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MEASUREMENT OF IMMUNOREACTIVE LEUKOTRIENE C_4 IN BLOOD OF ASTHMATIC CHILDREN

Toyokazu Isono*, Yasuko Koshihara*,[1], Sei-itsu Murota*,[2], Yutaka Fukuda** and Susumu Furukawa**

*Department of Pharmacology, Tokyo Metropolitan Institute of Gerontology, Itabashi-ku Tokyo-173, Japan

**Department of Pediatrics, Juntendo University School of Medicine, Bunkyo-ku Tokyo-113, Japan

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Peptide leukotriene (LT) such as LTC_4, LTD_4, LTE_4 have been considered to be major mediators of immediate type hypersensitivity reaction such as asthma. We have developed a rapid and simple extraction method using a Sep-Pak C_{18} cartridge for the measurement of LTC_4 by radioimmunoassay (i-LTC_4). In this extraction method, 91 % LTC_4 was recovered in a final methanol fraction. The identity was confirmed by the recovery test and by the dilution method. The amount of i-LTC_4 in plasma from asthmatic patients was determined by radioimmunoassay after the extraction. The order of the plasma level of i-LTC_4 was; severe asthma > slight or moderate asthma > asthmatic patient without attack > healthy adult. The highest level of LTC_4 was 0.27 ± 0.11 pmol/ml in severe asthmatic plasma.

Recently it has been found that slow-reacting substance is composed of peptide leukotrienes (LT) such as LTC_4, LTD_4 and LTE_4. Peptide-LTs have a strong bronchoconstrictive activity, especially to lung parenchymal strips (1, 2, 3). Lung strips have also been shown to produce peptide-LTs when challenged with antigen (4,5,6). These facts imply that peptide-LTs may be major mediators of asthma and other immediate type hypersensitivity (7). In order to investigate the role of peptide-LTs in human, we measured their level in plasma. Recently the amount of LTC_4 (5(S)-hydroxy-6(R)-S-glutathionyl-

[1] To whom reprint requests and other communications should be addressed.
[2] Present address, Faculty of Dental Sience, Tokyo Medical and Dental University, Bunkyo-ku, Tokyo-113, Japan.

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7,9-trans-11,14-cis-eicosatetraenoic acid) has been measured by radioimmunoassay. In this paper we describe measurement of LTC_4 levels in plasma from asthmatic children of various stages. To measure LTC_4 it must be extracted and concentrated, as LTC_4 level in plasma is low(8). Since LTC_4 is unstable, and easily degraded with acid(low pH), heat or light, a rapid and simple method is required for the extraction. Here we present a successful extraction method for LTC_4 from human plasma, and demonstrate the extracted LTC_4 fraction can be used directly in a radioimmunoassay without interference.

MATERIALS AND METHODS

Plasma preparation

Plasma was obtained from the peripheral blood of asthmatic children or healthy young adult human volunteers. Human blood was withdrawn by disposable syringe with a 3.8 % sodium citrate solution (1 part 3.8 % sodium citrate/9 parts blood). Plasma was separated by centrifugation at 1,500 rpm for 20 min. Buffy coats were removed from the plasma fraction.

Rapid extraction of LTC_4 from human plasma using Sep-Pak C_{18} cartridge

Four volumes of ethanol were added to plasma (1-3 ml) and stored at -80 °C until used. Stored samples were centrifuged at 10,000 rpm at 4 °C for 10 min to remove proteins and other precipitates. Ethanol in the supernatant fraction was removed by evaporation under reduced pressure. The aqueous solution was diluted with H_2O to 10 ml and adjusted to the desired pH with 1 N HCl. This sample was applied to Sep-Pak C_{18} cartridge, which had been pre-washed with 20 ml ethanol and 20 ml water as reported previously (9). After applying, the cartridge was successively washed with 20 ml H_2O and 20 ml petroleum ether in order to remove non-polar lipids and fatty acids. Finally peptide-LTs were eluted with 20 ml methanol. The methanol fraction was incompletely evaporated under reduced pressure to avoid LTC_4 sticking to the glass wall. For radioimmunoassay, the resulting residue was dissolved in 20 µl methanol and 180 µl radioimmunoassay buffer supplied by New England Nuclear. In the case of HPLC, the residue was suspended in 0.2 ml mobile phase which consisted of 65% methanol, 35% water and 0.1% acetic acid adjusted to pH 5.6 by NH_4OH (10). Reversed phase HPLC was performed using Bondapack C_{18} (4 mm x 30 cm) column at a flow rate of 1.0 ml/min, and 1.0 ml fractions were collected. Ultraviolet absorbance at 280 nm was monitored, and peaks corresponding to the retention time of authentic LTC_4, LTD_4 and LTE_4 were collected.

Radioimmunoassay of LTC_4

LTC_4 was measured by radioimmunoassay using an LTC_4-radioimmunoassay kit purchased from New England Nuclear, Boston,

MS, U.S.A. Every procedure was performed in accordance with the instruction supplied. Briefly, all materials were prepared in radioimmunoassay buffer consisting of 0.9% NaCl, 0.1% gelatin, 0.01 M EDTA,10 mM phosphate buffer,pH 7.4 and 0.1% sodium azide. Rabbit anti-LTC_4 serum (0.1 ml; 100-fold dilution of stock solution), standard solution of LTC_4(0.025 ng-1.6 ng) or samples (0.1 ml), ^3H-LTC_4 (approximately 5,000 cpm in 0.1 ml) were incubated at 4 °C for 16 hrs. After incubation, dextran coated charcoal was added to the incubation mixture to adsorb free ^3H-LTC_4, incubated on ice for 15 min, and the charcoal was removed by centrifugation. The supernatant (0.6 ml) containing antibody-bound LTC_4 was counted in a liquid scintillation spectrophotometer. The cross-reactivity of anti-LTC_4 serum used here was as follows: 100 % for(5S,6R) LTC_4, so called LTC_4, 100 % for (5R,6R)LTC_4, 55.3 % for LTD_4, 8.6 % for LTE_4, 0.006 % for LTB_4.

RESULTS AND DISCUSSION

Recovery of LTC_4 after various extraction methods

^3H-labeled LTC_4 in buffer (5,000 cpm, 50 ul) was added to plasma (1 ml) and then extracted as described in the Materials and Methods. Aliquots of each fraction were withdrawn and the radioactivity counted in a liquid scintillation spectrophotometer. The recovery of ^3H-LTC_4 in each fraction is summarized in Table 1. By adjusting the pH of deprotenized plasma to 4.0, 5.1, 5.6 just before applying the sample on to Sep-Pak C_{18}, the recovery of ^3H-LTC_4 in the methanol fraction from 83.6% to 93.7% could be determined. The recovery of ^3H-

Table 1. Recovery of LTC_4 from human plasma

	Recovery (%)		
	Plasma treated		
Fraction	pH 4.0	pH 5.1	pH 5.6
sample	100	100	100
80% ethanol	94.3 ± 3.8	98.4 ± 3.5	100.2 ± 3.8
Sep-Pak C_{18}	10.3 ± 3.5	3.9 ± 0.7	2.7 ± 0.6
Water	1.7 ± 0.7	1.2 ± 0.2	1.3 ± 0.3
Petroleum Ether	0.6 ± 0.3	0.3 ± 0.2	0.2 ± 0.1
Methanol	83.6 ± 2.1	93.7 ± 2.6	84.8 ± 7.6

(mean ± S.E.)

LTC_4 was the highest in the sample adjusted to pH 5.1. If the sample was acidified to pH 4.0, 10.3% of the LTC_4 in the deprotenized plasma passed through the Sep-Pak C_{18} cartridge. Washing the Sep-Pak C_{18} cartridge with H_2O and petroleum ether, the loss of ^3H-LTC_4 bound to the cartridge was small (less then 2 %) regardless of the pH of the sample.

During this extraction procedure, LTC_4 was not degraded and not converted to any other peptide-LTs, such as LTD_4 and LTE_4, as judged by reverse phase HPLC. Since plasma has been already reported to have the enzyme activity for degrading LTC_4(11), all the samples should be kept at 0 °C until the deprotenization procedure.

The identity of LTC_4 fraction extracted from plasma for radioimmunoassay

As shown in Table 1, the recovery of LTC_4 was 93 %. However our purpose was the measurement of LTC_4 in plasma by radioimmunoassay, therefore next we examined whether the fraction containing extracted LTC_4 interfered with the radioimmunoassay. A known amount of LTC_4 was added to plasma extracted as described in Materials and Methods, and the amount of LTC_4 in the extracted plasma determined by radioimmunoassay. The total recovery was approximately 89%. The extract scarcely inhibited the radioimmunoassay (11 % inhibition). The linearity of the radioimmunoassay was confirmed by assaying the extracted plasma, containing 0.8 ng LTC_4 at various dilutions. The amount of LTC_4 recovered as assayed by radioimmunoassay was more than 92.3 % at each dilution point (Fig. 1). At present, we have not confirmed by other methods whether or not the material in healthy donor's plasma that were cross-reactive to anti-LTC_4 serum really represented peptide-LTs. Addition of 32 ng LTC_4 to plasma (1

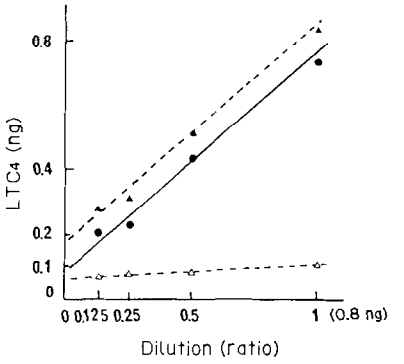

Figure 1. Recovery of LTC_4 added to plasma. Plasma was extracted as described in Materials and Methods. LTC_4 (0.8 ng) was added to the extracted plasma and diluted with radioimmunoassay buffer. The amount of LTC_4 was measured by radioimmunoassay. Δ---Δ, Extracted plasma (A); ▲---▲, Extracted plasma contained 0.8 ng of LTC_4 (B); ●——●, (B)-(A).

ml), followed by rapid extraction using a Sep-Pak C_{18} cartridge and reverse phase HPLC, showed approximately 60 % of the LTC_4 added was recovered as determined by radioimmunoassay.

Measurement of LTC_4 immunoreactive materials (i-LTC_4) in plasma of asthmatic children

We have measured by radioimmunoassay the plasma level of i-LTC_4 in peripheral venous blood from asthmatic children at various stages according to the criteria presented by Japanese Pediatrics Society. For example, in the case of slight asthmatic attacks, a doctor can hear whizzing from a patient, and in the case of severe asthmatic attacks, the mouth color of a patient turns to purple and a patient is stifling. The stage of moderate attacks is between stages of slight and severe attacks. The level of i-LTC_4 from patients with severe asthmatic attacks was 0.27 ± 0.11 pmol/ml plasma (mean ± S.D., n=3). While the average level of i-LTC_4 from patients with slight or moderate asthmatic attacks was 0.19 ± 0.10 pmol/ml plasma (n=13). The level of i-LTC_4 from asthmatic patients in remission, was 0.11 ± 0.03 pmol/ml plasma (n=8) and from healthy adult men was 0.08 ±

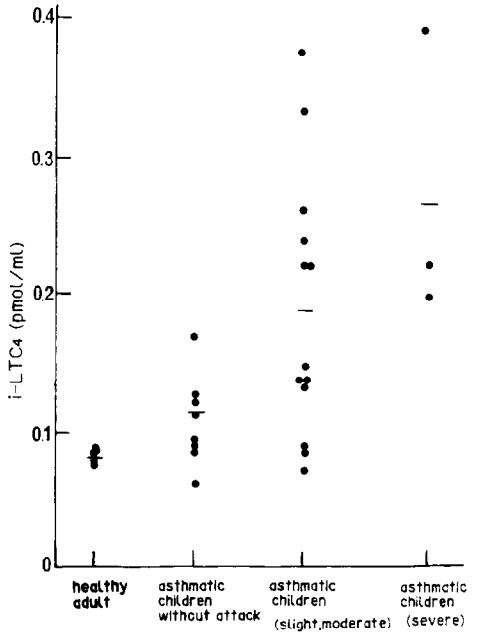

Figure 2. i-LTC_4 level in plasma from bronchial asthmatic children. Peripheral venous blood was withdrawn using disposable syringes containing 1/10 vol. of 3.8 % sodium citrate solution, and plasma was separated. i-LTC_4 in 2-3 ml plasma was extracted as described in Materials and Methods, and measured by radioimmunoassay. Statistical significance by student t-test : healthy adult and slight and moderate asthmatic children ($P<0.01$), slight and moderate asthmatic children and severe asthmatic children ($P<0.05$).

0.01 pmol/ml plasma (n=5) (Fig. 2). We have preliminary measured the average level of i-LTC_4 from childrens who did not have any inflammatory disease. It was 0.021 ± 0.015 pmol/ml plasma (n=4). the values were widely distributed from 0.007 to 0.058 pmol/ml. The plasma level of i-LTC_4 from severe asthmatic children was the highest of the plasma samples. The order of plasma level of i-LTC_4 was in the order of severe asthmatic children, slight and moderate asthmatic children, asthmatic children without attack, and healthy adult. Our result suggested that there was a significant correlation between the immunoreactive LTC_4 level in plasma and the severity of asthma attacks. This is the first paper presenting the plasma level of i-LTC_4 from asthmatic children, and suggesting a good correlation between

LTC_4 plasma level and asthma diagnosis. Plasma from healthy subjects also had a considerable amount of i-LTC_4. Whether this value was derived from LTC_4 itself or other immunoreactive materials is not clear yet. Morris, et. al.(8) have reported LTC_4 levels as high as 32 pmol/ml blood, which was found in only one of 18 asthmatic patients. However, since even very low amount of LTC_4, as low as 10^{-10} to 10^{-12} M can cause strong biological activities (12,13), the level of LTC_4 measured here, for example 1.9×10^{-10} M in slight and moderate asthmatic attacks, might be sufficient to cause smooth muscle contraction. As the anti-LTC_4 serum used in this experiment can show cross-reactivity to LTD_4 and LTE_4, we are now investigating the composition of i-LTC_4.

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