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Mild experimental exacerbation of asthma induced by individualised low-dose repeated allergen exposure. A double-blind evaluation

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Low doses of environmental allergens have been proposed to increase bronchial hyperreactivity in sensitised individuals, without causing immediate asthmatic reactions. The primary aim of the present study was to evaluate whether repeated low doses of allergen, that do not cause overt bronchoconstriction, cause augmented non-specific bronchial reactivity. A secondary aim was to evaluate whether any changes in reactivity are associated with increased variability of lung function, and whether signs of inflammatory activity could be found. To do this, mild asthmatic patients without regular symptoms, but with both immediate and late reactions in response to a high dose of inhaled cat allergen extract, were included in a double blind, placebo controlled, cross-over study in which a low dose of allergen was administered on four consecutive days (Monday to Thursday). The dose of allergen was individualised for each patient, and was calculated to be 25% of the total dose given to produce an immediate and late response at screening. Repeated low dose allergen exposure produced a significant increase in methacholine reactivity compared to placebo, whereas FEV_1 in the morning did not significantly change during the allergen week. Each low dose allergen exposure caused small changes in FEV_1 (approximately 7% drop), which was significant *vs.* placebo only on day 2 (Tuesday). During the allergen week, six of eight patients reported asthma symptoms on at least one occasion, and variability in lung function, measured with a portable spirometer, was increased. Repeated low doses of allergen also produced a significant increase of P-ECP *vs.* placebo, without a significant rise in circulating eosinophils. However, no significant changes in circulating CD3, CD4, CD8, CD19, or CD25 cells were found, evaluated by FACS analysis. We conclude that low doses of allergen produce signs of a mild exacerbation of asthma, including increased bronchial reactivity to methacholine. This clinical model may be useful to evaluate both the pathophysiological mechanisms of asthma, and the effects of novel anti-asthma drugs.

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Introduction

Two fundamental features of asthma are variability of airway calibre and increased bronchial hyperreactivity to non-specific stimuli (BHR)(1). The degree of BHR has been related to the number of inflammatory cells in the blood and airways, suggesting that the physiological dysfunctions in asthma are caused by an underlying inflammatory process (2–8). This is further supported in more recent studies, showing that the activation state of the inflammatory cells, especially eosinophils, is related to the degree of bronchial hyperreactivity and to the severity of asthma (9–15).

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Allergens are considered to be important inducers of BHR in allergic asthmatic patients (6). Following high-dose allergen exposure in an allergic patient, an early asthmatic reaction (EAR) occurs immediately after exposure, and after 3–7 h a late asthmatic reaction (LAR) may develop in some patients (16,17). The EAR is transient, often resolving spontaneously within 1 h. The LAR, on the other hand, is associated with influx of inflammatory cells into the blood and airways, as well as the increase of BHR, which sometimes can persist for several days (3,18–21). This high dose provocation model is often used to study pathophysiological mechanisms of allergic reactions, and to evaluate the effects of novel therapeutic agents developed for asthma (22). However, the model has also been criticised for being too experimental.

A more common way to be exposed to an allergen may be repeated or chronic exposures to low doses. For example, exposure to house dust mite may occur mainly when sleeping, and exposure to low doses of animal dander

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TABLE 1. Patient characteristics

Pat. #	Sex	Age (years)	FEV_1 (% pred)	SPT (skin prick test)	RAST (class)	PD20 allergen (SQ)
1	M	27	88	++++	4	120
2	F	32	96	++	3	40
3	F	36	112	++++	3	6
4	F	46	71	+++	3	10
5	F	22	87	++++	3	250
6	M	35	97	++++	3	2100
7	F	26	104	+++	3	50
8	M	26	95	+++	3	400
Mean		31·2	93·8			

FEV_1=Forced expiratory volume in 1 s.
SPT=Skin prick test.
SQ=standard quality units.

may occur in many public areas (23,24). It has earlier been suggested that experimental exposures to repeated low doses of allergen can increase BHR. These studies have however been unable to show any evidence of inflammatory changes (25,26), and the doses of allergen used were not individualised.

The aim of the present study was to develop a clinical experimental model of asthma, induced by lower doses of allergen than those causing an early and a late response. To do this, we gave patients with mild asthma either repeated inhalations of placebo, or four exposures of 25% of the individual dose of allergen known to cause an early and late response. We further asked whether this protocol would induce a mild exacerbation of asthma. To answer this, we measured bronchial methacholine reactivity, the daily variability in lung function, and the activation state of eosinophils (10–12,15–21).

Patients and methods

The study was approved by the Ethics Committee in Göteborg. Nine atopic patients mean age 31 years (range 20–46), with a history of asthma when exposed to cat, were included in the study, but one patient was excluded due to repeated accidental and symptomatic cat exposures. Characteristics of analysed patients are presented in Table 1. Skin prick test (SPT) and RAST were all positive to cat. SPT was graded in a plus system according to Northern standardisation, with histamine hydrochloride $10\ mg\ ml^{-1}$ as the positive reference (27). RAST was analysed as CAP-RAST (Pharmacia, Uppsala, Sweden). All patients had mild asthma with mean FEV_1 93% predicted (71–112%; Table 1), and did not require regular asthma therapy, although inhaled beta-2 agonists when needed was allowed at inclusion. At a screening day, high dose bronchial allergen challenge to cat allergen extract was performed. All patients included in the low-dose study presented both EAR and LAR at screening, which was defined as a fall in FEV_1 of at least 20 and 15% from baseline respectively. No overt allergen exposure or significant respiratory infection was allowed to be present within four weeks prior to the study, or during the study. Three patients had evidence of concomitant allergy to pollen, but were studied outside of the pollen season.

STUDY DESIGN

Screening

The study started with a screening day when history, a skin prick test and a high dose bronchial allergen challenge screening was performed. The allergen dose causing EAR was determined (FEV_1 decreased at least 20% from baseline, within 10 min from the allergen dose), and the presence of LAR was confirmed (FEV_1 decreased at least 15% 3–7 h after the last allergen dose). At the end of the screening day (at 7 h), all patients received 30 mg prednisolone as a single dose, to attenuate any prolonged inflammatory processes. Sixteen patients were screened, but seven of these presented no late response. The logarithmic allergen doses were plotted against the fall in FEV_1 and PD20 values for each patient was calculated from the cumulative doses by linear interpolation on the log-linear curve.

Repeated Low-dose Protocol

In a double-blind cross over design, the patients were at least 10 days after screening randomised to daily bronchial exposure to low-dose allergen (cat) or placebo for 4 days. 'Low-dose' was defined as 25% of the cumulative allergen PD20 on the screening day. The two periods of low-dose allergen or placebo inhalations, were separated by at least 10 days. On the Monday, (before the first allergen/placebo exposure), and on the Friday, approximately 24 h after the last allergen/placebo exposure, a bronchial methacholine

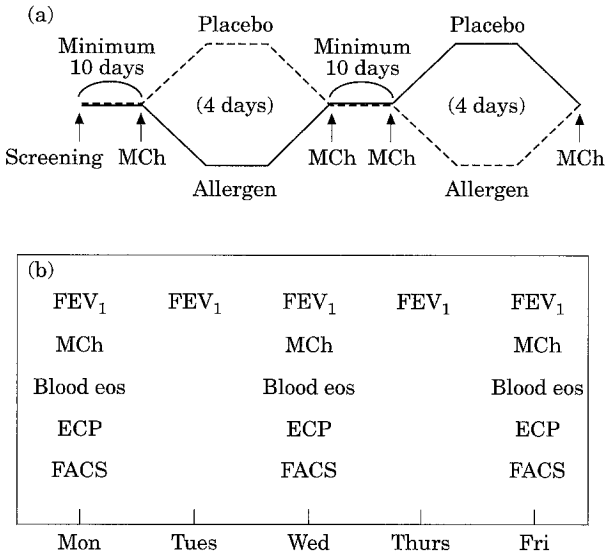

FIG. 1. (a) Protocol of the repeated individualised low dose allergen exposure. The study is double blind, placebo controlled and of a cross over design. (b) Protocol of measurements during the allergen/placebo exposure weeks. The individualised low dose allergen inhalation or placebo inhalation were given 1 h after the end of the methacholine challenge on the Monday. Subsequent low dose allergen or placebo inhalations were given on Tuesday, Wednesday and Thursday mornings, and the last methacholine challenge was performed approximately 24 h after the last inhalation of allergen/placebo.

challenge was performed. The single dose of an oral glucocorticoid (prednisone 30 mg) was given after the end of each treatment period. Blood samples for leucocyte-differential count were collected daily, and samples for ECP (eosinophil cationic protein, CAP, Pharmacia) were collected on Monday, Wednesday and Friday.

After the daily low-dose allergen/placebo exposure (Monday to Thursday), the patients used a home-spirometer (Spirobank, MIR) to register FEV_1 and symptoms every second hour on five occasions during the rest of the day.

BRONCHIAL CHALLENGE AND LUNG FUNCTION

Nebuliser/Dosimeter

The dosimeter ME.FAR MB3 (Brescia, Italy) was used for all bronchial challenges. The patient inhaled the dose of aerosol slowly by means of an inspiratory capacity breath, followed by 5 s of breath holding. The particle size of the aerosol is 0·5–5 μm, when driven at the used air pressure of 1·65 kg cm^{-2} and an air flow rate of 70–75 l min^{-1}. The nebulisation time was set to one second which was selected to obtain an output of 10 μl per breath. For each dose of methacholine or allergen, five inhalations were given. All bronchial challenges started with registration of basal FEV_1 (two measurements with at least 1 min between). If the measured FEV_1 was more than 65% predicted, the provocation was pursued with inhalation of diluent.

High Dose Allergen Screening

Allergen extract (cat) was purchased from the Allergological Laboratories (ALK), Copenhagen, Denmark. The 'high-dose' allergen challenge on the screening day started with inhalation of the extract vehicle. FEV_1 was measured after 4 and 5 min, and the best FEV_1 of these was used as the baseline value. The allergen provocation protocol was started with 32 standardised quality units (SQ), nebulised for 1 s (five times). The allergen doses were given at two-fold increasing doses. However, if FEV_1 dropped less than 5% *vs.* baseline, the next four-fold increasing dose of allergen was given. FEV_1 was measured after at 5 and 10 min (the best FEV_1 of two manoeuvres with 1 min between). The lowest FEV_1 at these respective time-points was regarded as the reaction to the given dose. The allergen provocation was stopped when FEV_1 had fallen >20% from baseline value, defined as the early asthmatic reaction (EAR). FEV_1 was then further measured after 15, 20, 30, 45 and 60 min (two FEV_1-manoeuvres at every time point with 1 min between). Hourly measures of FEV_1 was then made up to 7 h after EAR. An inhaled beta-2 agonist (Terbutaline or Salbutamol) and a single oral dose of 30 mg prednisolone was given to reverse the LAR.

Low Dose Allergen Challenge

The dose of allergen given to each patient, was calculated to be 25% of the cumulative dose causing and early and late reaction. Briefly, the concentration of allergen extract causing the early and late reaction was dissolved 1:4 in saline. This individualised concentration of allergen extract, or the vehicle (placebo), was given to each patient in the morning from Monday to Friday during the randomised weeks. FEV_1 was followed for 1 h after each low-dose allergen provocation (measured at 5, 10, 20, 30, 45 and 60 min after allergen). Prednisolone at a single dose of 30 mg was given after methacholine challenge on Friday, to end each allergen/vehicle period.

Methacholine Provocation Tests

Methacholine challenge tests were performed on the Monday, before the first allergen/placebo dose, and on Friday, approximately 24 h after the last allergen/placebo dose. The methacholine challenge test was always started at a concentration of 0·03 mg ml^{-1}, and then continued with two-fold increasing concentrations every fifth minute, with one FEV_1 manoeuvre 90 and 180 s after inhalation. The best FEV_1 after each concentration was used for calculations. When FEV_1 had fallen with at least 20%, the provocation test was stopped. On Mondays, the methacholine-induced change in FEV_1 was allowed to resolve spontaneously (within 30–60 min), before the low dose allergen/vehicle was given. On Fridays, the

methacholine-induced change in FEV_1 was reversed with inhaled beta-2 agonist (0·8 mg) salbutamol via Diskhaler). The logarithmic methacholine doses were plotted against the fall in FEV_1 and the methacholine PD20 was calculated by linear interpolation on the log-linear curve.

Home Spirometry

Ambulatory FEV_1 was measured five times (every 2 h), after leaving the clinic during the low-dose allergen/placebo days (Monday–Thursday), using a light weight mini flow turbine spirometer (Spirobank, Medical International Research, MIR, Roma, Italy). The spirometer alarm was set to go off every second hour, to make sure that the patients performed the manoeuvres. Furthermore, the time for each measurement was saved automatically by the spirometer. The variability in lung function was calculated as the best recorded ambulatory FEV_1 during the week, minus the second lowest ambulatory FEV_1 during the week, divided by the best FEV_1. The second lowest FEV_1 was used to avoid variability of data due to technically inappropriate recordings.

INFLAMMATORY PARAMETERS

Eosinophils/ECP

Samples for blood differential counts were taken every day, prior to allergen/placebo inhalation, and were subsequently analysed using a hospital standardised method. Samples for ECP-qualification were taken on Monday, Wednesday (before allergen), and on Friday, approximately 24 h after the last allergen/vehicle dose. ECP was measured with a fluoroimmunoassay method (Pharmacia ECP CAP System FEIA, Pharmacia Diagnostics AB, Uppsala, Sweden) and handled according to the manufacturer's instruction.

FACS-analysis

Sub-populations of lymphocytes in peripheral blood were determined by flow cytometry. The samples were stained with combinations of murine monoclonal antibodies, directly conjugated with fluorochromes. Fluorescein isothiocyanate-conjugated CD4 and CD8, phycoerythrin-conjugated CD25 and Per-CP-conjugated CD3 and CD 19 (Becton-Dickinson Inc., Mountain View, CA, USA) were used. One ml of whole EDTA-blood was treated with 10 ml of lysing solution (Ortho Diagnostic Systems Inc., Raritan, NJ, U.S.A.) and incubated for 10 min at room temperature. After washing once the cell concentration was adjusted to 4×10^6 ml^{-1} and 25 μl^{-1} was incubated with 10 μl of each monoclonal antibody for 15 min at room temperature. After washing twice, the cells were fixed in 1% paraformaldehyde in phosphate buffered saline (PBS) for 10 min in room temperature. The cells were stored at 4°C and were analysed the next day on a FACScan flow cytometer (Becton- Dickinson Inc.), calibrated with CALIBRETTEÔ beads (Becton-Dickinson Inc.) and AutoCOMPÔ software (Becton-Dickinson Inc.). A lymphocyte gate was set manually according to the location in the forward scatter versus side scatter diagram. Negative isotype controls (Becton-Dickinson Inc.) were used to set quadrant markers which delineated positive fluorescent staining from non-antigen specific staining. Dot plots and quadrant statistics from three-colour analysis were generated by the CellQuest software (Becton-Dickinson Inc.). The absolute number of blood lymphocytes was determined using a haematological cell counter (Sysmex-K 1000; TOA Medical Electronics Co).

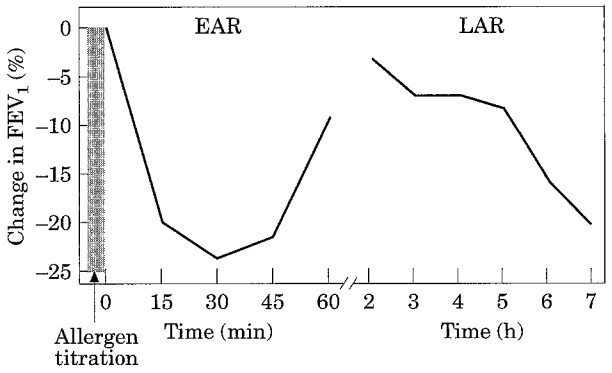

FIG. 2. Mean change in FEV_1 at the screening day, in the eight analysed patients with early and late reactions.

STATISTICS

The change in PD20 methacholine from Monday to Friday during allergen/placebo exposure weeks were decided to be the primary end-point in the present study. Secondary end-points are variability in lung function (home spirometry), recorded number of occasions with asthma symptoms, use of beta-2 agonist, change in peripheral blood eosinophils, change in P-ECP, and change in circulating inflammatory cell surface markers using FACS-analysis. A two-tailed Student's *t*-test for paired observations was used to determine significance. A P value $<0{\cdot}05$ is considered significant. The results are presented as mean values ± SEM.

Results

One of the nine randomised patients experienced several accidental and symptomatic cat exposures during the study, and was therefore excluded, leaving eight patients for analysis (Table 1). The mean per cent changes in FEV_1 at different time points during the high-dose allergen screening day are shown in Fig. 2. The induced EAR caused a mean maximal drop in FEV_1 of 29 ± 2%, and the LAR 21 ± 2%.

METHACHOLINE REACTIVITY

The mean baseline methacholine PD20 was slightly higher before the repeated low-dose allergen week compared to before the placebo week (46·6 ± 1·81 and 27·9 ± 1·84 μg

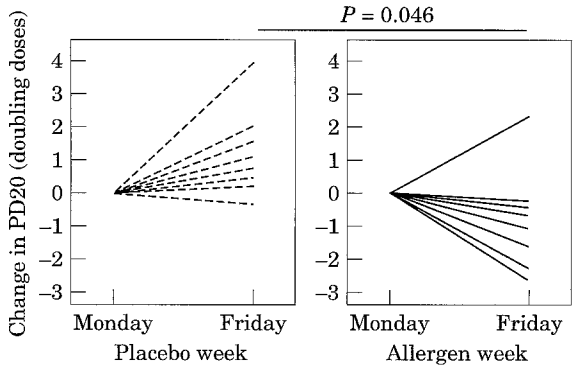

FIG. 3. Change in PD20 methacholine during the repeated low dose allergen exposure week and the placebo week (——, individualised low dose allergen; – – –, placebo). The change in PD20 methacholine from Monday to Friday was found to be statistically significant (P=0·046).

respectively). On the Friday after the repeated low-dose allergen week, the sensitivity to methacholine was increased compared with the placebo week (mean PD20 25·2 ± 1·7 and 59·5 ± 1·7 μg respectively), the change from the Monday being statistically significant between the two weeks (Fig. 3; P=0·046).

LUNG FUNCTION AND SYMPTOMS

No significant changes in baseline FEV_1 was found during the repeated low-dose allergen week *vs.* the placebo week [Fig. 4(a)]. The individual low dose allergen challenges caused small immediate decreases in FEV_1 [Fig. 4(b–e)] which were found to be significant *vs.* respective placebo exposure mainly on the second study day [Tuesday; Fig. 4(c)]. Ambulatory spirometry showed increased variability in lung function during the repeated low-dose allergen week *vs.* the placebo week [Fig. 5(a,b)], which also was associated with significantly increased number of episodes of asthma symptoms [Fig. 5(c)], but not significantly increased use of beta-2 agonists [Fig. 5(e)]. Six of eight patients reported episodes of asthma symptoms at least once during the repeated low-dose allergen week, and one patient reported asthma symptoms once during the placebo week.

INFLAMMATORY PARAMETERS

Baseline eosinophils and ECP were not significantly different prior to the repeated low-dose allergen and placebo weeks (P-ECP; 17 ± 3 and 21 ± 5 $\mu g l^{-1}$ before the low dose allergen and placebo weeks respectively). No significant changes in circulating eosinophils were found during the repeated low-dose allergen week *vs.* the placebo week [Fig. 6(a)]. However, P- ECP increased slightly but statistically significant during the repeated low-dose allergen week *vs.* the placebo week [Fig. 6(b)]. No significant correlation between the change in P-ECP and changes in methacholine reactivity was found (data not shown).

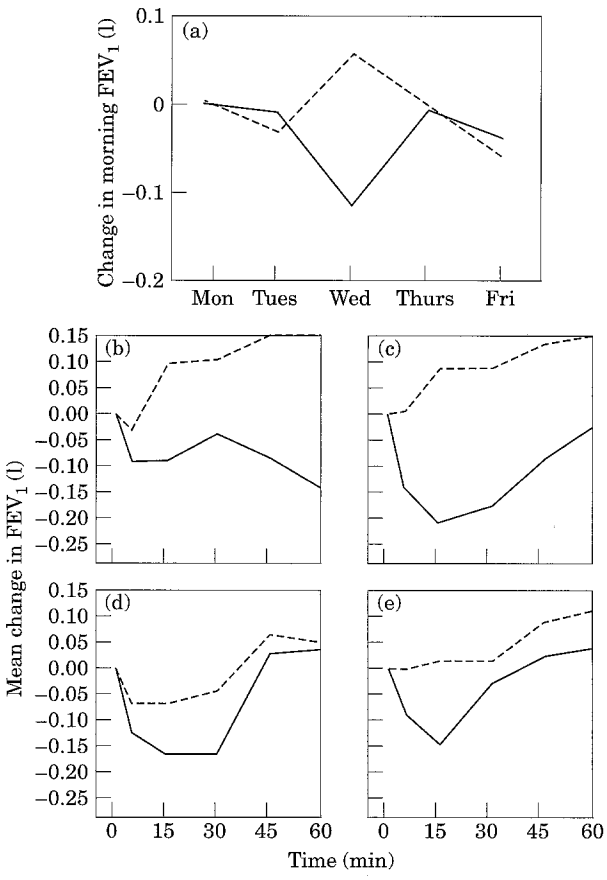

FIG. 4. (a) Baseline morning FEV_1 prior to allergen exposure during the placebo and individualised low dose allergen exposure weeks (——, individualised low dose allergen; – – –, placebo). (b–e) Mean per cent change in FEV_1 up to 1 h after the inhalation of placebo or individualised low dose allergen exposure on Monday to Thursday respectively (——, individualised low dose allergen; – – –, placebo). Significant drop in FEV_1 was found mainly on Tuesday (P<0·05).

We found no significant changes in circulating inflammatory T-cell or B- cell surface markers during the repeated low-dose allergen week *vs.* the placebo week, evaluated by FACS (Table 2).

Discussion

The present double blind and placebo controlled study shows that repeated individualised low doses of allergen produces increased bronchial reactivity, increased variability in lung function, increased asthma symptoms and discrete increases in ECP, suggesting that a mild asthmatic exacerbation was induced.

When planning this study, we decided to monitor methacholine reactivity, asthma symptoms, the use of beta-2 agonists, as well as daily variability in lung function,

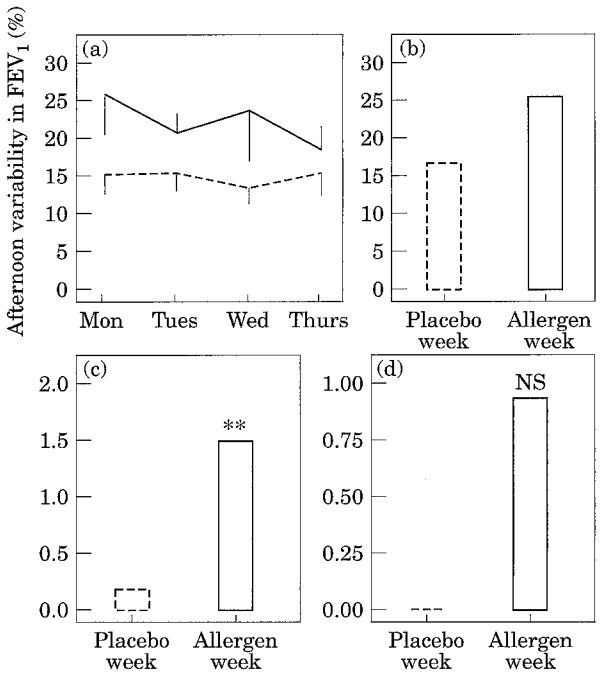

FIG. 5. (a) Mean afternoon variability in FEV^1 Monday to Thursday during repeated low dose allergen week *vs.* the placebo week (——, individualised low dose allergen; – – –, placebo). (b) Mean maximal variability in FEV_1 during the repeated low dose allergen week *vs.* the placebo week (——, repeated low dose allergen; – – –, placebo). The maximal variability in ambulatory FEV_1 was significantly higher during the low dose allergen week ($P<0{\cdot}05$). (c) Mean number of periods of asthma symptoms during the repeated low dose allergen week *vs.* the placebo week (——, individualised low dose allergen; – – –, placebo). Significantly higher number of periods of symptoms were induced during the low dose allergen week ($P<0{\cdot}01$). (d) Mean number of occasions of use of beta-2 agonist during the repeated low dose allergen week *vs.* the placebo week (——, individualised low dose allergen; – – –, placebo).

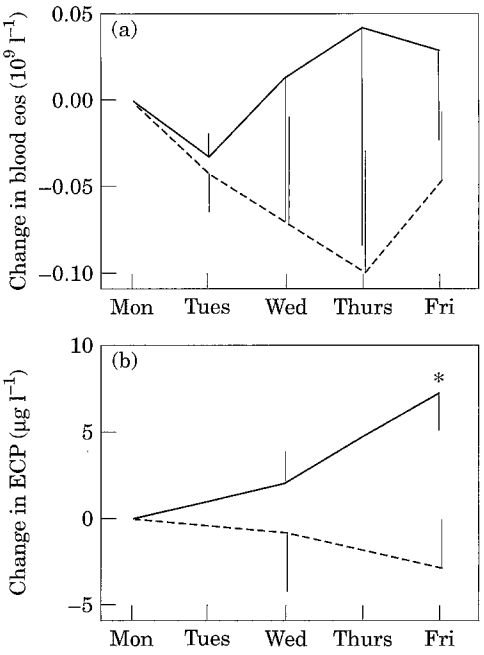

FIG. 6. (a) Mean change in blood eosinophil number during the repeated low dose allergen week *vs.* the placebo week (——, individualised low dose allergen; – – –, placebo). There was no statistically significant difference between the two weeks. (b) Mean change in P-ECP during the repeated low dose allergen week *vs.* the placebo week (——, individualised low dose allergen; – – –, placebo). The increase of P-ECP amounted to approximately 10 μg *vs.* placebo on the Friday, which is statistically significant ($P<0{\cdot}05$).

because increases in these variables would be signs of an exacerbations of asthma (13,29–31). The repeated individualised low doses of allergen induced a mean increase in bronchial reactivity to methacholine by approximately one doubling dose, and this increase in reactivity was paralleled by a small increase in the number of occasions of asthma symptoms over the 4-day exposure period, but only a non-significant tendency towards an increased use of beta-2 agonists. Worsening of asthma was however further confirmed by increased variability of lung function, measured by ambulatory spirometry every other hour during the study weeks. Thus, of these four clinical parameters, three statistically supported the hypothesis that the low-dose allergen exposure protocol would cause an exacerbation of asthma. Importantly, baseline lung function, prior to each low-dose allergen exposure, did not significantly deteriorate during the study week. Thus, no overt or prolonged airflow obstruction was induced by the low doses of allergen, and the induced changes in bronchial hyperreactivity, symptoms and variability of lung function were quite small, suggesting that the induced exacerbations are mild.

Asthma has been strongly associated with ongoing eosinophilic inflammation in the airways, illustrated by increased circulatory eosinophils, increased P-ECP and increased number of eosinophils in the airway wall and sputum (9–12,32). In the present study, we found that the low-dose allergen exposure slightly increased the level of P-ECP, although the number of circulating eosinophils were not significantly increased, further supporting that a mild exacerbation of asthma was induced. No increase in P-ECP was found in a previous study of low-dose allergen exposure (25), but in that case, the dose of allergen was not chosen on the basis of the patients own allergen-reactivity. In another study, in which the investigators gave low doses of allergen intranasally to patients with allergic rhinitis, ECP was found to be increased in nasal lavage, but not in the circulation, suggesting that the local inflammatory response may be stronger than implied in the P-ECP (28). This is further supported by a recent preliminary report, showing that repeated low-doses of allergen increases the relative amount of eosinophils in induced sputum (33).

The observed sign of discrete activation of circulatory eosinophils in the present study, was not paralleled by an

TABLE 2. Effects of repeated low dose allergen exposure on the total number of peripheral blod lymphocytes (× 106 per ml), measured by FACS analysis on Monday prior to allergen, and on Wednesday and Friday 24 h after the latest low dose allergen exposure (data shown as mean). The statistics are performed on the changed of total number of respective lymphocyte subset during the placebo and allergen weeks respectively (Delta)

	Placebo week				Allergen week				
	Mon	Wed	Fri	Delta	Mon	Wed	Fri	Delta	$P=$
CD3	1·33	1·23	1·4	0·07	1·2	1·22	1·23	0·03	0·86
CD3+4	0·68	0·65	0·79	0·11	0·62	0·61	0·66	0·04	0·71
CD3+8	0·23	0·32	0·33	0·10	0·24	0·25	0·31	0·07	0·72
CD4/CD8	3·78	2·31	2·61	− 1·17	4·41	4·00	3·54	− 0·87	0·94
CD3+4+25	0·28	0·34	2·61	0·08	0·28	0·29	0·26	− 0·02	0·30
CD3+8+25	0·02	0·02	0·02	0·02	0·02	0·02	0·06	0·04	0·61
CD19	0·12	0·12	0·01	0·01	0·10	0·08	0·08	− 0·02	0·77

increase of the analysed sub-populations of CD3, CD4, CD8, CD19 and CD25 lymphocytes in peripheral blood. This would imply that lymphocytes were not activated by the low-dose allergen exposure, at least not to a degree being reflected in the circulation.

This study has not in a randomised way compared the effects of repeated low-dose allergen exposure with a a single high dose challenge. However, our data in relation to the previous studies, imply that the degree of change of bronchial reactivity is quite similar in the two models (18,21). Furthermore, giving repeated low doses of allergen seems to be quite safe, because no or very small changes in lung function are induced with the low-dose protocol compared to the screening day, and the number of occasions with asthma symptoms are rare. Future studies, directly comparing the effects of a single high dose challenge with those of repeated low doses of allergen, will give us further insight into the differences and similarities of these two different ways of giving experimental allergen-challenge to allergic asthmatic patients.

The combination of data in this study, showing parallel increases in P-ECP, BHR, variability of FEV_1 and increased number of asthma symptoms during the low-dose allergen week, confirm that these parameters may be closely linked in asthma. Thus, it is possible, as has been proposed (3,4,10), that the activation of eosinophils is important for the increase in methacholine reactivity and increased variability of lung function, which in turn results in increased number of periods of asthma symptoms. It seems unlikely that the increased methacholine reactivity observed after repeated low-dose allergen exposure is independent of eosinophilic inflammation, as was implied previously (25).

This study suggests that repeated and individualised low dose exposure to allergen causes several features of an exacerbation of asthma in sensitive individuals, reflected in increased methacholine reactivity, increased symptoms of asthma, increased variability of lung function, and a small increase in P-ECP. These changes were, however, small in magnitude, suggesting that the induced exacerbations are quite mild. We believe that this model may become useful in the clinical evaluation of novel anti-asthma drugs, as a complement to the more commonly used high-dose allergen-exposure model (16–21).

Acknowledgement

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