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Determination of SRS-A release from guinea-pig lungs by a radioimmunoassay

David Aharony, Paul Dobson, Peter R. Bernstein[+],
Edward J. Kusner, Robert D. Krell and J. Bryan Smith*

Pulmonary Pharmacology and [+]Medicinal Chemistry Section,
Biomedical Research Department, Stuart Pharmaceuticals,
Division of ICI Americas Inc., Wilmington, DE 19897

*Center for Thrombosis Research, Temple University,
Philadelphia, PA 19140

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A sensitive radioimmunoassay for leukotrienes (LTs) has been developed. Rabbits were immunized with a conjugate of LTD_4 and bovine serum albumin, prepared by using 1,5-difluoro-2,4-dinitrobenzene as the coupling agent. The assay can detect 0.045 pmol LTD_4 at a final plasma dilution of 1:72. 50% displacement of bound ^3H-LTD_4 was obtained with 0.43±0.03 pmol LTD_4. LTC_4, LTE_4 and LTF_4 cross-react 159%, 57% and 85%, respectively, whereas LTB_4, 5-HETE and prostaglandins did not. The assay was validated by measuring the antigen-induced release of LTs from sensitized guinea-pig chopped lungs. High correlation (0.9434, $p<0.05$) was found when LTs were simultaneously determined by this assay and a bioassay on guinea pig ileum.

Peptide Leukotrienes, the principle components of slow reacting substance of anaphylaxis (SRS-A), are highly potent constrictors of airway smooth muscles (1) increase vascular permeability (2) and tracheal mucus secretion (3). These actions have implicated the LTs as major mediators of many allergic hypersensitivity-type reactions, including allergic asthma (1). Several radioimmunoassays (RIA) for LTs have been recently reported (4-7). These methods differ in their selectivity and sensitivity in regards to the LTs determined. Two of these methods (5,6) are highly selective towards LTC_4 whereas the other methods detect LTC_4 and LTD_4 with about equal sensitivity (4,7). Since no evidence for the predominance of any of the LTs in various diseases has been published it seemed that a highly-sensitive RIA that can detect all three peptide-LTs would be advantageous for in vivo studies.

This communication documents the development of a RIA that detects all three major LTs with greater (4) or equal (6,7) sensitivity compared with other available methods. The assay was validated by measuring LTs release from guinea-pig chopped

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lungs (GPCL) and correlated to data obtained by determining the LT content of the samples with a bioassay.

METHODS AND MATERIALS

LTC_4, LTD_4, LTE_4 and their analogs were synthesized in-house as published previously (8). LTF_4 was prepared biosynthetically as described (9). 15-hydroxy-LTs were prepared by reacting LT with 1mg/ml of soybean lipoxygenase type I (Sigma Chemical Co.). LTB_4 was prepared by Dr. Y.K. Yee. 5-HETE was prepared by Dr. K.H. Gibson of ICI-UK and 15-HETE was biosynthesized and purified as published (10). ^3H -LTD_4 (42.8 Ci/mmol) was purchased from New England Nuclear, Boston, MA. Arachidonic acid (>99% pure) was from Nucheck Prep., Elysian, MN. TxB_2 and 6-keto$PGF_{1\alpha}$ were purchased from the Upjohn Company, Kalamazoo, MI and lipoxygenase, PGE_2, glutathione, cysteine, ovalbumin (OA) bovine serum albumin (BSA) [N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid] (HEPES) and [2(N-morpholino)ethane sulfonic acid](MES) were obtained from Sigma Chemical Co., St. Louis, MO.

Preparation of antisera: LTD_4 (mixed with 0.05μ Ci ^3H-LTD_4) was coupled to BSA via 1,5-difluoro-2,4-dinitrobenzene (DFDNB) under conditions described previously (11). Approximately 10 moles LTD_4 were covalently bound to a mole of BSA as determined by analyzing the u.v. absorbance and radioactivity of the conjugate. 0.2mg of the conjugate dissolved in 1ml water was emulsified with 1ml of complete Freund's adjuvant. Each of three rabbits (New Zealand, White) was immunized (I.D.) at multiple sites on the back with 2ml of the emulsified conjugates. The rabbits were boosted with the same mixture every two weeks for the first three months and once a month thereafter.

Rabbits were bled from the ear artery. Blood was collected into sodium citrate solution (3.8% w/v final) and centrifuged at 3000xg for 30 minutes at 4°. The plasma was collected and kept frozen at -20° until assayed for anti-LTD_4 antibodies.

Radioimmunoassay Protocol: antiserum was diluted in buffered-saline (5mM MES, 5mM HEPES adjusted to pH 7.4 with 1N NaOH) and 50μl of the appropriate dilution was added to 50μl of sample and then mixed with ^3H-LTD_4 (8,000 dpm) in a total volume of 150μl. Incubations were carried out at 4° for 2 hours and terminated by addition of 0.5ml dextran-coated charcoal (20mg dextran, 200mg charcoal in 100ml of buffered-saline). After 5 minutes the mixture was centrifuged at 2,000xg for 5 minutes at 4°. 400μl samples of the supernatant (Ab~Ag complex) were removed, mixed with 5ml of Liquiscint (National Diagnostics, Somerville, NJ) and the radioactivity determined in a Beckman LS7500 spectrometer.

Release of SRS-A from sensitized guinea pig lungs: experiments which evaluated the antigen-induced release of LTs from GPCL previously sensitized with ovalbumin were conducted as published previously (12). LTs were assayed on guinea-pig ileum, treated with atropine and mepyramine ($5x10^{-7}$M each), as described elsewhere (13).

RESULTS AND DISCUSSION

After 6 months of immunization, antibodies were detected in one of the three rabbits. The anti-LTD_4 plasma bound 50% of the ^3H-LTD_4 at a final dilution of 1:72. The assay could detect 0.045 pmol LTD_4. The displacement of bound ^3H-LTD_4 by LTC_4, D_4 and E_4 is shown in Figure 1. The order of potency in displacing 50% of bound ^3H-LTD_4 was $LTC_4 > LTD_4 = LTF_4 > LTE_4$. The corresponding EC_{50} values were; 0.27±0.03 (N=3), 0.43±0.03 (N=6), 0.51±0.04 (N=3), 0.76±0.07 (N=3) pmol/tube (mean±S.E.) for LTC_4, D_4, F_4, and E_4, respectively. The cross-reactivity of LTC_4 and LTE_4 were significantly ($p<0.01$, t-test) different from that of LTD_4. The cross-reactivity of LTF_4 was not significantly different from LTD_4. These results indicate

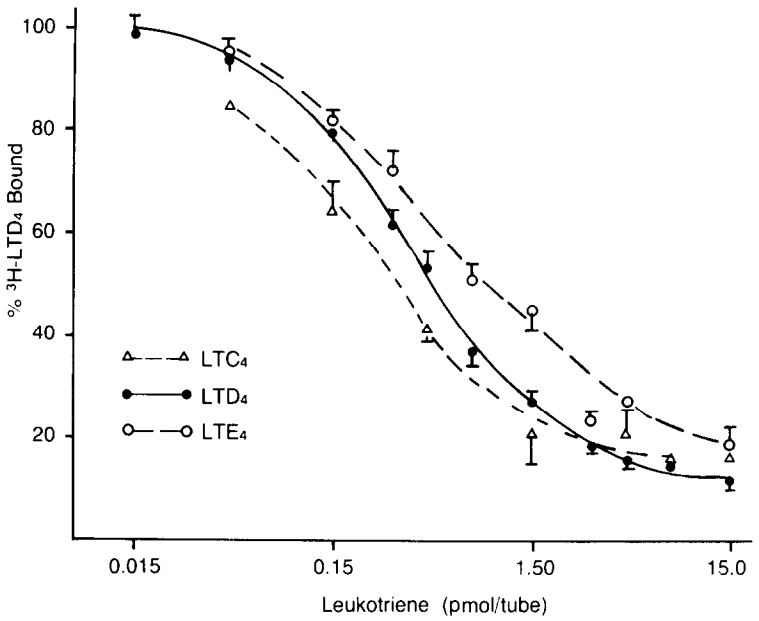

Figure 1 Displacement curve for ^{3}H-LTD$_4$. 8000 dpm of ligand as incubated with anti-LTD$_4$ plasma (final dilution 1:72). The assay was carried out as described in the text. Results are mean ± S.E. (N=3 experiments in duplicates).

that the peptidyl moiety plays an important part in antibody recognition. Further they appear to indicate that the degree of recognition (apparent affinity) is directly related to the number of amino acids in this region (i.e., LTC$_4$ has three amino acids, LTD$_4$ and F$_4$ have two amino acids, whereas LTE$_4$ has only one). In order to test the specificity of these antibodies, various LT analogs, prostaglandins (PG) and other metabolites of AA and potentially-interesting compounds were tested for cross-reactivity. Table 1 demonstrates that changes in the relative stereochemistry of the 5-hydroxyl and/or 6-peptidyl moieties (i.e., 5(R), 6(S), 5(R), 6(R) and 5(S), 6(S)-LTD$_4$ analogs) of LTD$_4$ (which has the 5(S), 6(R)-stereochemistry) had a relatively modest effect. In contrast, 15-OH-LTs, the hexahydro-LTC$_4$ analog and LTB$_4$ were devoid of any significant cross-reactivity. Glutathione and cysteine, which are the peptide-portions of LTC$_4$ and E$_4$, respectively, also did not compete with ^{3}H-LTD$_4$ for binding: nor did AA, 5-HETE and 15-HETE. In addition, TxB$_2$, 6-ketoPGF$_{1\alpha}$, PGE$_2$, histamine or the LT-antagonist FPL-55712 had no measurable cross reactivity (<0.03%). Reduction of the double bonds (hexahydro-LTC$_4$), or addition of a polar group (15-hydroxy LTs)

TABLE 1
SPECIFICITY OF THE RADIOIMMUNOASSAY FOR PEPTIDE-LT

COMPOUND	PICOMOLES REQUIRED TO DISPLACE 50% OF BOUND RADIOLIGAND	RELATIVE CROSS-REACTION (%)
LTC_4	0.27	159
9,11,14,Hexahydro-LTC_4	215	0.2
15-OH LTC_4	10.7	4.0
LTD_4	0.43	100
5(R),6(S)-LTD_4	0.77	56
5(R),6(R)-LTD_4	2.26	19
5(S),6(S)-LTD_4	0.74	58
15-OH LTD_4	15.9	2.7
LTD_3	1.05	41
LTE_4	0.75	57
LTF_4	0.51	85
15-OH LTE_4	15.36	2.8
LTB_4	1400	0.03
5-HETE	>1500	<0.03
15-HETE	>1500	<0.03
Arachidonic Acid	>1500	<0.03
TxB_2	>1500	<0.03
6-keto-$PGF_{1\alpha}$	>1500	<0.03
PGE_2	>1500	<0.03
Glutathione	>1500	<0.03
Glutathione disulfide	>1500	<0.03
Cysteine	>150,000	<0.03
Histamine	>1500	<0.03
FPL-55712	>1500	<0.03

Values are mean of results from 2-3 experiments, conditions are as described in Figure 1.

all dramatically reduced the activity. These studies indicate that recognition by the antibodies depends mostly on the unsaturated lipid backbone and on the presence of a peptidyl fragment at C-6.

The validity of the assay was tested in experiments where the release of LTs from sensitized GPCL was assayed by both the RIA and bioassay. Figure 2 demonstrates that when challenged with ovalbumin, GPCL released up to 82 ± 8 ng immunoreactive-LT or 75 ± 7 ng bioassayable-LTD_4 equivalents (mean $\pm$ S.E., N=3) per gm tissue within 30 min. Figure 2-A illustrates the linear regression analysis when values obtained

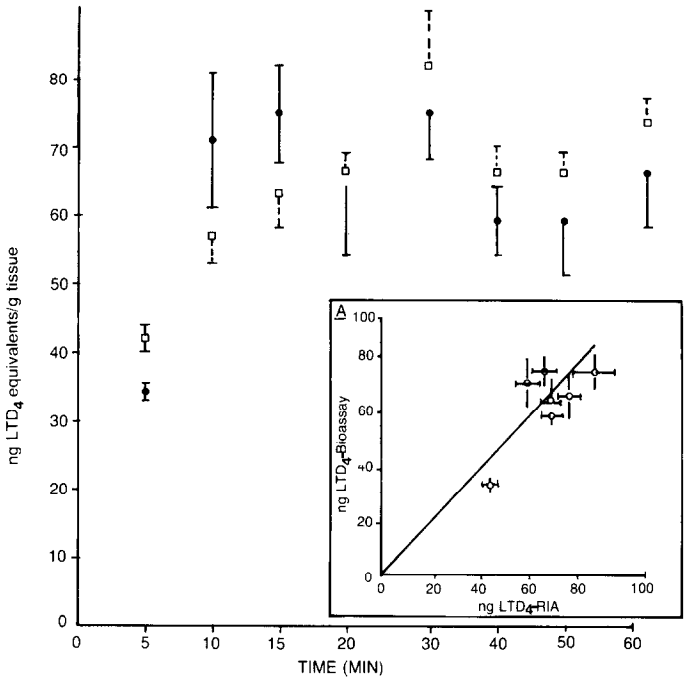

Figure 2 Time course for release of LTs from OA-challenged GPCL. Results are mean±S.E. (N=3) of ng LTD_4 equivalent as determined by a bioassay (open squares) and the RIA (dosed circles). 2-A; linear regression analysis of the data shown in Figure 2. Conditions and assays are as described in the text.

by the bioassay (ordinate) were plotted against values determined by the RIA (abscissa) for each point during the course of LTs release. The correlations between the two assays was 0.9343; ($p < 0.05$, Fig. 2-A).

In conclusion, the assay appears to be highly selective for peptide-LTs as compared to other metabolites of AA or mediators of inflammatory responses (e.g. histamine). The relatively-similiar cross reaction with all three major LTs makes the assay valuable for measuring LTs formation and inhibition in both isolated tissues and in vivo where a mixture of LTs most likely will be released due to active metabolism by gamma-glutamyl transpeptidase and amino-peptidase (14).

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