Release of leukotriene C_4 from human polymorphonuclear leucocytes as determined by radioimmunoassay

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Rabbits were immunized with a conjugate of leukotriene (LT) C_4 and bovine serum albumin prepared by coupling the single free amino group of the hapten to the protein using gluteraldehyde. Binding of [^{3}H]LTC_4 to the antibodies obtained is inhibited by 50% with 1.5 ng LTC_4. The relative cross-reaction of LTD_4 is 16% and of LTC_4-methyl ester 3.6%. The validity of the radioimmunoassay was demonstrated by comparison with bioassay using the isolated guinea pig ileum. Using the radioimmunoassay it could be shown that endogenous LTC_4 is released in a dose-dependent manner by human polymorphonuclear leucocytes stimulated with the divalent cation ionophore A23187.

Leukotriene C_4 *Radioimmunoassay* *Leucocyte* *Bioassay* *Slow-reacting substance* *Lipoxygenase*

1. INTRODUCTION

The biological activity of slow-reacting substances (SRS) [1,2] is now attributed to a group of arachidonic acid metabolites, the leukotrienes (LTs) [3,4]. Release of LTs from tissues like human lung [5] and various cell populations like neutrophils [6,7], basophils [8,9,10], eosinophils [11] and macrophages [12,13] has been described. For the quantitative determination of LTs biological, chromatographic and physicochemical methods have been used [14]. Recently, a radioimmunoassay of the LTs of slow-reacting substance of anaphylaxis (SRS-A) has been described [15]. A conjugate for immunization of rabbits had been prepared by coupling LTD_4 via the eicosanoid carboxyl group to bovine serum albumin (BSA). However, the antibodies exhibited only limited specificity with comparable affinities for LTC_4, LTD_4, LTE_4 and their 11-*trans* stereoisomers. More specific antibodies were obtained by immunization with a conjugate synthesized via the C-5-hydroxyl group [16]. Young et al. [17], in a study on the preparation of conjugates of LTC_4 with proteins, have pointed out that coupling procedures, which involve reactions on the free amino group of the glutamyl residue of LTC_4 should retain the most important parts of the hapten molecule unchanged. While these authors used either 1,5-difluoro-2,4-dinitrobenzene or 6-*N*-maleimidohexanoic acid chloride as coupling reagents, we here describe the synthesis of an immunogenic LTC_4–BSA conjugate via the single free amino group using glutaraldehyde as coupling reagent [18,19]. The antibodies produced by rabbits immunized with this conjugate were used to develop a radioimmunoassay for LTC_4. The sensitivity and specificity of the assay permit the quantitative determination of LTC_4 in biological material. Thus, while qualitatively the release of material identical with SRS-A [20] or LTC_4 [7], respectively, from human polymorphonuclear leucocytes (PMNs) stimulated by the divalent cation ionophore A23187 has been demonstrated, we have now used the radioimmunoassay to quantify the amounts of LTC_4 released under such conditions.

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2. MATERIALS AND METHODS

LTC_4 and LTD_4 were a generous gift of Dr J. Rokach (Merck-Frosst Labs., Pointe-Claire/Dorval, Quebec). Both LTs as well as LTA_4-methyl ester were also obtained from Ciba (Basle). The methyl esters of LTC_4 and LTE_4 were synthesized by Drs Spur, Crea and Falsone (University of Düsseldorf). Prostaglandins and thromboxane B_2 were a gift of Dr J. Pike (Upjohn Co., Kalamazoo MI). Arachidonic acid (purity ~99%) and glutathione were from Sigma. [14,15-^{3}H]LTC_4 (spec. act. 28 Ci/mmol) was purchased from New England Nuclear Co.

The LTC_4–BSA conjugate used for immunization of rabbits was synthesized using glutaraldehyde as coupling reagent. Briefly, 0.2 mg LTC_4 dissolved in 0.1 ml distilled water was added to a solution of 2 mg BSA (Sigma, A grade) in 0.1 ml phosphate buffer (0.2 mol/l, pH 7.5). Then 0.1 ml 0.21 mol/l glutaraldehyde (Sigma, grade I) was added dropwise under continuous stirring. The mixture turned yellow within a few minutes and was incubated in the dark at room temperature overnight. The reaction was stopped by the addition of 0.1 ml L-lysine–HCl (1 mol/l, pH 7.0) and the incubate was diluted to 1 ml with 0.1 mol/l phosphate buffer (pH 7.5). The conjugate was dialysed against 2 l phosphate-buffered saline. The preparation was then divided into portions and stored at –20°C until used. For immunization 0.5 mg of the conjugate (in terms of protein) was emulsified with an equal volume of complete Freund's adjuvant (Difco) and injected into the footpads of two rabbits (0.25 mg/animal). Booster injections with 0.1 mg immunogen/animal were given 1 and 3 weeks later and then at 3–6 weeks intervals. The rabbits were bled 10–14 days after booster injections. The blood was collected into a mixture of sodium EDTA and indomethacin as in [21]. Plasma was separated from the blood cells immediately by centrifugation at $1500 \times g$ at 4°C for 15 min.

For radioimmunoassay an appropriate antiplasma dilution as well as either standard LTC_4 or unknown samples were added to test tubes containing [^{3}H]LTC_4 (15 000 dpm) in a total volume of 0.6 ml. All dilutions were made in Tris–HCl buffer (pH 7.4, 0.01 mol/l, containing 0.14 mol/l NaCl and 0.1% gelatine). After incubation at 4°C overnight antibody-bound and free ligand were separated using 0.5 ml charcoal suspension (20 mg/ml). After centrifugation the supernatants were added to 11 ml Scintigel (Roth, Karlsruhe). Their radioactivity was determined in a liquid scintillation spectrometer. Bioassay of LTC_4 was performed as in [22] using the isolated guinea pig ileum treated with mepyramine (1 μg/ml) and atropine (0.2 μg/ml).

Human PMNs were obtained from heparinized blood of healthy donors and separated on a Ficoll–metrizoate gradient (Ficoll 400-Pharmacia, Uppsala; sodium metrizoate (75%) Nyegaard and Co., Oslo) followed by dextran sedimentation [23]. This method leads to >97% pure PMNs. The cells were then washed with TCM buffer and centrifuged at low speed ($300 \times g$) 3 times to remove the platelets. Human PMNs at various concentrations (1×10^5–1×10^7/500 μl) were incubated with the ionophore A23187. The divalent cation ionophore was obtained from Serva (Heidelberg). The compound (1 mg) was dissolved in ethanol. For cell triggering, a dilution of 5×10^{-6} mol/l in TCM buffer (pH 7.35) (mmol/l: Tris, 25; NaCl, 120; KCl, 4.0; $CaCl_2$, 0.6; $MgCl_2$, 1.0) was used. The incubation proceeded for 18 min at 37°C and was stopped with ice-cold TCM buffer (500 μl) [24]. The cells were then centrifuged at $350 \times g$ for 15 min and the supernatant was assayed for LTC_4 immunoreactivity. Cells incubated in the absence of ionophore served as controls.

3. RESULTS

Both rabbits immunized with the LTC_4–glutaraldehyde–BSA conjugate produced specific antibodies against the hapten. While an antiplasma obtained 12 weeks after first immunization binds 20% of the added radioactivity at a final dilution of 1:75, non-specific binding by the same dilution of rabbit plasma obtained before immunization was only 2%. Binding of label to the antiplasma was obviously completely due to γ-globulins, since the hapten–protein complexes could be precipitated by goat anti-rabbit–γ-globulin. The specificity of the radioimmunoassay for LTC_4 is shown in table 1. While 50% inhibition of binding of label to the antibodies is achieved with 1.5 ng of the homologous hapten, the crossreaction of LTD_4 is 16% and of LTC_4-methyl ester 3.6%. The other

Table 1

Specificity of the radioimmunoassay for LTC_4

Ligand	Nanograms required to displace 50% of bound label	Relative cross-reaction (%)
LTC_4	1.5	100.0
LTD_4	9.4	16.0
LTC_4-methyl ester	42.0	3.6
LTA_4-methyl ester	> 100.0	< 1.5
LTE_4-methyl ester	> 100.0	< 1.5
Glutathione	> 100.0	< 1.5
Arachidonic acid	> 100.0	< 1.5
PGD_2	> 100.0	< 1.5
PGE_2	> 100.0	< 1.5
6-Keto-$PGF_{1\alpha}$	> 100.0	< 1.5
TXB_2	> 100.0	< 1.5

compounds tested do not interfere significantly with the assay in amounts up to 100 ng. The sensitivity of the radioimmunoassay permits the detection of 165 pg LTC_4 (10% inhibition of binding of label to antiplasma).

The validity of the radioimmunoassay for LTC_4 was tested by comparison with bioassay (fig.1). For authentic LTC_4 a highly significant ($r = 0.98$, $p < 0.01$) correlation of results obtained by the two methods was observed.

Using the radioimmunoassay the release of substantial amounts of LTC_4 from human PMNs stimulated with the ionophore A23187 was detected. There is a clear correlation between the amounts of LTC_4 found in the incubation medium and the number of cells stimulated with the ionophore (fig.2).

4. DISCUSSION

Although the titer of our anti-LTC_4 antiplasma is not much higher than that of the antiserum in [15], the antibodies produced after immunization with the LTC_4–glutaraldehyde–BSA conjugate are more specific. These antibodies recognize both the glutathione and the fatty acid moiety as immunodominant parts of the LTC_4 molecule. This is demonstrated by the fact that LTD_4 (lacking the γ-glutamyl residue) as well as LTC_4 methyl ester (lacking the free carboxyl group of the eicosanoid) inhibit binding of label to the antibodies much less than the homologous hapten LTC_4 (table 1). The relatively high specificity of the antibodies elicited by immunization with the LTC_4–glutaraldehyde–BSA conjugate as compared to those in [15] may be caused by the different coupling procedures used for synthesis of the immunogens. Thus, our

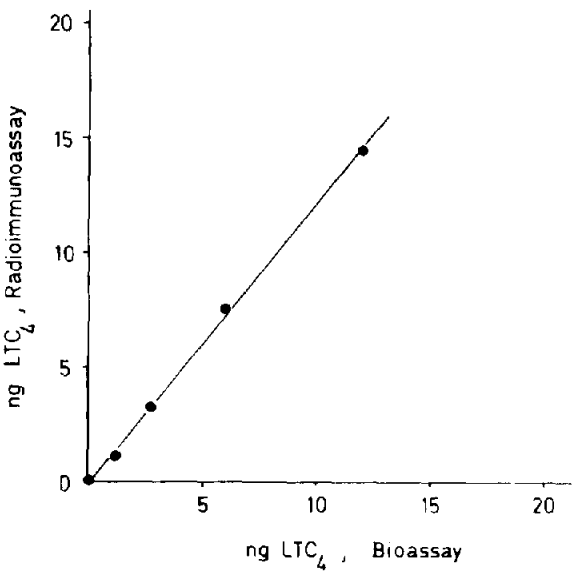

Fig.1. Correlation of data for authentic LTC_4 determined either by bioassay or radioimmunoassay.

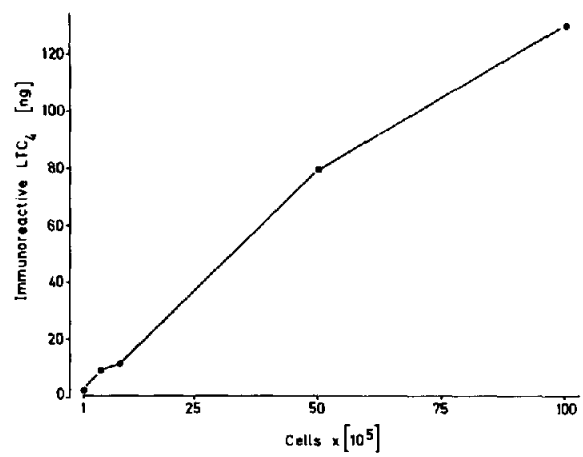

Fig.2. Relationship between number of human granulocytes incubated with ionophore A23187 (5 μmol/l) and release of LTC_4 determined by radioimmunoassay.

results support the view [17] that coupling the hapten via the single free amino group may be advantageous leaving the most characteristic parts of the hapten unchanged.

While an excellent correlation of results (fig.1) for the determination of authentic LTC_4 by radioimmunoassay and bioassay was observed, validation of radioimmunological data may be more difficult, when mixtures of various LTs are present in biological material. For determination of LTs in such material a chromatographic separation step before radioimmunoassay may be necessary [15]. On the other hand, the biological activity of SRS released from human PMNs incubated with ionophore A23187 was found to be mainly composed of 2 compounds, LTC_4 and smaller amounts of 11-*trans*-LTC_4 [7]. Similarly, synthesis of these two LTs as major constituents of SRS was observed, when LTA_4 as exogenous substrate had been added to human PMNs [25]. In such incubates containing just one major LT direct determination of the immunoreactive compound without prior separation seems feasible. Although we do not yet know the exact interference of 11-*trans*-LTC_4 with our assay system, our radioimmunological data are in good agreement with results of Jörg et al. [11] obtained by bioassay and ultraviolet spectrometry. These authors observed release of LTC_4 in the same order of magnitude from horse neutrophils stimulated by the ionophore A23187 as we report here for human PMNs.

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