

Nebulization and selective deposition of LTD₄ in human lungs

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The process of nebulization and deposition of LTD₄ was studied in detail. The concentration of LTD₄ in a saline solution decreased by approximately 90 % after 2 min of nebulization in a DeVilbiss 35B ultrasonic nebulizer. This decrease was prevented by diluting LTD₄ in a phosphate buffer, pH 7.4. Nebulization of tritiated LTD₄ in this phosphate buffer did not cause any appreciable deterioration of the leukotriene, as demonstrated by an unchanged ratio between radioactivity and LTD₄ concentration in the test solution before and after nebulization as well as in the condensed aerosol. The aerosol generated by the DeVilbiss 35B ultrasonic nebulizer was shown to generate particles with a mass median diameter of 1.3 microns (dry particle size). Interposition of a settling bag reduced the amount of large particles, reducing the mass median diameter to 0.84 microns (dry particle size). Nine healthy volunteers were challenged on separate days with 40 nmol LTD₄ or 100 µmol histamine, and the changes in FEV₁ and partial flow volume curves initiated at 50 % of vital capacity (V_{max30}) were measured. A relative diffuse deposition pattern was ensured by inhalation via a settling bag. These results were compared to challenges with a relatively central deposition pattern as ensured by inhalation directly from the nebulizer with brisk inhalation maneuvers. The diffuse deposition pattern caused minimal changes in FEV₁ but pronounced effect in V_{max30}. The effects of LTD₄ and histamine on FEV₁ and V_{max30} changed in parallel when the deposition of the mediators was changed to a more central pattern. This indicates that the two mediators do not differ with respect to any selective effects on different parts of the airways.

Key words: adsorption; bronchoconstriction; challenge; LTD₄; nebulization.

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CLINICAL ASPECTS

Nebulization of LTD₄ is demonstrated to depend on ionic strength or pH of the solvent, as stabilizing pH ensured the transport of intact LTD₄ from a solution to an aerosol, whereas nebulization of unbuffered solutions caused a decreasing concentration of LTD₄ in the solution, probably due to adsorption. This principle might apply to other mediators used for bronchial challenge, and would constitute an important confounding variable if not observed. The effect of bronchial challenge depends on the load of particles generated as well as the distribution of size. The aerosol generated by the common ultrasonic nebulizer DeVilbiss 35B is analyzed in these respects, and it is demonstrated how interposition of a settling bag can be used as a convenient means of reducing the particle size distribution in the aerosol. Since the heterodisperse aerosol will cause a relatively more central deposition in the airways than the monodisperse aerosol, the settling bag was used as a simple means of comparing the effect of histamine and LTD₄ on central versus peripheral airways.

Leukotriene (LT) C₄, D₄, and E₄ constitute the biological activity formerly known as slow reacting substance of anaphylaxis. Arachidonic acid

is transformed via a 5-lipoxygenase and conjugated with glutathion to yield LTC₄, and subsequently deaminated to LTD₄ and LTE₄ (18).

The elucidation of these structures started a surge of studies into their possible role in bronchial asthma. Human *in vivo* studies in several centers (2, 9, 13, 19, 23), as well as our own laboratory (3, 4), investigated the effect of inhalation of nebulized sulphidoleukotrienes. The unsaturated fatty acid, eicosatetraenoic acid, constitutes the backbone of these leukotrienes, and therefore it might be suspected that they would be oxidized or otherwise destroyed during nebulization. In the first part of the present study we addressed the question: can LTD₄ be transferred by ultrasonic nebulization to an aerosol without appreciable deterioration?

In the second part of the study we compared the distribution of particle sizes in aerosols of LTD₄, histamine and phosphate buffered saline nebulized from a 35B DeVilbiss nebulizer. Interposition of a settling bag was studied as a possible means of reducing the number of large particles in the aerosol, and thereby the sedimentation and impaction in central tracheo-bronchial airways.

In the last part of the study we addressed the question: will the reduction in FEV₁ and V_{max30} be affected similarly by LTD₄ and histamine when the deposition of aerosol is changed from a relatively diffuse to a relatively central pattern?

MATERIALS AND METHODS

Subjects

Five women and four men, aged 21–25 years, were studied. They had no history of atopic symptoms, nor had they a history of upper respiratory infections in the previous month. The subjects did not use drugs regularly or in the week prior to examination. They were non-smokers. All had participated in previous studies of the effect of LTD₄ in normal subjects (4). The subjects volunteered fully informed, and the study was approved by the local Ethics Committee.

Materials

LTD₄ (kindly supplied by Dr. J. Rokach, Merck-Frosst Research Laboratories, Quebec) was kept in phosphate buffered stock solutions under argon at –80°C until 1 h before use.

Phosphate buffered saline (PB-saline) pH 7.4 was used as solvent. The concentration of the leukotriene in the batches was regularly tested by UV spectral analysis. 14,15(N)-3H LTD₄ was purchased from Amersham (code No. TRK 771). KR-85 neutralizer, TSI model 3054; TSI diluter model 3302, and Aerodynamic particle Sizer model 3300: TSI incorp. St Paul, Minnesota, USA.

High pressure liquid chromatography (HPLC) of leukotriene D₄

The instrument comprised a pump model N-6000A, an automated injector WISP 710B and an absorbance detector 440, all from Waters Ass. 25 µl test solution was applied to a LiChrosorb RP-18 (5 µm) column (Merck, Darmstadt, FRG) in a mobile phase of 35 % acetonitrile and 65 % 0.02 M acetate buffer, pH 5.80, at a flowrate of 1.0 ml/min. UV-absorbance was measured as the height of the peak at the expected retention time as determined by the authentic LTD₄ stock solution.

Analysis of aerosol particles

Electrostatic charge on aerosol particles was neutralized by a Kr-85 radioactive source (TSI model 3054). The liquid droplets were thereafter evaporated in a diffusion dryer and led to a mixing chamber for dilution with dry air (TSI diluter 3302). The aerodynamic diameter could subsequently be analyzed in an aerodynamic Particle sizer, model APS 33 (5). The velocities of the individual particles in the airstream were measured by a two-spot laser velocimeter focusing a photomultiplier tube. A particle passing through the two beams produces two electrical pulses. The time interval between the two pulses is inversely proportional to the particle velocity and directly proportional to particle aerodynamic diameter. It should be noted that the aerosols were examined from dry particles instead of liquid droplets. Mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD) were calculated from the cumulative plot. The particle density was assumed to be 2.165 g/cm³.

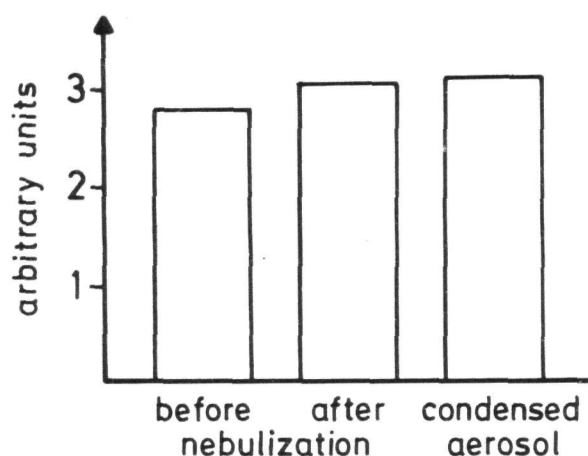


Fig. 1. Stability of LTD₄ during nebulization. Ratio between radioactivity and absorbance of a test sample of 14,15(N)-³H-LTD₄, before and after nebulization by a DeVilbiss 35B ultrasonic nebulizer, and in the condensed aerosol. The ratios are given in arbitrary units.

Challenge procedure

The test substances were dissolved in 2 ml PB-saline and added to a DeVilbiss 35B ultrasonic nebulizer. Nebulizer output 0.9 ml/2 min was determined by a weighing procedure. The test person wore a nose-clip and inhaled via an unidirectional valve to avoid aerosol disappearing into the atmosphere. The nebulization of the test solution was completed within 2 min, and the person inhaled for 4 min to ensure challenge by all the aerosol generated. The challenge was equated to the total amount nebulized. The aerosol could be led via a settling bag of 10 l and a 1 m-long hose. The persons were instructed to use tidal volume breathing only and to avoid coughing and sighing during the test period. On each day of examination the test persons inhaled 2 ml PB-saline prior to the inhalation of the test doses. The lung function examinations were performed before the inhalation of PB-saline (LF-I), immediately after the inhalation of PB-saline (LF-II) and immediately after the inhalation of the test dose (LF-III). The results of those performed after PB-saline defined the baseline. The individual lung function tests of LF-II and LF-III were always performed in the same sequence.

Lung function examination

The subjects were studied with computerized

Jaeger lung function equipment (transfer screen) which included a pneumotachygraph. All values were calculated at body temperature, pressure and saturation. The equipment was calibrated daily. Inspiratory vital capacity (VC) was measured three times and the highest value used. The highest value from three complete flow volume curves was used to calculate 1 s forced expiratory flow rate (FEV₁). Partial flow volume curves were initiated at RV + 50% VC, and the flow was read at RV + 30% VC (V_{max30}). The highest value from three attempts was used.

Statistics

Descriptive statistics were medians with regard to central tendency, and dispersion was indicated by the interquartile range, which contains the central 50% of data.

Protocol

Sub-study I was designed to study the possible deterioration of LTD₄ during nebulization. Test samples of LTD₄ dissolved in saline, acetonitrile or PB-saline to final concentrations of 10 µmol/l were nebulized for 2 min. Samples were taken from the solution left in the nebulization chamber before and after nebulization, and the relative change in absorbance determined on HPLC. Subsequently, 14,15(N)-³H-LTD₄ mixed with LTD₄ and dissolved in PB-saline to a final concentration of 10 µmol/l were nebulized for 2 min. Samples were taken from the solution left in the nebulization chamber before and after nebulization, as well as from the condensed aerosol. The changes in the ratios of absorbance to radioactivity were determined. All tests were done in duplicate.

Sub-study II aimed at a description of the distribution of particle aerodynamic diameters generated from solutions of LTD₄, histamine and PB-saline. Aerosols generated from LTD₄, 10 µmol/l, histamine chloride, 5 mmol/l, and PB-saline were led through a settling bag and analyzed. Finally, an aerosol of the LTD₄ solution was analyzed directly from the nebulizer. All tests were done in duplicate.

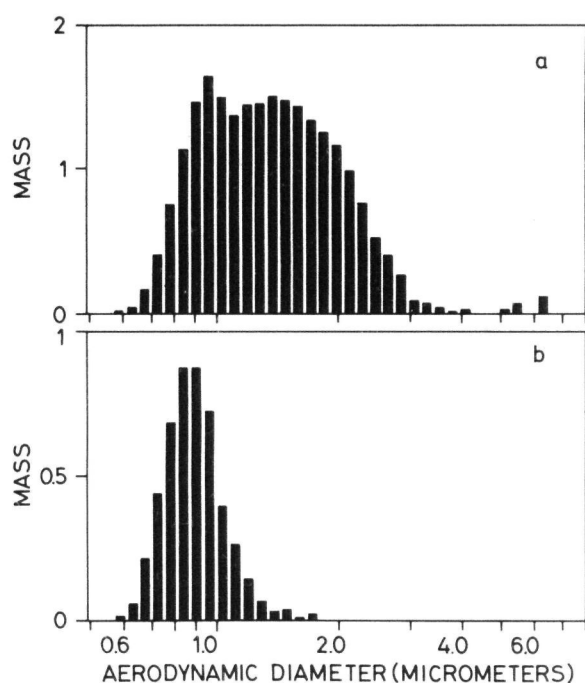


Fig. 2. (a) Distribution of dry particle sizes in an aerosol of PB-saline generated from a DeVilbiss 35B ultrasonic nebulizer and measured by the APS 33; mass median aerodynamic diameter 1.29 microns, geometric standard deviation 1.51. (b) Interposition of a 10 l settling bag reduces the total output, as well as the variability of particle sizes; mass median aerodynamic diameter 0.84 microns, geometric standard deviation 1.18.

Sub-study III was outlined to compare the effect of LTD₄ and histamine on FEV₁ versus V_{max30} when the aerosol deposition was changed from a relatively peripheral deposition to a relatively central deposition pattern. Nine volunteers were challenged on 4 different days with aerosols of LTD₄ (40 nmol), and histamine (100 µmol). Both mediators were given on 2 different days. One day a relatively diffuse deposition pattern was attempted by inhalation from the nebulizer via a settling bag, on a subsequent day a relatively central tracheo-bronchial deposition pattern was sought by inhalation directly from the nebulizer using rapid inhalation maneuvers. One week was interposed between the 4 test days.

RESULTS

Sub-study I. The LTD₄ absorbance in the saline solution left over after 1 and 2 min of nebulization relative to the absorbance in the test samples from the chamber before nebulization was 35% (range 35–36) and 9% (range 8–10) respectively. Comparable measurements on the

absorbance of LTD₄ in PB-saline showed percentages of 112% (range 95–130) and 106% (range 95–118), and similarly when dissolving LTD₄ in H₂O: acetonitrile 65:35; (range 100–105) and 109% (range 107–111).

The median ratio between radioactivity and HPLC absorbance of LTD₄ in the PB-saline solution from the nebulization chamber before and after nebulization as well as in the condensed aerosol is depicted in Fig. 1, demonstrating the consistency of this ratio.

Sub-study II. Fig. 2a, b depicts the results from the measurements of aerodynamic particle size of an LTD₄ aerosol measured directly from the nebulizer (a) and after interposition of a settling bag (b). The ultrasonic nebulizer generated particles with MMAD 1.29 µm, GSD 1.51 (dry particle size). It is apparent that the settling bag reduces the total amount of aerosol delivered as well as the particle size distribution mainly by filtering the large particles due to settling; MMAD 0.84 µm, GSD 1.18 (dry particle size). Aerosols generated from solutions of histamine and PB-saline did not differ from the aerosol generated from the LTD₄ and PB-saline solution.

Sub-study III. The effect of a relatively central tracheo-bronchial deposition versus a relatively diffuse deposition of LTD₄ and histamine on the change in FEV₁ and V_{max30} is shown in Fig. 3. The relatively central deposition of either mediator caused more pronounced change in both FEV₁ and V_{max30} than the relatively more diffuse deposition pattern. The change in the relative deposition pattern caused parallel changes in FEV₁ and V_{max30} for the two mediators.

DISCUSSION

The chemical structure of the sulphidoleukotrienes indicates that they are unstable compounds. A thioether linking the peptide moiety to the eicosanoid chain is labile to oxidation. Furthermore, the conjugated triene of the eicosatetraenoic acid is chemically labile. Oxidation on exposure to air can be expected.

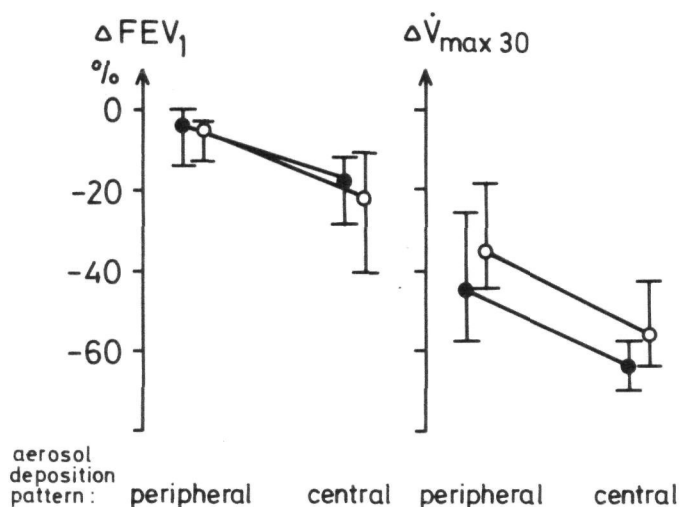


Fig. 3. Effect of 40 nmol LTD₄ (●) and 100 μmol histamine chloride (○) on FEV₁ and V_{max30} after peripheral versus central depositions. A relatively peripheral deposition pattern was ensured through inhalation via a settling bag, while a relatively central deposition pattern was ensured by rapid inhalations of the aerosol directly from the nebulizer. The effect of LTD₄ and histamine on FEV₁ and V_{max30} changed in parallel with changing the deposition pattern of the mediators.

Nebulization could therefore be suspected to cause oxidation of the sulphidoleukotrienes.

Crude preparations of SRS-A (17), and lately LTC₄ (24), are known to be unstable at low pH where they undergo decomposition to numerous products which cannot be observed by ultraviolet spectral measurements. Furthermore, sulphidoleukotrienes were demonstrated to adsorb reversibly to glass and plastic surfaces (24).

Previous studies have demonstrated a decreasing concentration of LTD₄ in saline solutions left in the nebulization chamber of a Wright jet nebulizer (2) and a DeVilbiss 35B ultrasonic nebulizer (3). Jet nebulization will normally concentrate the salt in a saline solution when unsaturated air is used, whereas ultrasonic nebulization does not appear to change the salt concentration in the nebulizer (8). A decrease in the LTD₄ concentration was consequently other than the expected inherent evaporation properties of the jet nebulizer and might indicate a deterioration of the LTD₄. The concentration of LTD₄ in the condensed aerosol from the ultrasonic nebulizer proved to be of a higher concentration than that originally

nebulized from a saline solution (3). However, as quantitative collection of the aerosol was not feasible this finding did not conclusively prove LTD₄ to be nebulized without deterioration. In the present study we found that the concentration of LTD₄ in saline solution showed a pronounced time-dependent decrease during nebulization. This decrease in concentration was prevented by the use of a phosphate buffer (pH 7.4) or water-acetonitrile mixture as diluents. Furthermore, we showed that LTD₄ in phosphate buffer was not appreciably deteriorated during ultrasonic nebulization, since the ratio between radioactivity of a sample of tritiated LTD₄ and the absorbance as measured by HPLC remained unchanged in the test solution before and after nebulization as well as in the condensed aerosol. Whether the decline in LTD₄-concentration when using saline as solvent is caused by deterioration and/or by adsorption to the plastic surfaces of the nebulizer cannot be concluded from the present study. We suspect, however, that we are dealing with an adsorption phenomenon, as the LTD₄ decline could be prevented by decreasing the polarity of the solvent by acetonitrile as well as by stabilizing the pH. If the decrease in the LTD₄ concentration was due to pH-dependent deterioration of LTD₄, it would be expected that acetonitrile-water-mixture and saline caused the same decrease in LTD₄-concentration. If however, the decrease in LTD₄ is due to adsorption to non-polar plastic-surfaces, one would suspect adsorption energy of this lipid substance to be lowered considerably when using a non-polar solvent like acetonitrile and by diminishing the degree of ionization in a neutral buffer, just as was found in the present study.

Use of unbuffered sulphidoleukotriene solutions might account for some of the variability in the reported potency of the substances as well as influencing the reproducibility of the results.

The aerodynamic diameter of the particles generated from solutions of PB-saline, LTD₄ or histamine did not differ. The DeVilbiss 35B ultrasonic nebulizer generated a heterodisperse aerosol with MMAD of 1,29 μm (dry particles). Interposition of a 10 l settling bag changed the

aerosol to a smaller size distribution with an MMAD of 0.84 μm , mainly by excluding large particles. The effects of the deposited particle mass in the lung may significantly depend on the fate of these large particles carrying a relatively high proportion of the total mass available. The distribution of inhaled particles between alveolar spaces and airways in the human lung depends on the aerodynamic diameter of the particles, the flow rate during inhalation, the volume of breath, breath holding, and any bronchoconstriction. The large particles are presumed to sediment and impact in the central tracheo-bronchial airways (1, 7, 8, 12, 21, 22). Sedimenting the large particles in a settling bag will consequently reduce the total amount of mediator delivered to the lungs, but especially the amount of mediator delivered to the central tracheo-bronchial airways. The aerodynamic particle diameters of the resulting aerosol will thus favor a diffuse deposition pattern, i.e., a relative increase in the amount of solute deposited in the peripheral parts of the airways (1, 7, 8, 12, 21, 22). Interposition of a settling bag was therefore used in the present study as a means of changing the deposition pattern of the solute from central towards more peripheral airways.

A relatively central deposition pattern was enforced by rapid inhalation manoeuvres. Increased inspiratory rate is held to increase the penetrance of turbulent flow down the airways, while slow inhalation will favor deposition in the more distal small airways (1, 12).

$V_{\text{max}30}$ is a sensitive measure of subclinical bronchospasm in the asymptomatic asthma patient, possibly as a reflection of small airway obstruction (15, 16). A similar situation was imitated by the inhalation of either of the two mediators via the settling bag: pronounced bronchial obstruction was revealed by $V_{\text{max}30}$, but not by FEV₁. The two mediators caused a parallel increase in the effect of both lung function parameters when deposited in a more central pattern. This indicated that there is no difference between LTD₄ and histamine with regard to any selective effects within the airways, thus supporting the notion previously indicated from human *in vivo* (2, 23), and *in*

vitro studies (10) but at variance with several animal *in vitro* (6, 11, 14) and *in vivo* (20) studies.

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