# A sensitive and specific radioimmunoassay for leukotriene $C_4$

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A sensitive and specific radioimmunoassay suitable for direct measurement of leukotriene $C_4$ was developed. Acetylated leukotriene $C_4$ was coupled to polyamino bovine serum albumin using 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide hydrochloride as coupling agent. The conjugate in complete or incomplete Freund's adjutant was injected into New Zealand white rabbits. At a final antiplasma dilution of 1 : 1050 the lowest detection limit of leukotriene $C_4$ was 0.046 pmol. The antiplasma cross-reacted <1% with leukotrienes $D_4$, $E_4$ and $F_4$, while high relative cross-reaction (86–100%) was obtained with leukotrienes $C_3$, $C_5$ and 11-*trans* leukotriene $C_4$. In experiments where known amounts of leukotriene $C_4$ were added to leukocyte suspensions, 67–100% of the added amount was recovered by the method. The radioimmunoassay was used to study leukotriene $C_4$ formation after stimulation of leukocyte suspensions with the ionophore A23187.

*Radioimmunoassay* *Leukotriene $C_4$* *6-Sulfidopeptide specificity* *Leukocyte suspension* *Ionophore A23187-stimulation* *Leukotriene $C_4$-formation*

## 1. INTRODUCTION

Leukotriene (LT)$C_4$ [1,2], a glutathione-containing derivative of arachidonic acid, constricts pulmonary airways and increases vascular permeability in the microcirculation with a potency, orders of magnitude greater than that of histamine [3,4]. $LTC_4$ is formed by enzymatic addition of glutathione to an epoxide derivative of arachidonic acid, $LTA_4$ [5,6], and is metabolized by stepwise elimination of glutamic acid and glycine through transpeptidation and/or hydrolytic reactions [7,8]. The leukotrienes $D_4$ and $E_4$ generated by the latter reactions have approximately the same biological potency as $LTC_4$ on lung strips [9] and on vascular permeability [4], in some species. $LTE_4$ is metabolized to $LTF_4$ [10] and other polar products, which are excreted in the urine and in the bile [11,12]. Leukotrienes $C_4$, $D_4$ and $E_4$ are the biologically active components of slow-reacting substance of anaphylaxis [13], which mediate allergic reactions [14].

For more detailed studies of the physiological roles of leukotrienes in, e.g., allergy and inflammation, the availability of sensitive and specific assay methods is of great importance. HPLC-chromatography combined with UV-spectroscopy has been used for quantitative determinations of leukotrienes. A drawback of this method is, however, the low sensitivity. A more sensitive but less specific method is bioassay. None of these methods is suitable for large numbers of samples since careful and time-consuming purification of each sample is necessary. Recently, radioimmunoassays for $LTB_4$ [15] and $LTC_4$ [16] have been reported. In addition a radioimmunoassay of the leukotrienes of slow reacting substance of anaphylaxis has been developed [17]. The present report describes a radioimmunoassay for $LTC_4$ which is superior to the previous methods both with regard to sensitivity and specificity.

## 2. MATERIALS AND METHODS

### 2.1. *Antiplasma*

The immunogen was prepared by coupling acetylated $LTC_4$ to polyamino-bovine serum albumin (PABSA). For the acetylation, 100 $\mu$g of $LTC_4$ in

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0.3 ml of pyridine was incubated with 10 $\mu$l of acetic anhydride for 15 min at 22°C under an atmosphere of argon. The solvent was removed by evaporation with a stream of nitrogen and the acetylated $LTC_4$ was redissolved in 100 $\mu$l methanol–water (1:1, v/v). PABSA was prepared by reacting 100 mg recrystallized BSA with 30 mg of recrystallized triethylene tetramine–HCl and 40 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) in 10 ml water adjusted to pH 4.0 with 1 M HCl for 16 h at 22°C, and was dialyzed against distilled water for 48 h at 4°C and lyophilized. Acetylated $LTC_4$ (100 $\mu$g) was incubated with 2.5 mg PABSA and 10 mg EDC in 0.3 ml distilled water (pH 4.0) for 60 min at 22°C and 180 min at 4°C under argon. Thereafter the conjugate obtained was dialyzed under nitrogen and used immediately for immunization. New Zealand white rabbits (4 kg) received 0.4 mg of conjugate in complete Freund's adjuvant in multiple subcutaneous and intradermal sites initially and 0.2 mg conjugate in incomplete Freund's adjuvant at 4, 8 and 12 weeks, and were bled at 16, 20 and 24 weeks. Before use the antiplasma was diluted 1:150 or 1:300 (v/v) with 0.05 M Tris–HCl (pH 8.0).

### 2.2. *Tritium-labelled leukotriene $C_4$*

[$^3H_8$]$LTC_4$ (10 Ci/mmol), prepared from [$^3H_8$]-arachidonic acid using the methods described for the synthesis of [$^3H_6$]$LTC_3$ [18], or [14,15-$^3H_2$]$LTC_4$ (34 Ci/mmol, New England Nuclear, Boston MA) were used as tracers.

### 2.3. *Unlabelled leukotrienes and related compounds*

$LTC_3$ [18], $LTC_4$ [1,2], 11-*trans* $LTC_4$ [19], $LTC_5$ [20], $LTD_4$ [7], $LTE_4$ [8] and $LTF_4$ [10] were prepared as described. 5*S*,12*S*-DHETE, $LTB_4$, 6-*trans* $LTB_4$ and 6-*trans*, 12-*epi* $LTB_4$ were prepared as in [21] and 20-OH $LTB_4$ and 20-COOH $LTB_4$ as in [22] and [23], respectively. 5*S*-HETE and 12*S*-HETE were prepared as in [24]. Arachidonic acid was from Nu-Check Prep. (Elysian MN) and $PGA_2$, $PGB_2$ and $PGE_2$ from The Upjohn Company (Kalamazoo MI).

### 2.4. *Cell preparations and incubations*

Human PMN leukocyte suspensions – $60 \times 10^6$ cells/ml of phosphate-buffered saline containing 0.87 mM $CaCl_2$ (pH 7.4) – were prepared as in [25]. One-ml aliquots of PMN leukocyte suspensions were preincubated at 37°C for 1 min prior to incubation with various concentrations (0–2 $\mu$M) of ionophore A23187 (Calbiochem, La Jolla CA) for 5 min or with 0.38 $\mu$M A23187 for various incubation times. The incubations were stopped by addition of 1 ml ice-cold 0.05 M Tris–HCl (pH 8.0) followed by rapid freezing of the samples at −70°C. After thawing the samples were centrifuged at $1400 \times g$ in order to remove the cells prior to radioimmunoassay. All samples were assayed directly without further purification.

### 2.5. *Radioimmunoassay*

To a series of test tubes were added 0.2 ml 0.05 M Tris–HCl (pH 8.0) followed by addition in triplicate of 0.1 ml of standard compounds ($LTC_4$: 0–5.86 pmol/0.1 ml; compounds tested for cross-reaction 0–3000 pmol/0.1 ml) or unknown samples. Thereafter 0.1 ml of antiplasma and 0.1 ml of radio-labelled ligand (8000 dpm) were added. Depending on the radioligand used ([$^3H_8$]$LTC_4$; 10 Ci/mmol or [14,15-$^3H_2$]$LTC_4$; 34 Ci/mmol) the antiplasma was diluted 1:150 (v/v) or 1:300 (v/v), respectively. For determination of total radioactivity added, 3 tubes received 0.1 ml of Tris–HCl instead of antiplasma. After addition of 0.2 ml of bovine gammaglobulin (5 mg/ml; Sigma) the mixtures were incubated at room temperature for 60 min. Thereafter the tubes were left at 0°C for 15 min prior to addition of 0.7 ml of ice-cold 25% (v/v) aqueous polyethylene glycol 4000 (Kebo AB, Stockholm) [26]. The contents were vigorously mixed and then centrifuged at $1400 \times g$ for 60 min at 4°C. One-ml aliquots of the supernatants were mixed with 0.5 ml $H_2O$ and 10 ml Instagel (Packard Instrument, Stockholm) in scintillation vials and the radioactivity was determined in a liquid scintillation spectrometer. Calculations were performed using off-line computerization [27]. All dilutions were made in 0.05 M Tris–HCl (pH 8.0).

## 3. RESULTS

An antiplasma against $LTC_4$ was obtained by immunizing rabbits with a conjugate of acetylated $LTC_4$ on polyamino-bovine serum albumin. At a final dilution of 1:1050, the antiplasma bound 50% of the radioactivity added. Ten percent dis-

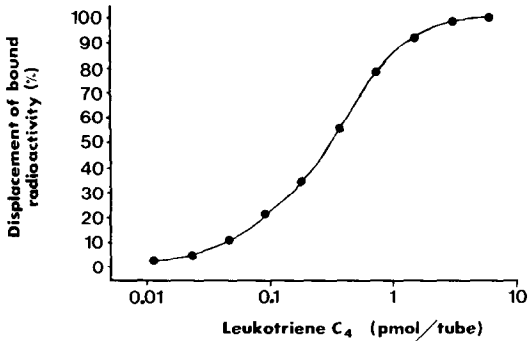


Fig.1. Standard curve for $LTC_4$. [$^3H_8$]$LTC_4$ (10 Ci/mmol) was used as tracer. Antiplasma dilution was 1:150 (v/v). Radioimmunoassay was carried out as described in the text.

Table 1

Specificity of $LTC_4$ antiplasma

| Compound | Picomoles required to displace 50% of bound radioactivity | Relative cross-reaction (%) |
|---|---|---|
| $LTC_4$ | 0.30 | 100 |
| 11-*trans* $LTC_4$ | 0.30 | 100 |
| $LTC_5$ | 0.33 | 90.9 |
| $LTC_3$ | 0.35 | 85.7 |
| $LTF_4$ | 64 | 0.47 |
| $LTD_4$ | 380 | 0.08 |
| $LTE_4$ | 410 | 0.07 |
| $LTB_4$ | >3000 | >0.01 |
| 20-OH $LTB_4$ | >3000 | >0.01 |
| 20-COOH $LTB_4$ | >3000 | >0.01 |
| 6-*trans* $LTB_4$ | >3000 | >0.01 |
| 6-*trans*,12-*epi* $LTB_4$ | >3000 | >0.01 |
| 5*S*,12*S*-DHETE | >3000 | >0.01 |
| 5*S*-HETE | >3000 | >0.01 |
| 12*S*-HETE | >3000 | >0.01 |
| $PGA_2$ | >3000 | >0.01 |
| $PGB_2$ | >3000 | >0.01 |
| $PGE_2$ | >3000 | >0.01 |
| Arachidonic acid | >3000 | >0.01 |
| Glutathione | >3000 | >0.01 |
| Glutathione disulfide | >3000 | >0.01 |
| Glutathione-*S*-paracetamol | >3000 | >0.01 |

[$^3H_8$]$LTC_4$ (10 Ci/mmol) was used as tracer. Antiplasma dilution was 1:150 (v/v). Radioimmunoassay was carried out as described in the text.

placement of bound activity was obtained after addition of 0.046 pmol (28 pg) $LTC_4$ while 50 and 90% displacement was achieved by 0.30 and 1.2 pmol, respectively. A typical standard curve is shown in fig.1.

Results of studies of the specificity of anti-$LTC_4$ binding showed that the relative cross-reaction of $LTD_4$, $LTE_4$ and $LTF_4$ was <1% for each compound, while 11-*trans* $LTC_4$, $LTC_3$ and $LTC_5$ cross-reacted 100, 86 and 91%, respectively (table 1). Other compounds tested, including $LTB_4$ and 5,12-di-HETEs, mono-HETEs, arachidonic acid, prostaglandins, glutathione and glutathione disulfide cross-reacted <0.01%.

To test the validity of the radioimmunoassay, known amounts of $LTC_4$ (0–100 pmol/ml) were added to 1-ml aliquots of human PMN leukocyte suspensions. After mixing, the samples were diluted with 1 ml of ice-cold Tris–HCl and rapidly frozen at −70°C. After thawing and centrifugation, the samples were analyzed by radioimmunoassay. There was a good correlation between added amounts of $LTC_4$ and the results obtained with the RIA (table 2). A background level of 1.1 pmol $LTC_4$/ml cell suspension was detected. After correction for the

Table 2

Radioimmunoassay of known amounts of $LTC_4$ added to leukocyte suspensions

| $LTC_4$ added; pmol/ml cell suspension | $LTC_4$ measured; pmol/ml cell suspension | Recovery (%) |
|---|---|---|
| 0 | 1.1 | |
| 0.5 | 1.6 | 100.0 |
| 1.0 | 2.0 | 95.2 |
| 5.0 | 4.3 | 70.5 |
| 10.0 | 7.4 | 66.7 |
| 50.0 | 46.1 | 90.2 |
| 100.0 | 74.8 | 74.0 |

Various amounts of $LTC_4$ were added in 10 μl EtOH to 1 ml PMN leukocyte suspensions. The samples were immediately mixed with 1 ml ice-cold Tris–HCl and rapidly frozen at −70°C. Radioimmunoassay was carried out as described in the text. Each value is the mean of 3 determinations on each of two samples. Recovery is determined as:

$$\frac{\text{amount } LTC_4 \text{ measured}}{\text{amount } LTC_4 \text{ added} + \text{background } LTC_4} \times 100$$

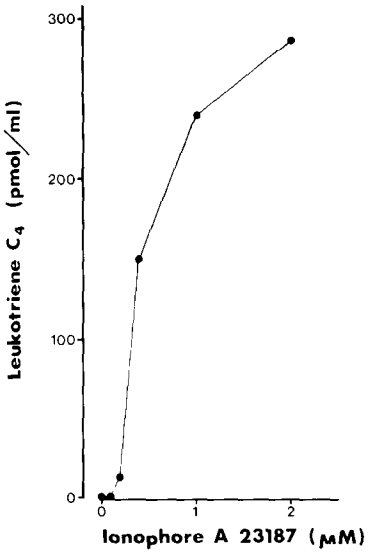


Fig.2. Dose–response curve for $LTC_4$ production by PMNL suspensions after addition of ionophore A23187 in various concentrations. The samples were incubated for 5 min at 37°C. Each point is the mean value of triplicate determinations on each of two samples. Radioimmunoassay was carried out as described in the text.

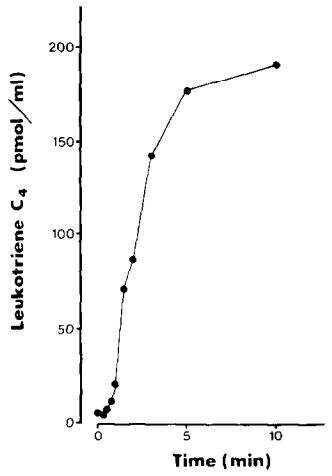


Fig.3. Time-course of $LTC_4$ production by PMNL suspensions after stimulation with 0.38 μM ionophore A23187 at 37°C. Each point is the mean value of triplicate determinations on each of two samples. Radioimmunoassay was carried out as described in the text.

background the recovery was 67–100% of added amounts.

The biosynthesis of $LTC_4$ by human PMN leukocyte suspensions stimulated with ionophore A23187 was determined by the radioimmunoassay. As shown in fig.2, 0.2 μM ionophore increased the levels of $LTC_4$ from a background level of 0.8 pmol/ml to 13.4 pmol/ml. Thereafter the concentrations of $LTC_4$ increased with increasing concentrations of ionophore A23187 reaching 286 pmol/ml at 2 μM of the ionophore. Fig.3 shows the time-course of $LTC_4$ production after stimulation of the PMN leukocytes with 0.38 μM ionophore A23187. After 45 s, elevated levels were detected and thereafter the concentrations of $LTC_4$ increased linearly and reached a plateau after about 5 min.

## 4. DISCUSSION

We describe here the development of a sensitive and specific radioimmunoassay for $LTC_4$. The antibodies were obtained from rabbits which had been immunized with a conjugate of acetylated $LTC_4$ on polyamino-bovine serum albumin. This conjugate was not further characterized and conceivably the 3 carboxyl groups may have given rise to 3 different conjugates.

The $LTC_4$-antiplasma obtained possessed a relatively high degree of sensitivity with a lowest detection limit for $LTC_4$ of 0.046 pmol (28 pg) at a final antiplasma dilution of 1:1050 with [$^3H_8$]$LTC_4$ (10 Ci/mmol; 8000 dpm) as tracer. When a $LTC_4$-tracer with higher spec. act. (34 Ci/mmol) was used, the lowest detection limit was decreased to 0.023 pmol (14 pg, not shown).

Specificity studies of the antiplasma revealed very high cross-reactivities of 11-*trans* $LTC_4$, $LTC_5$ and $LTC_3$. This indicates that the double bonds at C-11, C-14 and C-17 are weak immunodeterminants. On the other hand the antibodies specifically recognized changes within the 6-sulfidopeptide. Thus, $LTD_4$, $LTE_4$ and $LTF_4$ cross-reacted 0.07, 0.08 and 0.47%, respectively. Theoretically, these values could be explained by rapid destruction of the sulfidopeptide moiety during radioimmunoassay incubation. However, considerably higher cross-reactions for $LTD_4$ and $LTE_4$ under similar conditions have been reported [16,17], suggesting that the antibodies used in this study more specifically

recognize this part of the molecule. However, the sulfidopeptide alone does not cause immunorecognition since no significant cross-reaction was observed with glutathione-*S*-paracetamol. Because of the total cross-reactivity of the antiplasma, with 11-*trans* $LTC_4$, the radioimmunoassay will measure the sum of this compound and $LTC_4$. However, in comparison to the levels of $LTC_4$ only small amounts of 11-*trans* $LTC_4$ were found after stimulation of human PMN leukocytes with ionophore A23187 [28].

Because of the relatively high physiological concentration of glutathione disulfide in plasma and glutathione in certain tissues, it was important to determine the cross-reaction with anti-$LTC_4$ of these compounds at high concentrations. However, even at 1.5 mM, glutathione or glutathione disulfide failed to significantly displace bound tracer from the antiplasma. This shows that high concentrations of the compounds in samples will not interfere with the analyses of $LTC_4$.

After addition of known amounts of $LTC_4$ to PMN leukocyte suspensions around 100% was radioimmunologically recovered in samples containing low amounts (<2 pmol/ml) of the leukotriene. At higher concentrations (addition of 5–100 pmol $LTC_4$/ml) 67–90% was recovered by the method. The reason for the decrease in recovery is not known.

Determinations of $LTC_4$ production in PMN leukocyte suspensions after stimulation with ionophore A23187 show that the method can be used to measure $LTC_4$ formation from endogenous precursors by these cells. The amounts of $LTC_4$ reported here are of the same order of magnitude as those reported for human or horse PMNs after ionophore A23187 stimulation [16,29]. However, more physiological stimuli may give rise to lower levels of the leukotrienes. Thus, the amounts of $LTB_4$ produced after stimulation of PMN leukocyte suspensions with serum-coated zymosan were calculated to be 10–50-times lower than those produced by the same cell suspensions after stimulation with arachidonic acid plus ionophore A23187 [25]. A similar decrease of $LTC_4$ values, would however not prevent measurements by the present method. The radioimmunoassay described here seems suitable for direct determination of minute amounts of $LTC_4$ in small volume samples of various origin.

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