Radioimmunoassay for leukotriene E_4

Use for determination of total sulfidopeptide-leukotriene release from rat gastric mucosa

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A conjugate of leukotriene (LT) E_4 and bovine serum albumin (BSA) was prepared by covalently linking the free amino group of the hapten to the protein using dimethyl pimelindiimidate (DMP) as coupling reagent. Anti-LTE_4 antibodies were raised in rabbits immunized with the conjugate. Binding of [^{3}H]LTE_4 to the antibodies is inhibited by 50% with 0.63 ng LTE_4, while the relative cross-reaction of LTC_4 and LTD_4 is 46.3% and 12.6%, respectively. Using the radioimmunoassay release of sulfidopeptide-LT (SP-LT) from rat gastric mucosa incubated in vitro was determined after quantitative enzymatic conversion of SP-LT to LTE_4. It could be demonstrated that this method is suitable for determination of SP-LT in biological material.

Leukotriene E_4 *Leukotriene C_4* *Radioimmunoassay* *Dimethyl pimelindiimidate* *(Rat gastric mucosa)* *γ-Glutamyltranspeptidase*

1. INTRODUCTION

Leukotriene (LT) C_4, LTD_4 and LTE_4, now known to be constituents of a slow-reacting substance of anaphylaxis [1–3], are potent mediators of inflammatory and allergic reactions [4]. For the quantitative determination of low picomole amounts of sulfidopeptide-LT (SP-LT) in biological material a few radioimmunoassays have been developed [5–10]. Immunogenic conjugates were synthesized using either LTC_4 [6–9] or LTD_4 [5,10] as haptens. The antibodies raised by immunization of rabbits with such conjugates show various degrees of cross-reactivity with the heterologous SP-LT. While some of the radioimmunoassays are highly specific for LTC_4 [7,9], other assays can be used to measure the total amount of homologous LT plus the extensively cross-reacting related SP-LT [5,6,8,10]. The total amount of SP-LT present in biological material should be measurable more exactly by quantitative enzymatic conversion of SP-LT to LTE_4 followed by single step determination of this specific LT. A radioimmunoassay for LTE_4 has, however, so far not been described. Here we report the synthesis of an immunogenic LTE_4-conjugate using the bifunctional imidoester dimethyl pimelindiimidate (DMP) as coupling reagent. The reaction couples the single free amino group of the hapten to amino groups of the carrier protein under mild conditions and with high efficiency. The antibodies raised in rabbits by immunization with the conjugate could be used for a radioimmunoassay for LTE_4. An immunogenic LTC_4-conjugate was also synthesized by this method. The radioimmunoassay for LTE_4 was used to determine the ex vivo release of SP-LT from gastric mucosa of rats pretreated with the lipoxygenase inhibitor nordihydroguaiaretic acid (NDGA) or with vehicle prior to instillation of ethanol. Ethanol treatment has been demonstrated

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[11] to significantly increase the synthetic capacity for SP-LT of rat gastric mucosal tissue parallel to the induction of gastric damage.

2. MATERIALS AND METHODS

LTB_4, C_4, D_4 and E_4 were a generous gift of Dr J. Rokach (Merck Frosst Laboratories, Pointe Claire/Dorval, Canada). Prostaglandins, thromboxane B_2 and mono-hydroxyeicosatetraenoic acids (mono-HETEs) were a gift of Dr J. Pike (Upjohn Co., Kalamazoo, USA). Arachidonic acid (>99% purity), glutathione, L-cysteine, γ-glutamyltranspeptidase (GGT, crude, from porcine kidney, 7.5 U/mg) and bovine serum albumin were obtained from Sigma, St. Louis, USA. DMP was bought from Serva, Heidelberg, FRG. [14,15-^{3}H]LTE_4 (spec. act. 37 Ci/mmol) and [14,15-^{3}H]LTC_4 (spec. act. 37 Ci/mmol) were purchased from New England Nuclear Co., Dreieich, FRG.

The LTE_4-immunogen was synthesized using the bifunctional imidoester DMP as coupling reagent. Briefly, 0.2 mg LTE_4 and 0.05 μCi [14,15-^{3}H]LTE_4 were mixed in 0.1 ml Tris-HCl buffer (0.5 mol/l, pH 8.6). The mixture was added to an ice-cold solution of 2.0 mg BSA in 0.2 ml of the Tris-HCl buffer. The clear solution was stirred continuously and 7.7 mg DMP, dissolved in 0.1 ml of the Tris-HCl buffer, was added dropwise. The reaction was continued at 2°C overnight. The conjugate was separated from unreacted hapten by extensive dialysis against phosphate-buffered saline (PBS). After dialysis the conjugate was diluted with PBS to a final concentration of 2 mg/ml (in terms of bovine serum albumin (BSA)) and stored at −20°C until used. From determination of the radioactivity of the conjugate after dialysis it was calculated that 7.5 mol LTE_4/mol BSA had been coupled. For immunization 0.5 mg of the conjugate (in terms of BSA) was diluted with water to 1.0 ml, emulsified with an equal volume of complete Freund's adjuvant and injected into the foot pads of two rabbits. Booster injections (0.1 mg/animal) were administered as described previously [6]. Blood was collected from the ear artery 10–14 days after booster injections. Other rabbits were immunized with a LTC_4-BSA-conjugate that had been synthesized exactly as the LTE_4-conjugate. Binding experiments with 4 nCi/tube of [^{3}H]LTE_4 or [^{3}H]LTC_4 as tracers and radioimmunoassays were performed as described [6].

For enzymatic conversion of LTC_4 to LTE_4 authentic LTC_4 (30–1000 pg in 0.01 ml water) or biological samples (0.02 ml of incubation media of rat gastric mucosa, see below) were mixed with 0.01 ml (75 mU) crude GGT, dissolved in 50 mmol/l Tris-HCl buffer, pH 8.6. The total incubation volume was 0.1 ml and contained 0.1% gelatine. The mixture was incubated at 37°C for 30 min. The reaction was stopped by heat inactivation of the enzymes (100°C, 2 min). Radioimmunological analysis of the incubates was performed directly in the reaction tubes. Under the conditions used conversion of LTC_4 to LTE_4 by crude GGT was complete within 30 min as determined by high pressure liquid chromatography (HPLC) [11] and did not require additional use of aminopeptidase.

Male Wistar rats (200–250 g) were fasted for 24 h with free access to water. The rats were treated orally with NDGA (100 mg/kg, suspended in 0.25% carboxymethylcellulose) or with vehicle 30 min prior to instillation of 2.0 ml ethanol. The animals were killed 5 min later, gastric damage was assessed as ulcer index and fragments of gastric corpus mucosa were excised and incubated in oxygenated Tyrode solution as described [11]. Under these conditions rat gastric mucosa has been shown to release mainly LTC_4 with variable amounts of LTD_4 and LTE_4 [11]. The total amounts of SP-LT in the incubation media were determined by the newly developed radioimmunoassay for LTE_4 after incubation with crude GGT.

Means ± SE were calculated. Statistical analysis was performed using Student's *t*-test.

3. RESULTS AND DISCUSSION

Both rabbits immunized with the LTE_4-BSA-conjugate produced specific antibodies to the hapten within several weeks. An antiplasma obtained 8 weeks after first immunization bound 40% of the added tritium-labelled tracer at a final dilution of 1:1050. Under identical conditions non-specific binding of label by rabbit plasma obtained before immunization was less than 2%. The binding of [^{3}H]LTE_4 by the antibodies was in-

Table 1

Specificity of the radioimmunoassay for LTE_4

Ligand	Nanograms required to displace 50% of bound label	Relative cross-reaction (%)
LTE_4	0.63	100.00
LTC_4	1.36	46.32
LTD_4	5.02	12.55
LTB_4	80.00	0.79
5-HETE	>100.00	<0.60
11-HETE	>100.00	<0.60
12-HETE	>100.00	<0.60
15-HETE	>100.00	<0.60
PGE_2	>100.00	<0.60
PGD_2	>100.00	<0.60
$PGF_{2\alpha}$	>100.00	<0.60
6-Keto-$PGF_{1\alpha}$	>100.00	<0.60
TXB_2	>100.00	<0.60
Arachidonic acid	>100.00	<0.60
Glutathione	>100.00	<0.60
L-Cysteine	>100.00	<0.60

hibited by unlabelled LTE_4 in a competitive manner. 50% inhibition of binding of label was observed with 630 pg LTE_4 (table 1) and 10% inhibition, defined as detection limit of the assay, was obtained with 70 pg LTE_4. The relative cross-reaction of LTC_4 and LTD_4 was 46.3 and 12.6%, respectively (table 1). Various prostaglandins, thromboxane B_2, mono-HETEs, arachidonic acid, glutathione and L-cysteine do not interfere significantly with the assay in amounts up to 100 ng. LTB_4, on the other hand, which like the SP-LT contains the triene structure, exhibits a slight, but measurable interference of less than 1% (table 1). As a measure of assay precision the intra-assay and inter-assay coefficients of variation at 4 different amounts of LTE_4 (250, 500, 1000 and 2000 pg) were determined. At these levels the intra-assay coefficients of variation were 7.7, 3.2, 2.1 and 1.6%, respectively (n = 5 each), while the inter-assay coefficients of variation were 13.6, 14.5, 13.4 and 15.4%, respectively (n = 5 each). The anti-LTC_4 antibodies raised in rabbits by immunization with the LTC_4-DMP-BSA-conjugate recognized the homologous hapten LTC_4 best (50% inhibition of binding of label to antibodies by 285 pg), while the relative cross-reaction of LTD_4 and LTE_4 was 21.7 and 2.3%, respectively.

Imidoesters have been used so far for biochemical purposes such as modification of amino groups [12,13] or protein cross-linking [14,15]. The mechanism of the reaction of imidoesters with amines is well defined [12,16] resulting in the formation of a homogeneous type of conjugate under very mild conditions. The reaction is, therefore, suitable for the synthesis of immunogenic conjugates of biologically active haptens. DMP provides a 7 carbon spacer exposing the SP-LT molecule in a distance of about 10 Å from the BSA carrier. Consequently, the antibodies raised recognize not only differences in the fatty acid moiety, but also in the peptide moiety of the LT molecules. On the other hand, as LTC_4 cross-reacts more extensively with the anti-LTE_4 antibodies than LTD_4, part of the coupling reagent seems to resemble the 5 carbon chain of the γ-glutamyl residue of the LTC_4 molecule.

Quantitative conversion of LTC_4 to LTE_4 as determined by HPLC is observed after incubation with crude GGT at 37°C for 30 min. As shown in fig.1 values of immunoreactive LTE_4 measured after enzyme treatment of standard amounts of LTC_4 by the newly developed radioimmunoassay correlate excellently (r = 0.9989, n = 7, $p < 0.001$) with values of LTC_4 measured without enzyme

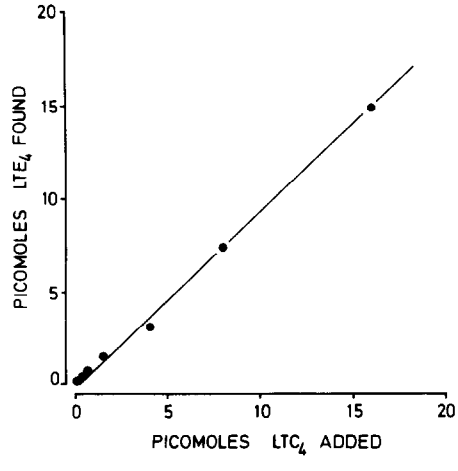

Fig.1. Correlation between standard amounts of LTC_4 and the corresponding amounts of immunoreactive LTE_4 resulting from treatment of LTC_4 with crude γ-glutamyltranspeptidase (75 mU/tube) at 37°C for 30 min.

treatment. The radioimmunoassay for LTE_4 was used for the quantitative determination of SP-LT release from rat gastric mucosa incubated ex vivo 5 min after ethanol instillation. As reported [11] SP-LT released under such conditions consist mainly of LTC_4 with smaller amounts of LTD_4 and LTE_4. Fig.2 illustrates values of immunoreactive LTE_4 observed in rat gastric mucosal incubation media after quantitative enzymatic conversion of SP-LT to LTE_4. Pretreatment of rats with the lipoxygenase inhibitor NDGA (100 mg/kg) decreases release of total SP-LT (determined as immunoreactive LTE_4) from gastric mucosa as compared to the mucosa of rats that had received the vehicle prior to ethanol. As shown in fig.2 and described in [11] inhibition of gastric mucosal SP-LT release is paralleled by gastric protection against the damaging effect of ethanol.

Our results show that quantitative enzymatic conversion of SP-LT followed by single step radioimmunological measurement of LTE_4 is a suitable method for determination of SP-LT in biological material. Alternatively, the various SP-LT could be separated by HPLC and quantitative determinations could be performed in eluted fractions [5,10,17]. This method is time-consuming and results in many more samples than determination of LTE_4 as an index of total SP-LT. The radioimmunoassay for LTE_4 might be of particular value, when the synthetic capacity for SP-LT is determined in tissues, which effectively convert LTC_4 to LTE_4 such as the human gastrointestinal tract [18]. Furthermore, since LTE_4 seems to occur in human bile [19] and is a urinary metabolite of LTC_4 in man [20], the radioimmunoassay for LTE_4 might be valuable for determination of the systemic biosynthesis and excretion of SP-LT under physiological and pathophysiological conditions in man.

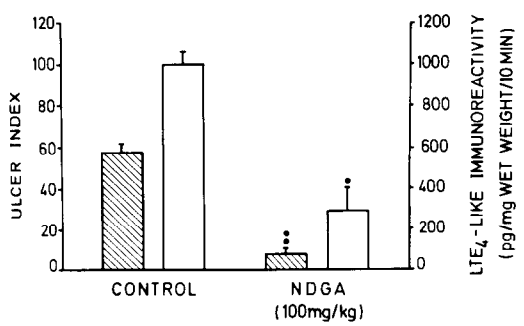

Fig.2. Ulcer index (hatched bars) and total amounts of SP-LT, determined as LTE_4-like immunoreactivity after quantitative enzymatic conversion of SP-LT to LTE_4 (open bars), released from rat gastric mucosa incubated at 37°C for 10 min ex vivo 5 min after oral instillation of 2.0 ml ethanol. NDGA (100 mg/kg), suspended in 0.25% carboxymethylcellulose (2.5 ml/kg) was given orally 30 min prior to ethanol, while control rats received the corresponding volume of 0.25% carboxymethylcellulose 30 min prior to ethanol. •• $p < 0.001$; • $p < 0.005$ as compared to control rats.

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