Serum leukotriene metabolism and Type I hypersensitivity reactions in different animal species

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SERUM LEUKOTRIENE METABOLISM AND TYPE I HYPERSENSITIVITY REACTIONS IN DIFFERENT ANIMAL SPECIES

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The peptidoleukotrienes, leukotriene C4, leukotriene D4, and leukotriene E4, (LTC4, LTD4, and LTE4) which are products of the 5-lipoxygenase pathway of arachidonic acid metabolism, play important roles in mediating hypersensitivity reactions. LTC4 is the primary leukotriene released from stimulated cells. A transpeptidase activity converts LTC4 to LTD4, which is subsequently converted to LTE4 by a dipeptidase activity. LTC4 metabolism was studied in human, mouse, guineapig, rabbit, horse and cow sera. LTD4 is the most potent of the peptidoleukotrienes. The relative amounts of LTD4 present after specific periods of incubation was compared in the sera of different species, using tritium labelled LTC4 (3H-LTC4). After specific incubation time, the lipids were extracted from the serum, and the leukotrienes separated by Reverse Phase High Pressure Liquid Chromatography. The leukotrienes in the different fractions were quantitated based on the radioactivity in the corresponding fractions. The profile of metabolism was different for each species in that the rate of formation of LTD4 (transpeptidase activity) and the rate of LTD4 metabolism (dipeptidase activity) varied in the different species. On the basis of these two enzymatic activities, the different animal species tested could be divided into four categories; (1) those with high transpeptidase and dipeptidase activity, which would result in large amount of LTD4 accumulation initially, with a rapid decline, as in horse and cow; (2) those with low activity of transpeptidase and dipeptidase, resulting in accumulation of formed LTD4, as in guineapig; (3) those with high transpeptidase activity and a low dipeptidase activity which would result in accumulation of large amounts of LTD4, as in humans; (4) those with low transpeptidase and high dipeptidase activity which results in very low or no accumulation of LTD4 as in mouse. The results show a relationship between the relative amounts of LTD4 observed and the manifestation of Type I hypersensitivity in the different species.
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INTRODUCTION

Arachidonic acid (a 20-C polyunsaturated fatty acid), is a 5,8,11,14-eicosatetraenoic acid. Arachidonic acid (AA) and its metabolites are known to play important roles in mediating hypersensitivity reactions like bronchial asthma, allergic rhinitis and psoriasis (24,32).

Arachidonic acid is usually present in membrane phospholipids (phosphatidyl choline, phosphatidyl inositol, etc.) esterified primarily to the second carbon (C-2) position. Release of AA from the C-2 position is mediated by different types of stimuli, which can be physiological or pathological (64). Hormones like angiotensin, bradykinin, epinephrine, antigen-antibody complexes etc. are physiological stimuli, whereas ischaemia, mechanical damage, membrane-active venoms (like mellitin from bee venom), Ca\(^{2+}\) ionophores (A23187), phorbol esters etc. are pathological stimuli. The membrane AA is released by the effect of two phospholipase: phospholipase C, which is specific for phosphatidyl inositol, and phospholipase A2, which is specific for phosphatidyl choline (Fig. 1). In general, all stimuli lead to changes in cell membrane, which lead to transport of ions across the membrane, especially a flow of Ca\(^{2+}\) ions from the extracellular to the intracellular
Fig. 1. Mechanisms involved in release of arachidonic acid.
compartment. The resulting increased intracellular Ca\(^{2+}\) concentration stimulate the cytoplasmic phospholipase C, which acts on phosphatidyl inositol to give inositotriphosphate (IP3) and diacylglyceride (DAG). DAG is acted upon by other lipases to a monoacylglyceride and AA. IP3 induces further Ca\(^{2+}\) mobilization from the endoplasmic reticulum to the cytoplasm, and this further activates phospholipase C. The DAG which is not converted to monoglyceride, stimulates other enzymes. Protein kinase C (PKC) is a major enzyme activated by DAG. PKC phosphorylates a phospholipase A2 inhibitory protein. This causes activation of phospholipase A2, which cleaves AA from phosphatidyl choline (64). The released AA has a very short half life. It is either metabolized, by a cycloxygenase or a lipoxygenase pathway, or is reincorporated into the membrane phospholipids. Very small amounts of AA are found in the free fatty acid pool. This low level is thought to be maintained by the action of an acyl-CoA transferase, which esterifies arachidonyl-CoA into phospholipids (25).

The metabolism of AA by the lipoxygenase and the cyclooxgenase pathways are together known as the arachidonic acid cascade (Fig. 2). Metabolism by the cyclooxgenase pathway leads to the formation of prostaglandins (PGs). The first PG to be formed is PG-G\(_2\). This is converted to PG-H\(_2\) from which other PGs and thromboxanes are formed.
Fig. 2. The arachidonic acid cascade.
The lipoxygenase pathways involve metabolism by 5-, 15- and 12-lipoxygenases. Lipoxins are produced by the 15-lipoxygenase pathway, and hydroxy eicosatetraenoic acids (HETES) are produced by the 12-lipoxygenase pathway. The 5-lipoxygenase pathway converts AA to 5-hydroperoxy, 6,8,11,14-eicosatetraenoic acid (5-HPETE). 5-HPETE can then undergo enzymatic conversion to 5-HETE or to 5,15-diHETE. Leukotriene A₄ (LTA₄) is formed from 5-HPETE by an LTA₄ synthase. LTA₄ is very unstable. The action of an LTA₄ hydrolase converts LTA₄ to LTB₄, which is relatively stable. LTB₄ can also be formed from 5-HETE, in the presence of cytochrome P-450 (64). Conversion of LTB₄ to its \( \omega \)-metabolites by Polymorphonuclear cells (PMNs) has been reported. 20-carboxy LTB₄ and 20-hydroxy LTB₄ are the products of \( \omega \)-oxidation.

LTA₄ undergoes enzymatic conversion to LTC₄ by the action of a glutathione-S-transferase, which is specific for LTA₄. Addition of a glutathione residue to the C-6 position by a sulfo-ether linkage, leads to the formation of LTC₄, which is the first peptidoleukotriene to be formed. LTC₄, along with its metabolites, LTD₄ and LTE₄, were formerly known as the Slow Reacting Substances of Anaphylaxis (SRS-A). Although SRS-A has been identified as early as 1930 it was only in 1980 that it was found to be a mixture of peptidoleukotriens (7). The main component of the SRS-A is LTD₄ (48). The name leukotriene refers to the
observation that they were seen to be released by leukocytes, and had three conjugated double bonds.

LTC\(_4\) is the form in which the peptidoleukotrienes are released into the extracellular medium. LTC\(_4\) is converted to LTD\(_4\) by transpeptidase reaction (64) that removes the glutamate from the glutathione residue. LTD\(_4\) is further converted to LTE\(_4\) by a dipeptidase activity (64), which cleaves the glycine from LTD\(_4\).

Several cell types are involved in the release of leukotrienes. LTB\(_4\) is released primarily from the PMNs (57,65). It is also released from bronchi and lung parenchyma (68). LTC\(_4\), on the other hand, is released predominantly from the eosinophils (57,65), mast cells, alveolar macrophages (24), and in lung, parenchymal tissue (68).

The metabolism of the peptidoleukotrienes has been studied in detail, both in vivo, and in vitro. In-vitro studies, showed conversion of LTC\(_4\) to LTD\(_4\) in Rat Basophil Leukemia (RBL-1) cells (42). The conversion of LTD\(_4\) to LTE\(_4\) was also first detected in RBL-1 cells (43). In fact, the SRS-A was first shown to be a metabolite of AA in RBL-1 cells (27). The metabolism of leukotrienes has also been studied in human plasma (1,31), and in the supernatant of PMNs (51). The transpeptidase activity responsible for the formation of LTD\(_4\) is released by PMNs, as seen by the formation of LTD\(_4\) from LTC\(_4\) in the supernatant of a
Fig. 3. Metabolism of the peptidoleukotrienes.
suspension of PMNs (51). The transpeptidase activity and dipeptidase activity were seen to be released from PMNs following immunological as well as nonimmunological stimuli (54). The transpeptidase activity was seen to be localized in the microsomal fraction of PMNs, by subcellular fractionation (51). This activity has also been detected in plasma (1), in the supernatant of alveolar macrophages, (15), and in the parenchyma of human and guinea pig lung tissue (53,54). The transpeptidase activity that converts LTC₄ to LTD₄ is thought to be due to a Y-glutamyl transferase, as glutamate was seen to be a substrate for this enzyme (23) and since inhibitors of Y-glutamyl transferase (L-serine borate, avicin) (43) inhibited the formation of LTD₄.

Other than conversion to LTD₄, LTC₄ can also undergo oxidative metabolism to biologically inactive products (33). This reaction was seen to take place during the respiratory burst in PMNs. Sulfoxides of LTC₄, like LTC₄ chlorsulfonium ion, are metabolites of this oxidative metabolism.

LTD₄ is converted to LTE₄ by a dipeptidase activity (64), which was associated with specific granules of PMNs (34), and plasma membrane of RBL-1 cells (51). This activity is present in alveolar macrophages, and in lung parenchyma, as shown by the presence of LTE₄ in these tissues following antigen challenge (17). L-cysteine is a dipeptidase inhibitor (43). The dipeptidase activity also
requires the presence of divalent cations. Substances like L-cysteine and dithiothreotol, which bind divalent cations, decreased the dipeptidase activity, which was subsequently restored by the addition of Co$^{2+}$, Zn$^{2+}$, or Mn$^{2+}$ into the system (52).

LTE$_4$ is relatively stable. It can undergo further conversion to N-acetyl LTE$_4$ in the presence of acetyl Co-A. This has been shown by the use of $^{14}$C-Acetyl Co-A (23). LTE$_4$ and N-acetyl LTE$_4$ are the main excretory metabolites of peptidoleukotriene metabolism in man and other mammalian species (6,60). N-acetyl LTE$_4$ can also be converted to 20-carboxy N-acetyl LTE$_4$ and 20-hydroxy N-acetyl LTE$_4$ (23). Addition of a glutamate residue to LTE$_4$ by a γ-glutamyl transferase leads to the formation of LTF$_4$, which is a γ-glutamyl-cysteinyll leukotriene (23).

Other than arachidonic acid, trienoic and pentaenoic acids were also seen to be metabolized to peptidoleukotrienes LTC$_3$ and LTC$_5$, as seen by the presence of LTE$_3$ and LTE$_5$ in RBL-1 cells. These leukotrienes, with 3 or 5 double bonds, were seen to cross react with anti-LTC$_4$ antibodies. The biological activity and enzyme specificities of these leukotrienes were seen to be similar to that of the leukotrienes with four double bonds (43).

The biological activities of the different leukotrienes have been described (55). LTB$_4$ is mainly chemotactic in activity. It facilitates migration of all leukocytes, but
the PMNs are mainly affected. LTB₄ also increases microvascular permeability. The peptidoleukotrienes, also referred to as the cysteinyl leukotrienes, have a smooth muscle constrictor effect, and thus induce bronchospasm. LTC₄, LTD₄ and LTE₄ are reported to be 100 to 1,000 times as potent as histamine, on a molar basis (48,55). Other than their direct constrictor effect on airway smooth muscle, they can also potentiate the action of other inflammatory mediators. LTC₄ and LTD₄ increase microvascular permeability and mucous secretion in bronchioles. These activities suggest that the cysteinyl leukotrienes are the major mediators of type I hypersensitivity reactions like bronchial asthma, allergic rhinitis and anaphylactic shock.

Four types of hypersensitivity reactions are recognized: Type I, Type II, Type III and Type IV (64). These are inflammatory reactions, mediated by the release of pharmacologically active substances like histamine and leukotrienes, from specific cell types, following antigen-antibody reactions. The Type I reactions are more acute, and cause more discomfort. This type of reaction is referred to as immediate hypersensitivity reaction, allergy or anaphylaxis. Antigen-antibody reactions cause release of AA and its metabolites directly from cell membranes of mast cells. AA metabolites, like leukotrienes, which are mediators of hypersensitivity reactions are also released directly by eosinophils, which are attracted to the site of
the reaction by chemotactic substances released by mast cells. These substances are called eosinophil chemotactic substances of anaphylaxis (ECF-A). The symptoms of Type I hypersensitivity can be attributed to the sum total of all the above factors. Bronchial asthma, which is a Type I hypersensitivity reaction, is characterized by severe prolonged bronchoconstriction and airway obstruction, both of which are due to the release of these mediators. In fact, several workers have reported the presence of elevated plasma levels of cysteinyl leukotrienes during the asthmatic attack (40,56,68). Eosinophils from asthmatic individuals have been reported to produce larger amounts of LTC₄ than those from normal individuals (59). The Type II hypersensitivity reaction results in cell destruction due to antigen-antibody reactions. Blood group incompatibilities fall into this group. The Arthus reaction, acute and chronic serum sickness which come under the Type III hypersensitivity reaction are produced by localized tissue damage due to deposition of immune complexes. Type IV hypersensitivity reactions are cell mediated reactions, in response to localized injection of antigen. It is mediated through lymphocytes and is called delayed hypersensitivity reaction (DHR).

The metabolism of LTC₄ in human blood has been studied (71). It was seen that LTC₄ slowly undergoes ω-oxidation, to 20-hydroxy and 20-carboxy LTC₄. There was no
difference in the rate of metabolism between normal and asthmatic individuals, suggesting that this pathway may not influence the outcome in a hypersensitivity state.

The cysteiny1 or peptidoleukotrienes have been implicated in a wide variety of hypersensitivity disorders eg., bronchial asthma (56,59), psoriasis (10) and allergic rhinitis (67). In an allergic condition, the eosinophils, which release the peptidoleukotrienes, are thought to be attracted to the tissue by the LTB$_4$ released by the PMNs (57). LTC$_4$ is the form in which the SRS-A is released from stimulated cells. Therefore, the high levels of LTD$_4$ detected in the plasma of individuals during the asthmatic attack (40), and the LTD$_4$ and LTE$_4$ seen following antigen challenge in human (17) and guinea pig (53) lung, must be due to the presence of metabolizing enzymes released into the medium. The release of these enzymes following antigen challenge have been shown by several workers (15). An elevated amount of leukotriene release has been reported from the lung (14), and stimulated blood cells (eosinophils) of individuals with allergic asthma and chronic bronchial asthma (59). This observation suggests that an increased release of leukotrienes might be the factor that predisposes these individuals to the development of hypersensitivity reactions.

A comparison of the urinary LTE$_4$ levels in normal and atopic individuals after antigen challenge, and in asthmatic
individuals during the asthmatic attack, showed that although the LTE4 levels were elevated in atopic individuals following antigen challenge, they were not elevated in individuals during the acute asthmatic attack, or in individuals having a severe episode of allergic rhinitis (60). These results suggest that if an increased release of LTs does occur during an acute asthmatic attack, there might be an alteration in the further metabolism of these leukotrienes to a biologically less active and excretable form, as increased levels were not detected in the urine during the acute attack.

Bronchial asthma is an anaphylactic reaction. Therefore, anaphylaxis induced in animal models should serve as an excellent model to study the role of leukotriene metabolism in bronchial asthma, and in hypersensitivity reactions as a whole. Although the phenomenon of hypersensitivity was observed in 1839 by Magendie, in rabbits, it was Richet, in 1902, who first recognized the reaction in dogs and named it "anaphylaxis" meaning "without protection" (50). Theobald Smith, in 1905, noted the ease with which guinea pigs developed anaphylaxis as compared to other animals (50). Thus guinea pigs became the experimental animal of choice to elicit the anaphylactic reaction in future experiments. The symptoms of anaphylaxis in guinea pigs were seen to resemble those in humans. The bronchoconstriction observed, was similar to that in
bronchial asthma (Burson, 1911, as quoted by Alexander, 1926) (3). Later, Chase reported that it was possible to elicit the cutaneous anaphylactic reaction in guinea pigs, just as in humans. Experiments conducted by Kabat in 1942 (28), indicated that very small amounts of antigen was sufficient to induce anaphylaxis in guinea pigs. Isolated guinea pig smooth muscle was also found to have the same amount of responsiveness to antigen as the intact animal. The amount of antigen required to produce fatal anaphylaxis was also found to be very small (29). Guinea pig smooth muscle was used as early as 1926 to elicit the Schultz-Dale reaction, in order to elicit the sensitivity of smooth muscle to antigen. This experiment is used widely, to study the effect of several mediators of anaphylaxis, because the symptoms of anaphylaxis are due to smooth muscle constriction. In many recent reports, the guinea pig has been used to elicit the anaphylactic reaction, in order to study the role of leukotrienes in anaphylaxis (30,53,62,63). Release of leukotrienes from trachea (53) and lung (62) of guinea pig following antigen challenge has been reported. These findings are also similar to reports of identical experiments conducted on human lung tissue (54).

The mechanism of anaphylaxis was studied in mice by Weisser and coworkers in 1941 (69). A series of experiments were conducted to determine the susceptibility of mice to develop anaphylaxis after active and passive sensitization.
The mice responded with little or no signs of anaphylaxis, and none showed fatal anaphylaxis. Hours after unilateral adrenalectomy, two mice had fatal shock, while the majority of animals died of shock after bilateral adrenalectomy. Sobey and Adams, in 1959, used a score, called anaphylactic score, to study the anaphylactic reaction in a batch of mother and daughter mice (58). This was based on symptoms like itching and decreased activity. A wide variation was found in the anaphylactic score in different mice. Based on these observations, they concluded that the anaphylactic potential of a mouse was determined by that of its mother and that this potential was under genetic control. The development of passive cutaneous anaphylaxis (PCA) was also found to be much slower in mice than in guinea pigs, and required a much larger dose of antigen (45). These findings were later supported by Levy in 1964 (35), who developed a technique to elicit PCA and reverse passive Arthus reactions simultaneously. A difference was noticed between three animal species. The mouse was found to be least sensitive to these cutaneous reactions, showing only a mild reaction to undiluted antigen. The rat was about 10 times more sensitive than the mouse, responding to 1/10 dilution, whereas the guinea pig was 1,000 times more sensitive than the mouse, since anaphylaxis was induced by a 1/1,000 dilution of the antigen. The difficulty in eliciting anaphylaxis and fatal shock in rats has also been described
Bilateral adrenalectomy was necessary to induce signs of fatal shock. The difficulty in inducing the Arthus phenomenon in rats, as compared to guinea pigs and rabbits has also been described (41).

The development of anaphylaxis in rabbits has been described as early as 1911 (Auer, as quoted by Coca in 1919) (16). The anaphylactic reaction in rabbits does not resemble that in humans. Although during anaphylaxis symptoms of respiratory distress were present, bronchoconstriction was not seen in rabbits. The cause of death in fatal shock was cardiac failure, due to increased pulmonary arterial pressure, as opposed to bronchiolar spasm in guinea pigs (50). This is explained by the fact that in rabbit, the pulmonary artery has a well developed smooth muscle layer, while the bronchiolar musculature does not. Manwaring used rabbit smooth muscle to study the response to antigens (36). It was found that although the animal developed fatal shock, smooth muscle contraction, as recorded by rise in intra cystic pressure, was very rare in rabbits, when compared to guinea pigs. Recent experiments have shown that LTC₄ and LTD₄ were seen to produce strong contraction in guinea pig lung parenchyma, while in rat and rabbit lung parenchyma, the dose required to produce a similar contraction was 3-4 fold higher (47).

Horse and cattle anaphylaxis are noted to have some similarity to human reactions (50). Dyspnoea, increased
peristalsis, and sweating are the main symptoms. In horses, the anaphylactic response is characterized by acute hypotension, followed by increased pulmonary arterial pressure and respiratory distress within 8-12 minutes. In cattle, similar symptoms are described, with severe reactions taking 15-20 minutes to manifest themselves. In both species death is reported to occur in 2-5 minutes (8). In the calf, abdominal distension is a marked feature. This was explained by the presence of a strong smooth muscle layer in the stomach compartments (50).

It is clear from the above data that a difference exists in the susceptibility of different species to develop anaphylaxis. The role of the peptidoleukotrienes in anaphylaxis has also been established. Studies have been done on in-vivo LTC metabolism in different species and differences in the metabolite that accumulates after specific periods of time have been reported (4, 23,44). In vitro studies have been done to study the products of metabolism and the comparative uptake of LTC by isolated organs and cells (23). The results of these studies showed a differential uptake by different organs. Metabolism of LTC₄ has been studied in human plasma. Although human plasma was seen to convert LTC₄ to LTD₄ and LTE₄ (1,31), LTD was not seen in the mouse or guinea pig tissues in in-vivo studies using LTC (4,22). In vivo studies on the metabolism of leukotrienes in different species has also shown that the
form in which the leukotrienes are excreted differed with the species. In humans, LTE₄ was the main excretory metabolite of LTC₄ metabolism (60,66), while in monkeys, N-acetyl LTE₄ was the form in which the peptidoleukotrienes were excreted (6). In rats, N-acetyl LTE₄ was the main metabolite (44). Considering these observations, it seemed likely that the profile of LTC₄ metabolism in the serum of these animal species would differ. This assumption was supported by observations made during our preliminary experiments using human and mouse serum (11). The profile of LTC₄ metabolism in mouse serum was seen to be very different from that in human serum, in that, after a specific incubation period, no LTD₄ was detected in mouse serum; most of the leukotriene detected was in the form of LTC₄, with small amounts present in the LTE₄ fraction. Under the same conditions, human serum showed presence of significant amounts of LTD₄ and LTE₄, with very little LTC₄. Since LTD₄ is known to be the most potent biologically among the peptidoleukotrienes, this observation correlated with the present knowledge concerning the susceptibility to the development of anaphylaxis in mice and humans.

This study is intended to compare the profiles in LTC₄ and LTD₄ metabolism in human, mouse, rabbit, guinea pig, horse, cow and fetal calf sera.
THESIS OBJECTIVES: The role of peptidoleukotrienes in anaphylaxis has been established. A species specific susceptibility to develop anaphylaxis has also been described. The main objectives of this study are to determine 1) if a species specific difference exists in the metabolic profile of LTC₄, which is the form in which the peptidoleukotrienes are released, and 2) if the anaphylactic susceptibility of an animal correlates with the profile of its LTC₄ metabolism.
MATERIALS AND METHODS

Collection of Animal Blood and Sera

Human blood collected from normal human volunteers, with no history of asthma or other allergic manifestations, was drawn from the cubital vein into heparin free vacutainers. The source of mouse blood was Balb-C mice in all experiments except the last one, in which the strain of mice used was Swiss Webster (SW) mice. The blood collected from several mice by way of the brachial artery, or the guillotine method was pooled. For rabbit blood, the New Zealand White strain of rabbit was used. Blood was collected by puncturing the marginal vein of the ear lobe with a 22 gauge needle. Guinea pig blood was collected from Duncan Hartley guinea pigs by cardiac puncture. The horse, as well as the cow were bled from the jugular vein into heparin free vacutainers. Fetal Bovine Serum (FBS) was purchased from Sigma Chemicals (Sigma Culture Reagents).

In all cases except with mice and rabbit, the blood was drawn into vacutainers. Pooled mouse blood was collected in centrifuge tubes, and rabbit blood from the marginal vein of the ear lobe was allowed to flow into the centrifuge tubes.

Clotting of Blood:

The blood was incubated at room temperature until
clotting occurred, (usually 1 hour to 90 minutes), and centrifuged using a clinical centrifuge (International Equipment) for 20 minutes at room temperature. The serum that was not used was transferred to vials and stored at -70°C.

Since changes in activity of enzymes were noticed using stored serum, a standard method was chosen for clotting procedures in order to ensure that all samples were being treated under the same conditions. The blood collected into heparin free vacutainers or conical centrifuge tubes was placed on ice until they could be placed in a 30°C water bath. Clotting was allowed to take place at 30°C for one hour. The clotted blood was centrifuged at 2,000 rpm for ten minutes at 4°C. The serum collected was maintained at 4°C until use. The serum that was not used immediately was stored in vials of 1 ml each at -70°C. The glassware, syringes, and all other instruments used were sterilized prior to bleeding.

**Enzyme Assays:**

Substrates: Synthetic unlabeled leukotrienes LTC₄, LTD₄, and LTE₄ were purchased from Cayman Chemicals, Ann Arbor, Michigan. The preparations contained 0.1 mg/ml of leukotrienes, dissolved in 0.25 ml of 1:1 ethanol/PBS. Tritium labeled leukotrienes were purchased from DuPont-NEN products. The specific radioactivity of the leukotrienes was 39.3 Ci/m mol, (2.2 x 10⁶ dpm/µl) in 0.5 ml of 50%
ethanol, 0.01 M \(2K_{2}PO_{4}\), at a pH of 6.8.

Unlabeled leukotriene (160 pmols) mixed with 22,000 dpm of tritium labeled leukotrienes was used as a substrate.

Hartmann's Solution (Lactated Ringer Solution):

- Potassium chloride - 0.03 gms
- Sodium chloride - 0.6 gms
- Calcium chloride - 0.02 gms
- Sodium Lactate - 0.31 gms

Made up to 100 ml using dd H\(_2\)O.

**Assay Mixture:**

The assay mixture consisted of 100 μl of undiluted serum (unless otherwise mentioned). A stock solution of the leukotriene mix was made up in Hartmann's solution containing 1% Triton X-100 so that each sample received 160 pmols (22,000 dpm) of leukotriene and 25 μl of 1% Triton X-100. This gave an effective dilution of 0.1% Triton X-100 in the assay mixture), the volume of which was made up to 250 ul with Hartmann's solution. Incubation was carried out at 37°C, for specific time periods. The reaction was stopped by the addition of two volumes of methanol at -20°C, containing butylated hydroxy toluene (BHT) as an antioxidant. After the addition of methanol, the samples were mixed well, and kept at -70°C for at least one hour prior to centrifugation, in order to precipitate proteins. The mixture was then centrifuged at 2,000 rpm for ten minutes at 4°C. The supernatant was transferred to conical centrifuge tubes, evaporated to dryness under vacuum at
40°C. Samples were stored at -70°C until HPLC analysis. The glassware used for the assays were siliconized using Sigmacote (Sigma Chemicals), in order to prevent adherence of LTs to the surfaces, and hence loss of the leukotrienes during extraction.

**RBL-1 Cells:**

Rat Basophilic leukaemia cells were purchased from American Tissue Culture Collection. The cells were maintained in Iscove's modified Dulbecco's medium containing l-glutamine, in the presence of 10% fetal calf serum.

**Stimulation of RBL-1 Cells:**

RBL-1 cells were washed twice with 1 ml of serum free media. The cells were resuspended in Phosphate Buffered Saline (PBS, Sigma Diagnostics) at a concentration of approximately 10^6/ml. The cells were stimulated using lipopolysaccharide (LPS) purchased from Ribi Immunochem 10 ngms/ml, and calcium ionophore (A-23187, Sigma Chemicals) 10 ngms/ml, for 30 minutes at 37°C in the presence of 5 mM calcium chloride (2). After stimulation, leukotrienes were added, and the samples incubated for 30 minutes at 37°C before lipids were extracted.

**Lipid Extraction:**

Modified Bligh and Dyer procedure: This procedure was used in preliminary experiments using RBL-1 cells. After the specific incubation time, the cells were pelleted by
centrifugation in a clinical centrifuge. To the supernatant, two volumes of methanol were added, mixed well, and the proteins allowed to precipitate at -70°C. The protein precipitate was pelleted by centrifugation at 2,000 rpm at 4°C, and to the supernatant, were added two volumes of chloroform, mixed well for one minute, centrifuged, and the chloroform fraction was separated by centrifugation in a clinical centrifuge. The chloroform fraction was collected in a conical centrifuge tube, evaporated to dryness under vacuum, and stored at -70°C until analyzed by reverse phase-high pressure liquid chromatography (RP-HPLC).

Sep-Pak procedure: After protein precipitation using two volumes of methanol, the supernatant was made up to contain 20% methanol, using sodium phosphate buffer (0.1 M, pH 7.4). The sample was run through a C-18 cartridge (Bakerbond spe, J.T. Baker Inc.), pre-washed with 20 ml methanol and 20 ml water. The cartridge was then washed with 5 ml of 20% methanol in sodium phosphate buffer, followed by 5 ml of water. The leukotrienes in the cartridge were then eluted with 3 ml of 80% methanol in distilled water. This eluent was dried down, and analyzed by RP-HPLC.

Direct evaporation technique: The Sep-Pak procedure resulted in low recovery of leukotrienes in the different fractions. So we developed a method, in which direct evaporation of the supernatant, after protein precipitation, was done. The evaporated sample was stored at -70°C, until
it was resuspended in solvent and analyzed by RP-HPLC. This procedure involved minimal loss of radioactivity in different fractions, and less loss by adherence to glassware. The presence of other substances with UV absorbance at 280 nm did not interfere with the analysis, since calculation were based only on radioactivity in different fractions.

**Reverse Phase High Pressure Liquid Chromatography (RP-HPLC) Analysis**

Solvent: The solvent system used was: acetonitrile: methanol: water: acetic acid (1280 / 800 / 1916 / 4, V/V/V/V). The pH was adjusted to 5.5 using ammonium hydroxide (5). The samples that had been evaporated to dryness were resuspended in 250 μl of solvent and injected into a Nucleosil 5-C18 (25 x 4.6) column (Phenomenex), maintaining a flow rate of 1.0 ml/min. A pressure of 60 psi was applied. The sample was eluted from the column, for a total time of 25 minutes. The column was then rinsed with 100% methanol for 15 minutes. The solvent was then allowed to run through the column for 15 minutes to equilibrate the column before the next sample was injected. The peptidoleukotrienes have a max at 279 nm (38).

The elution times of the different leukotrienes were marked (according to UV absorption peaks at 280 nm) using standard LTC₄, LTD₄, LTE₄, LTF₄ and N-acetyl LTE₄. In initial
experiments, to quantify the relative amounts of leukotrienes present after each assay, the UV absorption peaks of the different leukotrienes at the characteristic elution times were matched with the radioactivity in the corresponding fractions. The fractions were collected using a Gibson model 203 micro fraction collector. The radioactivity in the fractions were then determined in a Beckman 7500 scintillation counter. The different leukotrienes were quantitated based on the percentage of total recovered radioactivity in each peak. In later experiments, a Flo-one Beta In-line radioactive flow detector (Radiomatic) was used in which the UV absorption, as well as the radioactivity of the eluted leukotrienes were simultaneously displayed.
RESULTS:

Evaluation of analytical procedures

In the initial experiments with RBL-1 cells, a modified Bligh and Dyer technique of lipid extraction was compared to a technique using C-18 cartridges (Sep-Pak) (26) and one involving a direct evaporation of the methanol fraction after protein precipitation. The recovery of the leukotrienes by the Bligh and Dyer technique was 58.13% (S.D. 10.43, n=10), while the recovery using C-18 cartridges was 71.48% (S.D. 12.6, n=10), and that by the direct evaporation technique was 89.52% (S.D. 8.61, n=20). The elution profile for a mixture of the different leukotrienes is by RP-HPLC using the solvent system previously described, is shown in Fig. 4.

Leukotriene Metabolism in RBL-1 Cells

Initial experiments to study conversion of \(^3\)H-LTC\(_4\), standardization of lipid extraction and enzyme assay procedures were done using RBL-1 cells. RBL-1 cells, stimulated by bacterial lipopolysaccharide, in the presence of Ca\(^{2+}\) and A-23187 (calcium ionophore), were seen to produce both \(^3\)H-LTD\(_4\) and \(^3\)H-LTE\(_4\) from \(^3\)H-LTC\(_4\) (Fig. 5).
Fig. 4. Relative elution times of standard leukotrienes - LTC₄, LTD₄, LTE₄, LTF₄, and N-acetyl LTE₄ by RP-HPLC.
Fig. 5. Metabolism of $^3$H-LTC$_4$ in the supernatant of RBL-1 cells. RBL-1 cells were resuspended to a concentration of approximately $10^6$ cells/ml and stimulated with LPS and A 23187 (calcium ionophore) in the presence of calcium. 1ml of the supernatant was assayed for transpeptidase activity and leukotrienes separated by RP-HPLC. Radioactivity was calculated at elution times corresponding to LTC$_4$, LTD$_4$, and LTE$_4$. Data is expressed as percent radioactivity at different elution times.
Leukoreiene Metabolism in Human Serum

After procedures were standardized using RBL-1 cells, human serum from a normal volunteer was used. Table I shows the mean value and standard deviation of values obtained for the percent conversion of LTC₄ after 30 min. incubation and for LTD₄ after 15 min. incubation in six different serum samples. Conversion of ³H-LTC₄ or ³H-LTD₄ was not seen in serum at 4°C, Hartmann's solution at 37°C, or following denaturation of proteins (Fig. 6, 7). ³H-LTC₄ was converted to ³H-LTD₄, which was then converted to ³H-LTE₄ as seen by the relative amounts of the different leukotrienes present after 15 and 30 minutes incubation (Fig. 8). The experiment was repeated using serum from clotted blood, and plasma from heparinized blood. Heparinized blood showed a decrease in the formation of LTE₄, compared to serum (Fig. 9).

Thereafter, all experiments were conducted using serum.

The effect of storage of serum on the conversion of LTC₄ and LTD₄ was studied using serum stored at 4°C, -20°C and -70°C. Prolonged storage resulted in significant loss of the LTD₄ conversion at 4°C, but not at -70°C (Fig. 10). The experiments that followed were all done using fresh serum, or serum that had been stored at -70°C immediately after collection of blood.

The percent conversion of ³H-LTC₄ to ³H-LTD₄ and ³H-LTE₄ was seen to have a linear relationship with the volume of serum used (Fig. 11). The conversion of ³H-LTC₄
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Table I. Mean and S.D. values for percent conversion of $^3$H-LTC$_4$ conversion (following 30 minutes incubation) and $^3$H-LTD$_4$ (following 15 minutes incubation) in six serum samples.
Fig. 6. Chromatogram showing LTC₄ profile after 90 minutes incubation in (A) serum at 4°C (B) Hartmann's solution at 37°C and (C) protein denatured serum.
Fig. 7. Chromatogram showing LTD₄ profile after 45 minutes incubation in (A) serum at 4°C (B) Hartmann's solution at 37°C and (C) protein denatured serum.
Fig. 8. $^3$H-LTC₄ metabolism in human serum. 1 ml of fresh human serum was incubated with $^3$H-LTC₄ for 15 and 30 minutes. The reaction was stopped using 2 volumes of methanol. Leukotrienes were extracted using C-18 cartridges. The recovered leukotrienes were separated by RP-HPLC. Data shows percent radioactivity at different elution times corresponding to the different leukotrienes.
Fig. 9. Comparison of $^3$H-LTC₄ metabolized in clotted and heparinized blood, placed at 4°C for approximately 24 hours. Assay procedure was as described in Figure 6. Figure shows relative amounts of LTC₄, LTD₄, and LTE₄, present in clotted and heparinized blood, as percent of total radioactivity after 60 minutes incubation.
Fig. 10. Comparison of $^{3}$H-LTC$_{4}$ metabolism in fresh serum stored at 4°C for 4 days, -20°C for 7 days and -70°C for 7 days after 60 minutes incubation. Procedure was same as described in Figure 8. Data expressed as percent of total radioactivity at different elution times corresponding to the different leukotrienes.
Fig. 11. Relationship between enzyme concentration and \(^3\)H-LTC\(_4\) metabolism in human serum. Varying amounts of human serum made up to 1ml using phosphate buffered saline, were assayed for transpeptidase activity using procedure described in Fig.8. Data shows percent of LTC\(_4\) metabolized (left y-axis) and picomoles of LTC\(_4\) converted (right y-axis).
Fig. 12. Relationship between incubation time and $^3$H-LTC$_4$ metabolism in human serum. 0.5 ml serum was made up to 1 ml using PBS. Assay procedure as described in Fig 8. Data is expressed as percent LTC$_4$ converted (left y-axis) and picomoles LTC$_4$ converted (right y-axis).
Fig. 13. Relationship between substrated concentration and $^3$H-LTC$_4$ metabolism. Varying amounts of $^3$H-LTC$_4$, of known specific radioactivity were used as substrate. Assay procedure was as described in Fig 8. Data expressed as picomoles of LTC$_4$, converted/ml/min. Incubation period was 15 minutes.
(to $^3$H-LTD$_4$ and $^3$H-LTE$_4$) showed a linear relation to time (Fig. 12). The conversion of LTC$_4$ also showed a linear increase with respect to substrate concentration (Fig. 13).

**Leukotriene metabolism in mouse serum**

Using $^3$H-LTC$_4$ as a substrate, the profile of LTC$_4$ metabolism was compared in mouse and human serum. The results, as shown in Fig. 14, show a great difference. After a 30 min incubation, with 0.5 ml of serum, 50% of the radioactivity was in the LTD$_4$ and LTE$_4$ fractions in human serum, 47% being present as LTD$_4$. Whereas in mouse serum, all the radioactivity in the 15% of converted $^3$H-LTC$_4$ was present in the region corresponding to the elution time of LTE$_4$. A time course study using $^3$H-LTC$_4$ showed a much higher activity in human serum than in mouse serum (Fig. 15). As this observation suggested a difference in the rate of LTD$_4$ metabolism, metabolism of $^3$H-LTD$_4$ was compared in human and mouse sera (Fig. 16). The mouse serum was seen to have a much higher activity, 85% of the $^3$H-LTD$_4$ being converted in the first 10 minutes of incubation time, while in human serum, only 17% was converted under identical conditions. The metabolism of $^3$H-LTD$_4$ in both human and mouse sera was linear with relation to time and substrate concentration (Figs. 16 and 17). A difference was seen in the rate of conversion with relation to the concentration of enzyme (volume of serum) used, as shown in Fig. 18. Serial dilution of sera were used in this experiment, because the
rate of conversion of $^3$H-LTD$_4$ was seen to be much faster than that of $^3$H-LTC$_4$. The rate of conversion of $^3$H-LTD$_4$ did not decrease with increasing dilutions in human serum. This suggested the presence of some factor in the serum that inhibited the enzyme activity in the undiluted form, or in lower dilutions. Heating the sera to 65°C in a water bath for 30 min decreased the activity in both human and mouse serum. The activity in the heated sera of both species was the same (21%) as shown in Fig. 19. The effect of heat inactivated human and mouse serum on unheated human and mouse serum was studied (Fig. 20,21). The results show that while heat inactivated human serum was able to decrease LTD$_4$ conversion in normal human and mouse serum, heat inactivated mouse serum did not show the same effect.
Fig. 14. Comparison of profiles of $^3$H-LTC$_4$ metabolism in human and mouse serum after 30 minutes incubation. Procedure as described in Fig 8. Data expressed as percent radioactivity at different elution times.
Fig 15. Effect of incubation time on $^3$H-LTC$_4$ conversion in mouse and human serum. 100 μl serum, brought to a total volume of 250 μl with Hartmann’s solution and at 0.1% Triton X-100, was incubated in the presence of 160 picomoles $^3$H-LTC$_4$ (22,000 dpms) at 37°C. Data expressed as percent of LTC$_4$ converted (left y axis) and picomoles LTC$_4$ converted (right y axis).
Fig. 16. Comparison of dipeptidase activity in human and mouse serum. Procedure as described in Fig 15, except $^{3}\text{H}-\text{LTD}_4$ was used as substrate. Data expressed as percent $\text{LTD}_4$ converted (left y-axis) and picomoles $\text{LTD}_4$ converted (right y-axis).
Fig. 17. Relationship between $^3$H-LTD$_4$ conversion and substrate concentration expressed as picomoles/ml. Experimental procedure as described in Fig. 16, except that different substrate concentrations of varying specific radioactivity were used. Data expressed as picomoles converted/ml/min. Incubation period was 15 minutes.
Fig. 18. Relationship between LTD₄ conversion and amount of serum present. 100 ul of 2-fold serial dilutions for human and mouse serum were used. Experimental procedure as described in Fig. 16. Incubation period was 15 minutes. Data expressed as percent LTD₄ converted (left y axis) and picomoles LTD₄ converted (right y axis).
Fig. 19. Dipeptidase activity in heat treated and untreated human and mouse serum after 30 minutes incubation. The dipeptidase activity was assayed as per procedure described in Fig 16.
Fig. 20. Effect of increasing amounts of heated human serum on the dipeptidase activity in normal human serum after 30 minutes incubation. Data expressed as percent conversion (left y-axis), as well as picomoles of LTD₄ converted (right y-axis). Different amounts of heat inactivated serum were added to 100 µl of 1/8 dilution of normal human serum brought to 250 µl with Hartmann's solution and 0.1% Triton X-100 and incubated with 160 picomoles of [³H]-LTD₄ (22,000 dpm).
Fig. 21. Comparison of dipeptidase activity in normal human and mouse serum in presence of heat inactivated human or mouse serum after 30 minutes incubation. Procedure as described in Fig 20. Data expressed as percent LTD4 converted (left y-axis) and picomoles LTD4 converted (right y-axis).
Leukotriene Metabolism in different animal species

The metabolism of $^3$H-LTC$_4$ and $^3$H-LTD$_4$, in human, mouse, guinea pig and rabbit sera were compared by time course studies. No studies were done to compare the conversion of LTC$_4$ and LTD$_4$ with relation to the volume of serum present, or substrate concentration. Fig. 22 and Fig. 23 show the difference in the profile of $^3$H-LTC$_4$ and $^3$H-LTD$_4$ metabolism in the four species. Fig. 24 shows the relative amount of $^3$H-LTD$_4$ present in the converted $^3$H-LTC$_4$ in the four species after specific incubation times. Fig. 25 shows the amount of LTD$_4$ present as a percent of the total $^3$H-LTC$_4$ metabolized. The wide variation of this value in the four species and the correlation of this value with the susceptibility to develop anaphylaxis made it necessary to repeat the experiment. In the following experiment, in which fresh serum was used, horse and cow serum and fetal bovine serum (FBS) were included. Fig 26 and Fig. 27 show the time course of LTC$_4$ and LTD$_4$ conversion in the different species. Fig.28 shows the relative amount of $^3$H-LTD$_4$ present, as a percentage of the total converted $^3$H-LTC$_4$, after 15, 60 and 90 min incubation. Fig. 29 shows a comparison of the relative amounts of a radioactive metabolite of $^3$H-LTD$_4$, metabolism, which has not been identified. The values mentioned in this experiment are an average of the results from experiments done in duplicate (Table II).
Fig. 22. Comparison of transpeptidase activity in human, mouse, guinea pig and rabbit serum. Procedure as described in Fig 15. Data expressed as percent LTC converted (left y-axis) and picomoles LTC converted (right y-axis).
Fig. 23. Comparison of dipeptidase activity in human, mouse, guinea pig and rabbit serum. Procedure as described in Fig 16. Data expressed as percent LTD₄ converted (left y-axis) and as picomoles LTD₄ converted (right y-axis).
Fig. 24. Comparison of $^3$H-LTC$_4$ conversion in human, mouse, guinea pig and rabbit serum. The amount of LTC$_4$ converted is expressed as relative amount of LTD$_4$ and LTE$_4$ present after 20 min and 90 min. incubation (percent conversion on left y-axis, picomoles converted on right y-axis).
Fig. 25. Comparison of LTD₄ present in the converted ³H-LTC₄ as a percentage of total converted LTC₄, after 90 minutes incubation.
Fig. 26. $^3$H-LTC₄ conversion in 100 ul of horse, cow, fetal bovine, human, rabbit, mouse, and guinea pig as a time course study. Procedure as described in Fig 15. Data expressed as per cent LTC₄ converted (left y axis) and picomoles LTC₄ converted (right y axis).
Fig. 27. Conversion of $^3$H-LTD$_4$ in 100 ul of horse, cow, fetal bovine, human, rabbit, mouse, and guinea pig as a time course study. Procedure as described in Fig 16. Data expressed as per cent LTD$_4$ converted (left y axis) and picomoles LTD$_4$ converted (right y axis).
Fig. 28. Relative amounts of $^3$H-LTD$_4$, present in 100 ul of horse, cow, human, rabbit, mouse, and guinea pig sera after 15, 60 and 90 minutes. Data expressed as per cent of LTD$_4$ present in the total converted $^3$H-LTC$_4$. 
Fig. 29. Relative amount of the unknown radioactive substance present in the total converted LTD, after 45 minutes incubation.
### % LTC4 Conversion

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### % LTD4 Conversion

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Table II. Duplicate values obtained for percent conversion of $^3$H-LTC$_4$ and $^3$H-LTD$_4$, after varying periods of incubation in the sera of the different species tested.
DISCUSSION:

Arachidonic acid metabolites have been associated with a variety of inflammatory reactions. Products of the lipoxygenase pathway of metabolism, the peptidoleukotrienes (formerly referred to as SRS-A) are important mediators in immediate hypersensitivity reactions (24,54). Elevated levels of peptidoleukotrienes were noticed in bronchial asthma and are thought to be responsible for the symptoms in bronchial asthma, which is an immediate hypersensitivity reaction. Elevated leukotriene release from lungs of animals, during experimentally induced anaphylactic reactions have been reported (18,30,53,62). The profile of leukotriene metabolism in different species of animals were studied to see if a correlation exists between the leukotriene metabolism profile, and the relative species susceptibility to develop anaphylaxis.

Using $^3$H-LTC$_4$ as substrate, supernatant from RBL-1 cells were seen to convert LTC$_4$ to other radioactive substances, which eluted, during RP-HPLC, at times corresponding to synthetic LTD$_4$ and LTE$_4$. The conversion of $^3$H-LTC$_4$, was enzymatic since it was not seen to take place in a buffer (Fig. 5).

Lipid extraction and analytical procedures, that gave satisfactory results with RBL-1 cells, were employed to
extract the leukotrienes from human serum. $^{3}\text{H-LTC}_4$ was metabolized by human serum, with the production of new radioactive products, which eluted with synthetic LTD$_4$ and LTE$_4$. Conversion of $^{3}\text{H-LTC}_4$ or $^{3}\text{H-LTD}_4$ was not observed in Hartmann's solution at 37°C, serum at 4°C, or in protein denatured serum (Fig. 6, 7). The metabolism of $^{3}\text{H-LTC}_4$ in human serum was dependent on time, amount of enzyme present (volume of serum) and on the substrate concentration (Fig. 11, 12, 13). In the time course study (Fig. 8), more radioactivity was seen in the LTD$_4$ region with shorter incubation periods. As incubation time increased, the amount of radioactivity decreased in the LTD$_4$ region and increased in the LTE$_4$ region, showing that LTC$_4$ was first converted to LTD$_4$, which was then converted to LTE$_4$. These findings are in accordance with the current knowledge about LTC$_4$ metabolism; that LTC$_4$ is first converted to LTD$_4$ and that LTD$_4$ is then converted to LTE$_4$ (24, 54).

The enzymatic activity in serum was influenced by storage conditions. Prolonged storage at temperatures above -70°C were seen to decrease the conversion of LTD$_4$ to LTE$_4$ (Fig. 10). A difference in LTD$_4$ conversion was also noticed using heparinized versus clotted blood. When blood was heparinized, the LTD$_4$ conversion was the same as in clotted blood, when assayed immediately; however, when heparinized blood was stored overnight at 4°C, prior to separation of plasma, the amount of LTD$_4$ converted was seen to be reduced.
(Fig. 9). The LTD₄ conversion requires Ca²⁺, Mg²⁺, and Mn²⁺ for optimal activity (39,52). The presence of substances like L-cysteine and dithiothreitol, which remove divalent cations from the medium are known to inhibit the LTD₄ conversion (39). Subsequent addition of these cations at 1 mM concentration was seen to restore the lost activity (39). Although the anticoagulant effect of heparin is not due to the removal of Ca²⁺, it is possible that it is acting as an inhibitor of LTD₄ conversion because it has the property of activating protease inhibitors, which in turn prevent the conversion of prothrombin to thrombin (70).

Initially, leukotriene metabolism in serum of normal Balb-C mouse was compared with that in human serum. The profile in normal mouse serum was seen to be very different from that in humans (Fig.14,15). After 30 minutes incubation, 0.5 ml of normal human serum showed 67% conversions, with 34.6% recovered as LTD₄ and 15.1% as LTE₄. The mouse serum showed only 13.4% conversion, and all the radioactivity of the metabolized ³H-LTC₄ was recovered as LTE₄. It was presumed from these results, that the formation of LTD₄ must be a much slower process in mouse than in humans, and that the LTD₄ formed was immediately converted to LTE₄. Subsequent experiments with ³H-LTD₄ as substrate, showed that the amount of LTE₄ formed during LTD₄ conversion was 4.2 times more in mouse than in humans (Fig.16). These findings correlate with results of
experiments by Applegreen and Hammarstrom (4), who found that 20 minutes after intravenous injection of $^3$H-LTC$_3$ into mice, the radioactivity was recovered as LTC$_3$ in liver and other organs. $^3$H-LTE$_3$ was detected only in the lung, after 60 minutes. Recovery of $^3$H-LTD$_3$ was not reported in any tissue. LTC$_3$ has been reported as having similar biological activity and enzyme specificity as LTC$_4$ (21); therefore the profile of LTC$_3$ metabolism should reflect that of LTD$_4$. Since LTD$_4$ is biologically the most potent peptidoleukotriene, the absence of LTD$_4$ accumulation in mouse serum, correlates with the reported resistance to the development of anaphylaxis in the mouse (35,45,69).

The conversion of LTD$_4$ was studied in mouse and human sera, in the same way as the LTC$_4$ conversion, using $^3$H-LTD$_4$ as substrate. The rate of conversion of LTD$_4$ increased with time, as well as with substrate concentration (Fig.16 and 17). However, the rate of reaction did not decrease in a linear manner, with increasing dilutions (Fig. 18). The rate of conversion increased up to a dilution of 1/8, and then declined. This observation implied that the serum contained another factor, the presence of which was reducing LTD$_4$ conversion, in the undiluted form. Initial dilutions could have diluted the effect of this factor, leading to an increase in the LTD$_4$ conversion.

The presence of an inhibitor of LTD$_4$ conversion has been mentioned briefly, by Raulf and coworkers in 1985
They suggested that a rapid decline in LTD₄ conversion in supernatant of stimulated RBL-1 cells was probably due to the presence of inhibitors. An attempt was made to study the nature of this "inhibitor" in serum. The enzyme activity in the serum was destroyed by immersing serum in a 65°C water bath for 30 minutes (Fig. 19). This temperature was chosen, because temperatures above 65°C solidify the serum, due to coagulation of serum proteins, making it unfit for enzyme assay. Addition of heat inactivated human serum to unheated human serum was seen to decrease the LTD₄ conversion by the unheated serum, in a concentration dependent manner (Fig. 20). The effect of heat inactivated human and mouse serum, on unheated human and mouse serum, was compared. The heat inactivated human serum, seemed to contain larger amounts of the "inhibitory substance", as shown by the dramatic reduction of activity in normal mouse serum (which showed high rate of LTD₄ conversion), as compared to the reduction of activity in human serum. Heat inactivated mouse serum did not have as much an inhibitory effect on mouse serum, or on human serum (Fig. 21). Based on the results of this experiment, human serum was thought to contain a higher concentration of an unknown heat stable substance, which decreased the rate of conversion of LTD₄. This "inhibitory factor", could have increased the accumulation of ³H-LTD₄ in human serum. Absence, or a less amount of this substance in mouse serum
might contribute to the higher resistance offered by mice to the development of anaphylaxis. Further studies, leading to characterization of this "inhibitory factor" are now in progress.

The interesting results obtained by comparing human and mouse serum, with respect to leukotriene metabolism, and the correlation of these results with the anaphylactic reaction in these two species, lead us to conduct similar experiments on sera of other animal species. Guinea pigs and rabbits are reported to develop severe anaphylactic reactions (9). Anaphylactic reactions in horse and cattle have also been described (8). In an effort to identify a basis for the differences in hypersensitivity reactions, $^3$H-LTC$_4$, and $^3$H-LTD$_4$, were used as substrates to study the profile of leukotriene metabolism in human, mouse, guinea pig and rabbit serum. The profile of LTC$_4$ conversion different in all four species (Fig. 22). It was highest in human serum, 32% being converted by 100 ul of serum after 60 min incubation. Rabbit and mouse sera showed a similar profile, 6% and 4% being converted after 60 min, while guinea pig serum showed least activity, 0% LTC$_4$ being converted after 60 min. LTD$_4$, is the most potent of the peptidoleukotrienes, with respect to biological activity. Therefore the relative amount of LTD$_4$, present as a percent of the total converted $^3$H-LTC$_4$, was determined (Fig. 24 and 25). Larger amounts were present in human, guinea pig and rabbit sera, 25, 65
and 72\% being present respectively. In the mouse serum, only 4\% of the converted $^3$H-LTC$_4$ was present as $^3$H-LTD$_4$. The difference in the relative amount of LTD$_4$ present suggested a difference in the metabolism of LTD$_4$. Using $^3$H-LTD$_4$ as a substrate, LTD$_4$ metabolism in the four species was compared (Fig.23). The mouse serum showed highest activity, which accounted for the negligible amounts of LTD$_4$ accumulation in mouse serum. The susceptibility to the development of anaphylaxis, in the different species described by earlier workers (9,50), seemed to be related to the rates of synthesis and metabolism of LTD$_4$; i.e., the lower the rate of LTD$_4$ synthesis, or the higher the rate of LTD$_4$ conversion, less prone was the animal to develop a severe anaphylactic reaction.

The above experiment was done using sera stored at -70°C immediately after collection. Another experiment was designed using fresh serum from each species. In this experiment, which was done in duplicate, the serum was placed at 4°C (for 1 hour maximum time) until the assay was done. Sera from horse, cow and fetal bovine were also included. The mouse serum in this experiment came from Swiss Webster (SW) mice, whereas in the previous experiment it was collected from Balb-C mice. Sera from horse, cow and FBS showed maximal activity, more than 50\% of LTC$_4$ being converted in the first 15 min of incubation (Fig.26).
showed similar patterns as those described above. However, there were differences in the relative amounts of LTD₄ produced. The relative amounts of LTD₄ present after 15, 60 and 90 min. incubation are shown in Fig. 28. In mouse serum, 100% of the converted LTC₄ after a 60 min incubation was seen as LTD₄, in contrast to the complete conversion to LTE₄ in the previous experiment. Nevertheless, only 6.7% of the ³H-LTC₄ was metabolized. Moreover, a high rate of LTD₄ conversion was observed in the mouse serum, which could lower the LTD₄ level as soon as it arises. A significant decrease in the LTD₄ levels was seen after 90 min. This difference seen between the two experiments, may be due to a strain difference. Since development of anaphylaxis is thought to be genetically controlled (58), it could be that a strain to strain difference exists in the susceptibility to develop anaphylaxis. A strain difference in asthma models in rats has been reported (37). Experiments dealing with induction of anaphylaxis in different strains of mice are necessary to confirm this view.

The addition of ³H-LTC₄ to rabbit serum resulted in the accumulation of a large amount of ³H-LTD₄ even after 90 minutes incubation (Fig. 28). This correlated with the susceptibility of the animal to develop anaphylaxis. In the presence of a high rate of LTC₄ conversion, a slower rate of LTD₄ conversion would be expected, in order to maintain high levels of LTD₄ even after 90 minutes incubation with ³H-LTC₄.
Surprisingly, rabbit serum showed a high rate of $^3$H-LTD$_4$ conversion (Fig. 27). This observation was different from that in the previous experiment. In human serum, a large amount of LTD$_4$ was present after 60 and 90 min incubation (Fig. 28). This appeared to result from a high rate of LTC$_4$ metabolism and a lower rate of conversion of LTD$_4$ to LTE$_4$.

This metabolic profile might maintain a high level of LTD$_4$ for prolonged periods of time. This pattern of leukotriene metabolism might, therefore, contribute to the prolonged symptoms of anaphylaxis in humans, (eg., the symptoms of bronchial asthma). The slow rate of metabolism of LTD$_4$ might account for the high amounts of LTD$_4$ in the supernatant from alveolar macrophages in asthmatics individuals, as compared to those from normal individuals, reported by Damon and coworkers (17). However, Schwartzberg and coworkers (56), and Isono and coworkers (26) found only LTC$_4$ in the serum during an acute asthmatic attack in children. These findings suggest that during an acute asthmatic attack, the metabolism of leukotrienes in the lung might be different from that in the blood.

The LTC$_4$ conversion as well as the LTD$_4$ conversion activity in guinea pig serum seemed to be the lowest of all sera tested (Fig. 26, 27). Although the rate of conversion of LTC$_4$ was low, the slow rate of LTD$_4$ conversion might be responsible for accumulation of the converted $^3$H-LTC$_4$ as LTD$_4$. These observations correlate with those of in vivo
experiments conducted by Keppler et al (30), where LTD₄ was the main metabolite in bile following anaphylactic shock in the guinea pig. Clancy and coworkers (15) have reported LTD₄ as the main metabolite in the supernatant of guinea pig lung tissue after incubation with LTC₄. De Nucci and coworkers (18) have identified LTD₄ as the main metabolite in perfused lung tissue, after antigen challenge. Guinea pig and mouse sera showed a similarity in the profile of ³H-LTC₄ metabolism. On comparing the relative amounts of LTD₄ following ³H-LTC₄ metabolism, 100% of the metabolized ³H-LTC₄ was recovered as LTD₄ after 60 minutes incubation. However, the rapid rate of LTD₄ conversion in mouse serum brought about a reduction in the ³H-LTD₄ levels after 90 minutes incubation (Fig 28). This suggests that LTD₄ conversion is more important than LTC₄ conversion in controlling the accumulation of LTD₄.

Although guinea pig and rabbit sera are similar in the relative amounts of LTD₄ present, the anaphylactic reaction is different in the two species. In guinea pig, the anaphylactic reactions mimic that in humans, and the respiratory distress is similar to that seen in bronchial asthma in humans (50). These symptoms are due to narrowing of bronchioles due to smooth muscle constriction. Although respiratory distress occurs in rabbit, it is not due to bronchoconstriction, but rather due to constriction of the pulmonary artery. This has been shown by experiments in
which a rise in pulmonary arterial pressure was demonstrated (16). The pulmonary artery of the rabbit has a more
developed smooth muscle layer, whereas the bronchioles have
less smooth muscle than in the guinea pig. After fatal
anaphylaxis, a difference in lung pathology supports the
fact that in rabbit, death is due to cardiac failure due to
raised pulmonary arterial pressure, whereas in guinea pig it
is due to bronchoconstriction (50). For these reasons, the
guinea pig is known to be a better experimental animal model
for bronchial asthma.

In horse and cow serum, both LTC₄ and LTD₄ conversion
were seen to be high, reaching almost maximal levels after
15 min incubation (Fig.26,27). Although large amounts of
³H-LTD₄ are formed in the initial stages, these levels
decline rapidly due to increased LTD₄ conversion. This
observation correlates with the description of the
anaphylactic reaction in horse and cattle (8), where the
symptoms of respiratory distress develop within 8-15
minutes, and then subside spontaneously. The horse and cow
serum appeared to be similar to human serum in that
significant amounts of LTD₄ were present after 15 min
incubation. This might be one of the factors responsible
for the similarity in symptoms of anaphylaxis between
humans, horse and cattle (50).

The relative amounts of LTD₄ present were expressed
as a percentage of the total converted ³H-LTC₄.
radioactive substances other than LTD$_4$ were not always confined to the LTE$_4$ region. In certain species, as in horse, and in FBS, an additional radioactive peak was seen immediately after the elution of LTE$_4$. The elution time of this metabolite did not coincide with that of standard preparations of LTC$_4$, LTD$_4$, LTE$_4$, N-acetyl LTE$_4$, or LTF$_4$. The formation of this unknown substance did not appear to be related to the rate of LTD$_4$ conversion reaction (Fig 29), as it was not seen in rabbit and mouse serum which showed high rate of conversion of LTD$_4$. Hammarstrom (23) has reported the presence of eight LTC$_4$ metabolites in the feces of rats after subcutaneous injection of $^3$H-LTC$_4$. Several of these have not yet been identified. Until this substance has been identified, and its biological activity assessed, it cannot be concluded if the formation of this substance enhances or decreases the symptoms in an anaphylactic reaction.
REFERENCES:


33. Lee, Chong W., Robert A. Lewis, E.J. Corey, Alan


44. Orning, Lars, Elisabeth Norin, Bengt Gustafsson and


65. Verhagen, Jan, Pieter L.B., Bruynzeel, Johannes A. Koedam, G. Aryan Wassink, Martin de Boer, Gerber K.


