the assay was used to detect the presence of endogenous LTs in blood sampled from volunteers (Figure 4). Immunoreactivity was observed in the LTD_4 running position (0.67 fmoles and 1.3 fmoles; one-sixth sample assayed) and in the LTC_4 and LTE_4 positions (5 fmoles and 13 fmoles LTC_4, 1 fmole and 1.5 fmoles LTE_4). The low crossreactivities of the antisera towards LTC_4 and LTE_4 (7% and 17%) would of course amplify any measurement errors for these LTs.

The assay described here has the ability to detect LTD_4,LTE_4 and LTC_4 in physiological samples, and at levels orders of magnitude lower than those generated in cell culture preparations. Preliminary experiments bear out the capability of the assay to deal with post-HPLC human sera and the suitability of the assay for the screening of biological samples.

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