

Reproducibility of leukotriene D₄ inhalation challenge in asthmatics

Effect of a novel leukotriene D₄/E₄-antagonist (SR 2640) on leukotriene D₄-induced bronchoconstriction

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We have studied the reproducibility of a bronchial leukotriene (LT) provocation test in asthmatics, and the effect of prior treatment with an oral leukotriene D₄/E₄ antagonist (SR 2640) on LTD₄-induced bronchoconstriction in nine asthmatics in a double-blind placebo-controlled randomized cross-over trial. The reproducibility of the bronchial leukotriene provocation test was high. For a specific patient, the replication variance is 0.2303, and the standard deviation is thus 0.4799, corresponding to 48%, i.e. one halving of the dose or half doubling of the dose. SR 2640 antagonised LTD₄ induced bronchoconstriction causing a mean shift of 48% to the right of the dose-response curve as compared with placebo (95% confidence interval being 11-137%). This study demonstrates that bronchial LTD₄ provocation test is a safe and reproducible method in asthmatics, and that the method can be used to detect LT-antagonism; furthermore that SR 2640 is a weak LTD₄-antagonist in asthmatics.

Key words: leukotriene D₄ inhalation; leukotriene D₄/E₄ antagonist; reproducibility.

Accepted for publication 19 October 1990

Leukotrienes (LT) C₄, D₄, and E₄ constitute the biological activity formerly known as slow reacting substance of anaphylaxis (21).

LT are synthesized by cells involved in inflammatory reactions (17). Their actions include contraction of airway smooth muscles. On a molar basis, LT are 100-1000 times more active in contracting human airways than histamine (15). Since LT are released *in vitro* from antigen-challenged human lung tissue at concentrations 100th that of histamine (26) and inhalation of LTD₄ in human volunteers leads to marked bronchoconstriction, the sensitivity to LTD₄ being much higher in asthmatics than in normal subjects (8, 24), a physiologic role of LT as mediators of human asthma is suggested. Reproducibility data con-

cerning leukotriene bronchial provocations are few, and accessible in normal subjects only (3, 4, 5, 7, 20, 25).

A novel compound, SR 2640 (2-[3-(2-quinolylmethoxy)phenylamino]-benzoic acid), has been developed and studied with regard to LT-antagonistic activity *in vitro* and *in vivo* (1, 16).

The purpose of this study was 1) to evaluate the reproducibility of a bronchial leukotriene provocation test in asthmatics; 2) to investigate the effect of SR 2640 on bronchoconstriction induced by a provocation with inhaled LTD₄ in patients with asthma; 3) to record lung function on a daily basis during a 7-day treatment period with SR 2640; and 4) to evaluate safety and tolerability during this short-term treatment.

PATIENTS AND METHODS

Study population

Nine asthmatics (22) (7 men and 2 women) participated in the study after informed consent had been obtained. Mean age: 32 years (range 23–45 years). All patients were non-smokers with lung functions $>75\%$ of predicted value, a PC_{20} histamine <4 mg/ml (9), and were familiar with respiratory function testing. None were taking regular medication except β_2 -agonist spray, and non-steroid anti-inflammatory drugs such as aspirin were not allowed 1 month before and during the study period.

During the study, the patients were allowed to use β_2 -agonist spray concomitantly with the test medication.

Safety screening

Patients were initially screened to ensure that they were healthy except for the diagnosis of asthma. The screening included a full physical examination, routine haematology, biochemistry, chest radiograph, and electrocardiogram. The safety checks apart from the chest radiograph and electrocardiogram were repeated at each visit.

Lung function measurements

Forced expiratory volume in 1 sec (FEV_1) was measured on a calibrated dry wedge spirometer (Vitalograph Ltd., Buckingham, UK) and at least two technically correct forced maximal expiratory manoeuvres with a variation $<5\%$ in FEV_1 were obtained: the highest value was used for further calculation. Results were reported at ambient temperature, atmospheric pressure and saturated with water vapour (ATPS). Determination of peak flow was performed using a Mini Wright Peak Flow Meter, and patients were instructed to perform a forced maximal expiratory effort through the mouth after a maximal inspiration. The best of three readings with a variation of $<10\%$ was considered the true peak flow and used for calculations.

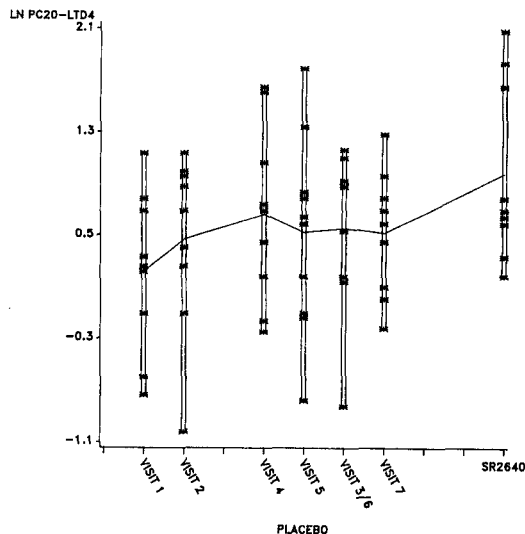


Fig. 1. The $\ln PC_{20}$ - LTD_4 during placebo and 1 week's treatment with SR 2640 in nine patients. The intra-patient replication standard deviation of PC_{20} - LTD_4 was 0.48 corresponding to 48%, i.e. one halving of the dose or half doubling of the dose. A shift to the right mean 48% as compared with placebo (95% confidence interval (11–137%)) was seen after treatment with SR 2640 ($P < 0.05$).

Aerosol generation

The aerosol was generated by a jet nebulizer (Mallinckrodt Diagnostica, Holland) (6, 7). The nebulizer was filled with 0.5 ml of solution and driven by compressed nitrogen at a pressure of 4 bar for 1 min, and the aerosol was spread into a drying chamber (a 30 l collapsible plastic bag). Under these conditions the mass median aerodynamic diameter (MMAD) of the saline aerosol was $2.5 \mu\text{m}$. The aerosol evaporated rapidly to dry particles MMAD $0.6 \mu\text{m}$ (ASASP-6, particle measuring system, Bolder, Nevada, USA). Subsequently,

the aerosol in the drying chamber was inhaled through a three-way valve with a mouthpiece by tidal breathing for 3 min, while oxygen was supplied into the mouthpiece at a flow of 4 l/min.

Prechallenge procedures

Bronchial challenge took place at the same time of day once a week during the study period. Immediately before challenge, blood samples were drawn for determination of the serum concentration of SR 2640 and the metabolite AMN 504 (16). Test medication was to be taken in the morning 3-6 h prior to the provocation together with a meal. Patients were instructed to refrain from coffee-containing beverages from midnight on the day before bronchial challenge.

Challenge protocol

From the LTD₄ stock solution 2-fold serial dilutions (25 µg/ml-0.78 µg/ml) were prepared

immediately before use, along with two further dilutions, 0.078 and 0.0078 µg/ml. Dilutions were kept on ice under nitrogen, until the nebulizer was filled. The challenge was carried out as a dose-response study. After an initial 0.5 ml solvent (buffered phosphate 0.01 mol/l, NaCl 0.9%, pH 7.4) inhalation, 0.5 ml of LTD₄ (0.0078 µg/ml) was given, the absolute dose ranging from 0.0078 - 25 nmol LTD₄, increasing with increments of a factor 2 every 15 min. Due to the slower onset of action of LTD₄ compared with histamine (3), FEV₁ was measured 0, 5, 8, and 15 min after inhalation to ascertain measurements of FEV₁, until the lowest value for FEV₁ for each dose had been recorded. The challenge was interrupted when a decrease of at least 20% of the post-solvent FEV₁ was observed. A PC₂₀ leukotriene was calculated by interpolation between the two last points on the log dose-response curve. The lowest value of obtained FEV₁ values (0, 5, 8, 15 min) was used for calculation. Interpolation between FEV₁ solvent and FEV₁ threshold was never performed. The cut-off level, if no decrease in FEV₁ of more than 20% occurred, was the highest dose of LTD₄ (25 nmol).

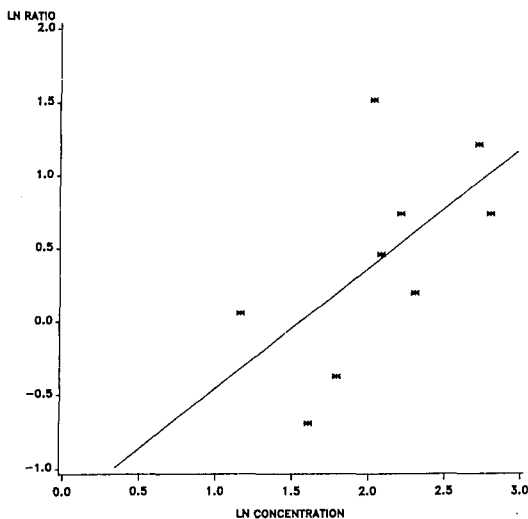


Fig. 2. The ln ratio of PC₂₀-LTD₄ after SR 2640 and placebo ($\ln \frac{PC_{20} \text{ 2640}}{PC_{20} \text{ placebo}}$) versus ln serum concentrations. ln ratio = $-1.27 + 0.82 \ln (\text{concentration})$. $r = 0.59$, $P = 0.09$ (non-significant).

Study design

It was a randomized, double-blind, placebo-controlled cross-over study consisting of the following phases:

A 1-week baseline assessment, hereafter 1 week during which the patients received double-blind treatment with either active SR 2640 tablets or matching placebo tablets, followed by a 2-week wash-out period, and a 1-week period during which the patients received the reverse treatment followed by a 1-week wash-out period. During the whole study, patients received SR 2640 placebo tablets except for the 1-week treatment with SR 2640, 250 mg day (Leo Pharmaceutical Products, Denmark), and a PC₂₀-LTD₄ was obtained every week.

To ensure complete wash-out, the leukotriene PC₂₀ measured each week during the wash-out period was to have returned to the

baseline value obtained within one doubling of the baseline assessments. If this was not the case, the wash-out period was prolonged an extra week. Throughout the entire study, the patients were asked to record asthma symptoms (0-3, 3 = maximum), morning and evening peak flow values, and use of β_2 -agonist spray, in a diary card.

The study was approved by the local ethics committee.

Statistical analysis

Results are given as means with ranges in brackets. Since provocation concentrations resulting in a 20% decrease in lung function (PC_{20} -LTD₄) varied over several orders of magnitude, the natural logarithm of the results was used for calculations. Student's t-test was used for paired observations. The significance of the treatment $\times$ period interaction, period effect and treatment effect were tested. Interdependence was described by least square regression. Reproducibility was calculated by estimating the intra- and inter-subject variations (2). A 95% confidence interval for the mean difference between observations was calculated, and a 95% confidence interval of the treatment effect was calculated as well. Correlation between response and measured serum concentrations of SR 2640 was made. $P < 0.05$ was considered statistically significant.

RESULTS

Nine patients were included and completed the study. There was no significant difference in baseline lung functions obtained at each visit in any individual. Totally, 65 provocations were performed; nine provocations after treatment with SR 2640, and 56 provocations after placebo; seven patients with six values and two patients with seven values, as the latter two patients had to receive an extra challenge to ensure return to baseline within $\pm$ one doubling after the wash-out period.

Reproducibility of the LTD₄ challenge (Fig.1) was calculated from 56 observations.

No evidence of treatment $\times$ period interaction or period effect was found (paired t-test) and, no development of tachyphylaxis could be demonstrated. The intra-patient replication standard deviation of PC_{20} leukotriene was 0.48, corresponding to 48%, i.e. one halving of the dose or half doubling of the dose. PC_{20} -LTD₄ was statistically significantly increased after treatment with SR 2640 compared with challenge after receiving placebo (analysis of variance $P < 0.05$ (Fig.1)). The 95% confidence interval (CI) for the increase of PC_{20} -LTD₄ was 11%-137%. The relative change in PC_{20} -LTD₄ was not statistically significantly correlated ($P = 0.09$) to the plasma concentration of SR 2640 (Fig.2). The plasma concentration varied from 3 to 16 μ g/ml, and the time from latest taken dose to sampling was median 207 min (180-315). The onset of the LTD₄ effect after the threshold dose had been given was calculated from the 56 placebo provocations. Nine patients were positive (achieved a PC_{20} -LTD₄) measured 0 min after inhalation, 40 patients were positive 5 min after inhalation, seven patients were positive 8 min after inhalation, and none were positive 15 min after inhalation. The subjectively assessed symptom scores and daily peak flow recordings during the study period did not show any statistically significant differences between treatments, nor was any decrease in lung function observed as expressed from peak flow values the evening and morning after challenge. No adverse effects or significant changes in routine haematology and biochemistry were reported.

DISCUSSION

In this study a method for bronchial challenge with LTD₄ has been described (7), and found reproducible. A significant although small shift in the dose/response curve of LTD₄ to the right after treatment with SR 2640, a novel LTD₄/E₄ antagonist, as compared with placebo, has been demonstrated. The logarithmic transformed plasma concentration of SR 2640 was not significantly correlated with the logarithmic relative change in PC_{20} -LTD₄. SR

2640 had no effect on lung function or symptom scores in the asthmatics.

In previous studies, leukotriene challenges in the bronchi have been carried out and reproducibility data have been presented in normal subjects (3, 4, 5, 7, 20, 25). In this study the reproducibility was investigated in patients with mild bronchial asthma and found to be one halving of the dose or half doubling of the dose for a single determination. A previous study (4) used 3.2-fold increasing concentration steps (5 doublings) of LTD₄ and found a $\frac{1}{2}$ log 10 change in concentrations for PC₃₅-sGaw with the slope of the regression lines being 0.88, but regression lines do not give any information about intra- and inter-subject variation (2). Another study (25) gives a correlation coefficient of $R > 0.86$ as an expression of a highly reproducible method for bronchial provocation with LTD₄. Finally, one study assumes (6) equal reproducibility of a methacholine challenge test compared with LTD₄ challenge test, as a significant correlation was found between the two of them being ± 1.6 doubling dose. PC₂₀-FEV₁ has been used in this study, whereas others have reported PC₃₅-sGaw. A significant correlation between PC₁₀-FEV₁-LTD₄ and PC₃₅-sGaw-LTD₄ has been reported (25). The measurement of FEV₁ is easily performed, highly repeatable and reproducible (11, 12).

The jet nebulizer used has recently been developed and evaluated (6, 7), and is suitable for the administration of high doses of precious substances such as LTD₄. Other studies have used a Wright jet nebulizer and have demonstrated a decreasing concentration of LTD₄ in isotonic saline solutions left in the nebulization chamber (3), and a similar decrease is found for a De Vilbiss 35B ultrasonic nebulizer (8). These nebulizers are driven by compressed air or a ventilator, which might account for the oxidation of the sulphido-leukotrienes. Compressed nitrogen was used in the method described, thus preventing oxidation. Sulphido-leukotrienes have been demonstrated to adsorb reversibly to glass and plastic surfaces (28), which can partly be prevented by decreasing the polarity of the solvent and by stabilizing

the pH. A neutral buffer has been used as solvent in order to diminish the degree of ionization, and to reduce variability in the potency of the substance, furthermore the nebulization chamber contains only the amount to be nebulized and is filled immediately before nebulization in order to minimize adsorption. The dosimeter delivers the aerosol into a drying reservoir from where the patient inhales. The optimal aerodynamic particle size will result in maximal deposition in the lungs, and in this study the particles were equally distributed and within the optimal limits (7, 23). A previous study has shown a deposition in the lungs comparable to deposition in a perspex lung phantom on an average of 53% (40-62%) (7), and comparing the technique in this study with a standardized 2 min tidal breathing method (14), the position of the dose-response curve to metacholine did not differ between the two methods. Peak flow (PEF) measured in the evening and morning after challenge did not indicate any late-phase reaction, which is the case in the maximal response of the dose-response curve to metacholine (7). This might be due to persistence of some mucosal swelling, but the measurements were few, and performed with a peak flow meter by the patients themselves, whereas other lung function tests might have detected such a late-phase reaction, if any.

Comparing the LTD₄ challenges on the days of placebo and SR 2640 (Fig. 1), a significant although small increase in PC₂₀-LTD₄ after treatment with SR 2640 was demonstrated with a 48% mean increase of threshold dose (95% confidence interval being 11-137%). The dose-response curves demonstrated a parallel shift in keeping with the drug being a competitive antagonist. The shift of the dose-response curve was moderate (48%) indicating a weak antagonist, a too low dose and plasma levels of SR 2640 obtained, or that the smooth muscle is not the only effector organ for LTD₄ *in vivo*. LTC₄ is not antagonized by SR 2640, and may play an important part in the first sequence of events leading to bronchoconstrictions, since it is the first S-cysteinyl substituted leukotriene formed by LTA₄. This might ac-

count for the very moderate antagonism seen in this study concerning the provocation test as well as data from symptom score and peak flow recording. No side effects usually associated with LT antagonists, e.g. abdominal pain and diarrhoea, were observed, which might indicate that the maximum tolerated dose was not obtained. Plasma levels of SR 2640 demonstrated a large inter-subject variation, although the study was designed so that the time at which the concentration of LTD₄ causing bronchoconstriction was inhaled, should correspond approximately to the time at which peak plasma levels had previously been demonstrated (1, 16). Furthermore, steady state was apparently not achieved after 1 week's treatment with the dose used. No significant ($P = 0.09$) relationship between plasma drug levels and degree of antagonism of LTD₄ induced bronchoconstriction could be demonstrated, although a tendency was observed (Fig. 2), but the number of patients was small, and the time from dosing to sampling varied (median 207 min, range 180–315). No information is available as to whether plasma or tissue levels of the drug are important in humans, neither is it known whether the metabolite is active.

This study demonstrates a reproducible method for bronchial challenge with leukotriene D₄ in asthmatics. Further, it shows that an oral LT antagonist, SR 2640, may specifically block bronchoconstriction induced by inhaled leukotrienes, the drug being less potent than would be expected from *in vitro* studies (1), and less potent than other newly investigated antagonists. SR 2640 had no effect on lung function or symptom scores in asthmatics.

ACKNOWLEDGEMENTS

We thank Mr. Claus Bay, mathematical statistician, for assistance with statistical calculations, Mrs. Anne-Marie Kissmeyer for determinations of plasma concentrations of SR 2640, and Mrs. Anne Larsen for valuable secretarial assistance.

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