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Anti-inflammatory effects of inhaled beclomethasone dipropionate in nonatopic asthmatics

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ABSTRACT: The effects of inhaled beclomethasone dipropionate (BDP) on asthma symptoms and infiltration of the bronchial mucosa by inflammatory cells were investigated in an open study of 10 patients with mild-to-moderate nonatopic bronchial asthma.

Asthma scores were recorded in an asthma diary. Peak expiratory flow (PEF), PEF diurnal variation (PEF%), forced expiratory volume in one second (FEV_1%), methacholine airway hypersensitivity (minimum dose of methacholine) (D_{min}) were measured. Biopsy of the bronchial mucosa was performed before and after 8 weeks of treatment with BDP (400 $\mu g \cdot day^{-1}$). The following inflammatory cells were immunostained: eosinophils with anti-EG2; mast cells with AA1; neutrophils with NP57; T-lymphocytes with anti-CD3, CD4, and CD8; and activated T-lymphocytes with anti-CD25.

There was a significant improvement in the asthma symptom score, PEF%, FEV_1%, and D_{min} after BDP therapy and the number of EG2-, AA1-, CD3-, CD4-, and CD25-positive cells decreased significantly.

We conclude that inhaled beclomethasone dipropionate inhibited inflammatory cell infiltration of airway tissue and that associated with this there was an improvement of symptoms in this open study of inhaled beclomethasone dipropionate in a group of nonatopic asthmatic subjects.

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Bronchial asthma is perceived as a chronic bronchial inflammatory disease associated with infiltration of eosinophils, mast cells and T-cells into the airway wall. This is demonstrated by biopsy of the bronchial mucosa [1, 2], and by examination of bronchoalveolar lavage (BAL) fluid [3], regardless of severity [4, 5].

Asthma can be divided. according to its nature, into two types: atopic (extrinsic) and nonatopic (intrinsic). The former is characterized by a type I allergic reaction, thought to result from the reaction of specific immunoglobulin E (IgE) antibody on mast cell or basophil with antigen, and where atopic symptoms are the result of congenital factors. In the latter, the antigen, if any, remains unidentified and the pathogenic factor is still unknown, although viral infection [6], receptor anomaly [7] and autoantibody [8] have been considered to be possible responsible factors. Type III and/or IV allergic reactions are also thought to play a causative role in the manifestation of bronchial asthma [9]. Nonatopic asthma is apt to occur in middle-aged and older individuals and often becomes intractable in these patients.

The infiltration of inflammatory cells into airway tissues is noted regardless of the type of asthma, although it is more severe in nonatopic asthma [10]. Therefore, the treatment of asthma is often aimed at the alleviation of chronic airway inflammation due to such cellular infiltration: for this purpose, combinations of bronchodilators such as β_2-agonist [11] and xanthine [12], but also anti-inflammatory agents, are variously used.

Use of corticosteroids was reported by WALSH and GRANT [13] to be highly effective for the treatment of asthma. Beclomethasone dipropionate (BDP), developed as a steroid for inhalation, was reported to be a useful treatment for asthma and exhibited minimal side-effects [14]. For long-term treatment, steroid inhalation is effective and safe in asthma if it is used at low dosage [15], or at high dosage for short-term treatment [16, 17]. Histopathologically in atopic asthma, steroid inhalation has been reported to increase the number of ciliated cells [18], suppress the infiltration of inflammatory cells [19, 20], and improve airway epithelial damage [21].

The present open study was designed to evaluate the effects of BDP inhalation both clinically and histopathologically in patients with mild-to-moderate nonatopic asthma, as this has not been previously studied. We find that BDP inhibits inflammatory cell infiltration of tissues in association with improvement both of clinical symptoms and lung function.

Patients and methods

Patients

The study included a total of 10 patients with mild-to-moderate nonatopic asthma (three females and seven males, aged 47 yrs on average). Asthmatic patients were defined as: 1) those who had intermittent chest

tightness, wheeze, cough, shortness of breath; 2) those who had reversible airway obstruction (variation in forced expiratory volume in one second (FEV_1) or peak expiratory flow (PEF) of 20% or more); and 3) those who had increased airway hypersensitivity. The severity of asthma was graded as: mild, where the patient complained of mild intermittent dyspnoea which occurred once or twice a week; moderate, where the patients had dyspnoea occurring three times or more a week and an nocturnal attack of asthma occurring twice or more a month; and severe, where continuous exacerbation of dyspnoea and frequent nocturnal attacks were noted in spite of treatment.

Five of the asthmatics took intermittent inhaled β_2-agonist alone, five took oral theophylline and β_2-agonist in addition to inhaled β_2-agonist. None received oral or inhaled corticosteroids or cromolyn.

The patients were characterized by negative skin-prick tests to common allergens, as well as IgE radioallergosorbent (RAST) using various antigens, such as house dust mite, grass pollens, cat fur, dog hair and fungi. Their total serum IgE values were all within normal range. Additional antigen inhalation tests were carried out with 11 antigens to ensure their nonatopic status. Antigen extracts for skin-prick test were diluted in normal saline to prepare a 10 fold series of dilutions (10^{-3}, 10^{-2} and 10^{-1}). However, the highest fungal concentration for inhalation was 10^{-2}. The antigen dilutions were inhaled by a DeVilbiss 646 Nebulizer (DeVilbiss Co., Somerset, PA, USA) at a constant airflow of 5 $L \cdot min^{-1}$. FEV_1 was measured after 10 min following a 2 min administration of saline. The FEV_1 obtained was recorded as the baseline FEV_1 value. For each of the antigens, FEV_1 was measured in the same way as the baseline FEV_1 in ascending order of concentration. The measurement was repeated to confirm whether the parameter would decrease by 20% or more from the baseline value. The parameter was measured 20, 30, 60 and 120 min after inhalation at the highest concentration of each antigen. Reduction in FEV_1 did not exceed 20% in any of the patients (table 1).

Informed consent was obtained prior to the study, which was performed under the control of the Ethics Committee of our Institute. Permission was not given for a placebo-controlled arm to this study, which was therefore "open" in design. Patients acted as their own control and were biopsied both before and after treatment with the corticosteroid.

Study design

Following a 2 week observation period, the patients underwent bronchoscopy and were, subsequently, treated with BDP at 400 $\mu g \cdot day^{-1}$ for 8 weeks. BDP inhalation was delivered from a pressurized metered-dose inhaler through a 750 mL volumatic spacer device (Glaxo Co., Tokyo, Japan). Patients were each directed to make a daily record of their use of BDP inhalation, β_2-agonist inhalation, theophylline and oral β_2-agonist in their patient diary. During the observation and treatment periods the patients recorded the severity of attack and cough, expectoration of sputum, disturbance in daily activities and sleep due to asthma, and the consumption of the given drug in their patient diary.

PEF was measured with an "Assess Peak Flow Meter" (HealthScan, Cedar Grove, NJ, USA) three times every morning and evening. The highest value was recorded. The diurnal variation in PEF was expressed as a ratio (%) of the difference between PEF_{max} and PEF_{min} to PEF_{max}.

Bronchoscopic examination was performed twice: 1) at the end of the observation period; and 2) at the end of the 8 week period of BDP treatment. One week before the bronchoscopic examination (or at 1 week into observation and 7 weeks into treatment), airway hypersensitivity and lung function were measured and, for the patients who used theophylline, the serum level of theophylline was determined.

Scoring of symptoms

Six symptom scores were recorded daily on the diary card: 1) wheeze during the day using a graded scale as none=0, mild=3, moderate=6, severe=9; 2) amount of

Table 1. – Patient details

Pt No.	Sex	Age yrs	FEV_1 % pred	D_{min} Unit	IgE $IU \cdot mL^{-1}$	IgE RAST score	SPT	AIT	Mean symptom score before BDP
1	M	58	76	2.03	71	0	-ve	-ve	6.4
2	M	50	65	0.73	67	0	-ve	-ve	7.5
3	F	30	72	1.41	31	0	-ve	-ve	3.2
4	F	58	70	0.39	89	0	-ve	-ve	9.4
5	M	42	70	0.63	79	0	-ve	-ve	1.1
6	M	48	75	0.29	66	0	-ve	-ve	1.3
7	F	42	60	0.35	60	0	-ve	-ve	12.8
8	M	51	59	0.21	49	0	-ve	-ve	1.8
9	M	55	76	1.40	15	0	-ve	-ve	5.6
10	M	41	72	0.70	40	0	-ve	-ve	4.5
Mean±SEM			70±6	0.81±0.58	65.2±19.9	0			5.4±3.8

Pt: patient; M: male; F: female; FEV_1: forced expiratory volume in one second; D_{min}: minimum dose of methacholine as the indicator for bronchial sensitivity; IgE: immunoglobulin E; RAST: radioallergosorbent test; SPT: skin-prick test; AIT: allergen inhalation test; -ve: negative; BDP: beclomethasone dipropionate; IU: immunizing unit; Unit: equal to 1 min of a 1.0 $mg \cdot mL^{-1}$ aerosol inhalation of methacholine.

sputum was as graded small=1 or large=2; 3) expectoration of sputum as good=0 or bad=1; 4) limitation of daily activity as none=0, mild=6, moderate=12, severe=18; and 5) nocturnal wheeze or nocturnal cough as none=0, mild=3, moderate=6, severe=9. The results were expressed as the daily mean of the sums of the six scores. The mean initial symptom score was calculated from the scores recorded for the 2 week observation period. In the same way, the mean posttreatment symptom score was calculated from those which were recorded for the 2 weeks during weeks 7–8 of treatment.

Functional assessment

Airway hypersensitivity. The use of any bronchodilator was discontinued for 12 or more hours before the examination of methacholine-induced airway hypersensitivity. The examination was performed by the method of TAKISHIMA *et al*. [22] using an Astograph (Chest Co., Tokyo, Japan) direct-writing recorder, measuring dose-response curves of respiratory resistance (R_{rs}), during continuous inhalation of methacholine at stepwise incremental concentration. Methacholine hydrochloride in isotonic saline was gradually increased to 49, 98, 195, 390, 781, 1,563, 3,125, 6,250, 12,500 and 25,000 µg·mL^{-1}. At first, saline solution was inhaled and after 1 min was followed by successive inhalations of increased methacholine concentration. The minimum dose of methacholine (D_{min}) was used as the indicator of bronchial sensitivity, *i.e*. the amount of the cumulative dose at the inflection point where the reciprocal of the R_{rs} decreased linearly. D_{min} was scaled by a unit equal to one min of a 1.0 mg·mL^{-1} aerosol inhalation of methacholine.

Biopsy of bronchial mucosa. Biopsy specimens were obtained during a bronchoscopy performed according to published guidelines [23]. The patients were premedicated with atropine sulphate (0.6 mg *i.m*.) and diazepam (5 mg *i.v*.) for sedation, and then received two puffs (200 µg) of salbutamol. Four percent lidocaine was sprayed for local anaesthesia of the pharynx and the larynx. The bronchoscope (BF-20, Olympus Co., Tokyo, Japan) was inserted *via* the mouth following surface anaesthesia of the trachea and the bronchi with 2% lidocaine. Oxygen was administered *via* a nasal cannula at 4 L·min^{-1} and arterial oxygen saturation was monitored with a finger oximeter (Biox 3740, Pulse Oximeter, Ohmeda, Louiville, KY, USA). Using alligator forceps (FB-15C, Olympus), bronchial biopsy was performed at: 1) the right main bronchus upper lobe bronchus bifurcation, 2) the bifurcation of the right middle lobe; and 3) the right B6 inlet. During the second biopsy, tissue samples were obtained from the equivalent sites in the contralateral lung.

Histopathological examination. The tissue specimens were covered in ornithine carbamyl transferase (OCT) compound, rapidly frozen in dry ice-acetone and stored in a deep freeze at -70°C. Specimens from all the tissues were prepared by staining with haematoxylin and eosin (H&E). Cryostat slices 4 µm thick were placed on albumin-coated slides, allowed to air dry for 60 min, and then fixed for 15 min in cold acetone (-20°C). According to the method of NAKANE and PIERCE [24], the prepared tissue slices were washed for 5 min in phosphate buffered saline (PBS) five times and then treated with 10% normal porcine serum at room temperature for 30 min. After these procedures, the preparations were reacted with monoclonal antibodies as the primary antibody. To detect activated eosinophils, tissue sections were immunostained with anti-EG2 (Nichirei, Tokyo, Japan), mast cells with AA1 (Dako Ltd, High Wycombe, UK), neutrophils with NP57 (Dako Ltd), T-lymphocytes with CD3 (Becton-Dickinson, Cowley, UK), CD4 (Becton-Dickinson) and CD8 (Becton-Dickinson), and activated T-lymphocytes were identified using anti-CD25 (Dako Ltd). All these antibodies were diluted to 1:50, except for NP57 (1:100). Staining reactions were performed at 37°C for 1 h. After washing in PBS, sections were reacted with peroxidase-labelled mouse anti-immunoglobulin G (IgG) (5 mg·mL^{-1}) as a secondary antibody at room temperature for 1 h, and washed again with PBS, which contained NaN_3 (65 mg·dL^{-1}) in order to avoid nonspecific reaction caused by the endogenous peroxidase of eosinophils and neutrophils.

Colour development was performed for 5 min with 3,3'-diaminobenzidine·4 HCl. Methylene green was used for nuclear staining, rinsed in running water, dehydrated with alcohol, and cleared with xylol. For negative controls, tissues were prepared omitting the primary antibodies and using mouse IgG2a myeloma protein as a substitute for the primary antisera. All tissue samples were stained within 24 h after collection.

The specimens were all coded to blind the observer to treatment and sampling times: each section was observed by optical microscopy at ×400 magnification (BH2, Olympus). The same blinded observer counted the number of cells in five different fields and calculated the number of cells per field. The stained cells were counted only in intact mucosa, excluding epithelium, glands, blood vessels and muscle. The contour of the tissue in which cells were counted was traced with computer software (NEC Co., Tokyo, Japan) on a video display (ITC-370, Olympus) and the area was calculated to determine the number of cells per unit area (mm^{-2}) tissue.

Statistical analyses

The Shapiro-Wilk test was used, whether data distribution was normal or not [25]. Two-tailed Wilcoxon's signed-rank test was used to assess differences in paired data for the mean symptom scores, PEF_{max}, and diurnal variation in PEF. Two-tailed paired Student's t-test was used for FEV_1%, D_{min}, and serum levels of theophylline. Wilcoxon's signed-rank test was used for statistical comparison between the number of cells per unit area before and after BDP inhalation. All data are expressed as mean±SEM. Differences at p-value less than 0.05 were considered to be statistically significant.

Results

There was no problem with regard to the safety of the bronchoscopic procedure in our series of patients. After

the first bronchoscopic study, two patients complained of mild dyspnoea: an additional β_2-agonist inhalation was administered and 10 mg oral prednisone was added for 2 days.

Clinical and functional examination

Symptom scores decreased significantly ($p<0.05$) after 8 weeks of BDP treatment, from an initial score of 5.4±3.8 to 2.3±2.0 as recorded during the observation period. The percentage of diurnal variation in PEF decreased significantly ($p<0.05$) from 24±4 to 13±4%. PEF increased from 413±97 $L \cdot min^{-1}$ in the observation period to 478±72 $L \cdot min^{-1}$ after treatment. FEV_1% increased significantly ($p<0.05$) from 70±6% to 74±5%, and D_{min} from 0.81±0.58 to 1.56±0.86 units. The serum level of theophylline did not change significantly, being 4.6±2.1 $\mu g \cdot mL^{-1}$ in the observation period and 4.0±1.7 $\mu g \cdot mL^{-1}$ after treatment (fig. 1).

Histopathological observation

For the counting of stained cells, the specimen showing the least damage at biopsy was selected from the tissue samples taken from the three different anatomical sites. Figure 2 shows representative findings on EG2- and CD4-stained specimens.

After treatment with BDP at 400 $\mu g \cdot day^{-1}$ for 8 weeks, the number of EG2-positive cells decreased significantly from an initial average of 45.3±16.4 to 15.4±7.1 cells·mm^{-2}, AA1-positive mast cells decreased from 24.0±2.6 to 18.6±3.0 cells·mm^{-2}, CD3-positive T-cells from 180.6±41.9 to 92.3±18.6 cells·mm^{-2}, CD4-positive T-cells from 90.1±22.3 to 44.3±11.9 cells·mm^{-2} and CD25-positive T-cells from 10.4±5.1 to 3.9±2.3 cells·mm^{-2} (all $p<0.05$). However, the number of NP57-positive neutrophils and CD8-positive T-cells did not change significantly (figs. 3 and 4).

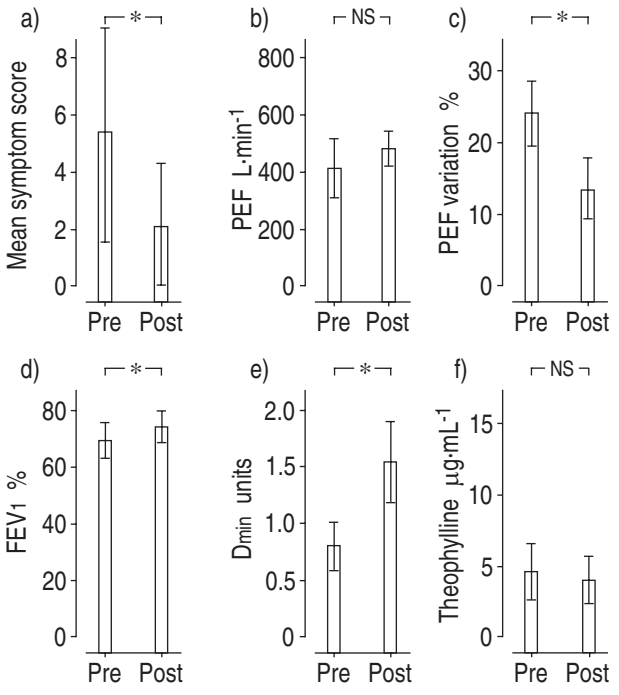

Fig. 1. – a) mean daily symptom scores; b) best peak expiratory flow (PEF); c) diurnal PEF variation, before (Pre) and after (Post) 8 weeks treatment with BDP. Measurements of: d) FEV_1%; e) D_{min} methacholine; and f) serum theophylline value, were performed 7 days before treatment and before the second bronchoscopy (7 weeks of treatment). Data are expressed as mean±SEM. *: $p<0.05$. BDP: beclomethasone dipropionate; FEV_1: forced expiratory volume in one second; D_{min}: minimum dose of methacholine as indicator for bronchial sensitivity; NS: nonsignificant.

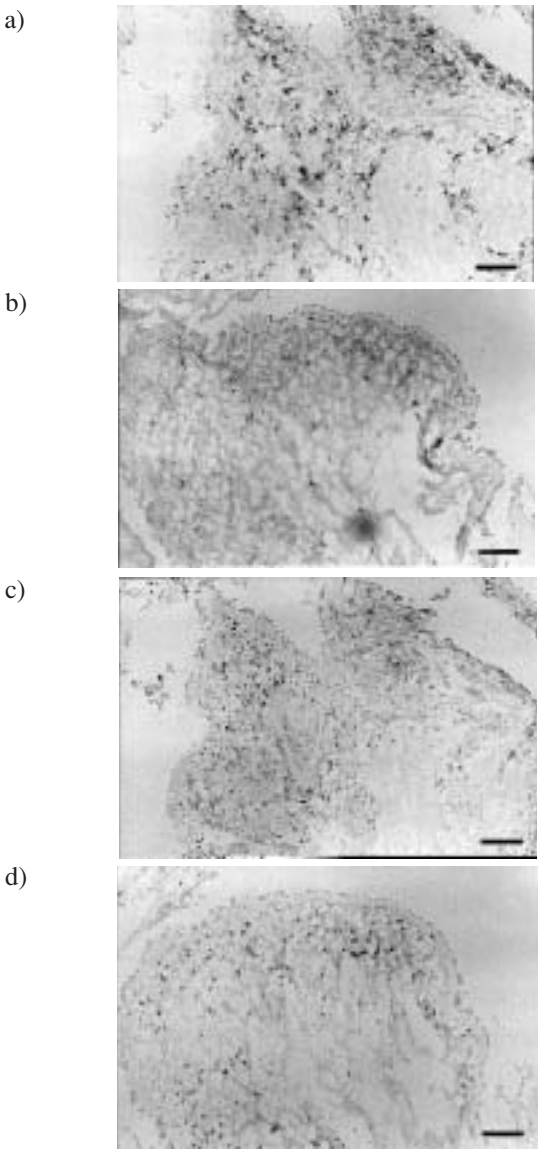

Fig. 2. – Bronchial mucosa from an asthmatic patient. Two sections demonstrating EG2-positive cells: a) before and b) after BDP treatment; and CD4-positive cells; c) before and d) after BDP treatment. BDP: beclomethasone dipropionate. (Original magnification for all four panels is ×150; internal scale bar=0.1 mm).

Discussion

The present study was uncontrolled due to the constraint of the Ethics Committee of our Institute, which felt that the use of a placebo arm instead of an effective inhaled steroid was inappropriate in a symptomatic patient population. Subjects with severe asthma were not included for the same reason. Consequently, we cannot

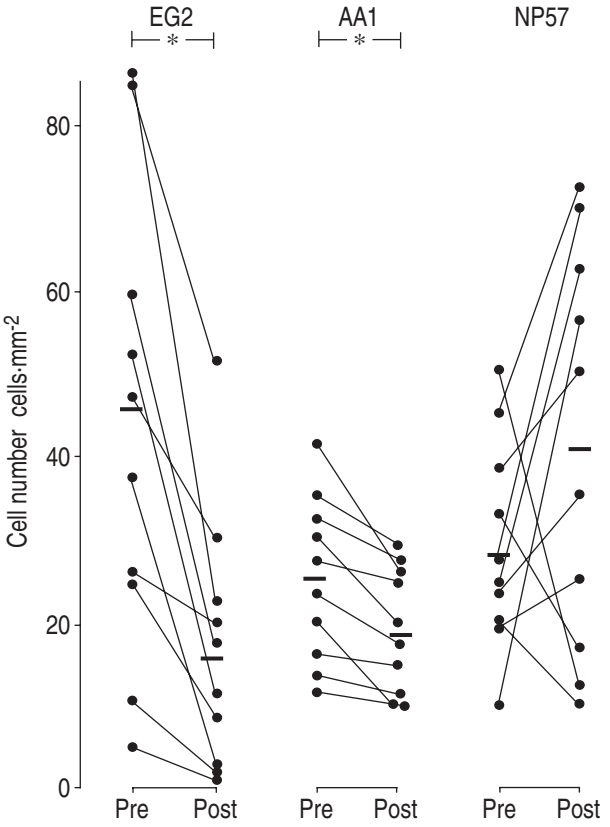

Fig. 3. – Numbers of EG2-positive cells (eosinophils), AA1-positive cells (mast cells) and NP57-positive cells (neutrophils) per square millimetre mucosa in individual cases. Mean values are represented by the horizontal bars. Pre: pretreatment; Post: posttreatment with beclomethasone dipropionate (BDP). *: $p<0.05$.

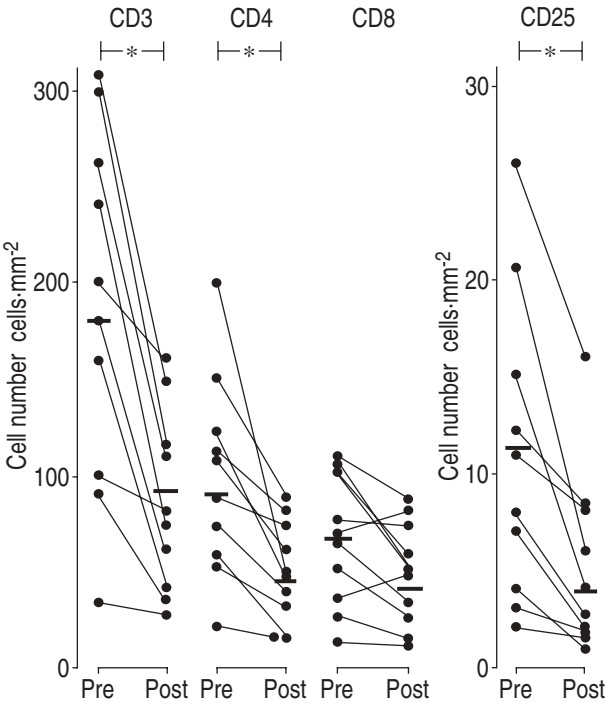

Fig. 4. – Numbers of T-lymphocyte (CD3, CD4, CD8) and activated T-lymphocyte (CD25) count per square millimetre mucosa in individual cases. Mean values are represented by the horizontal bars. Pre: pretreatment; Post: posttreatment with beclomethasone dipropionate (BDP). *: $p<0.05$.

be certain that the improvement in asthma was totally as result of BDP inhalation. However, the clinical and histopathological results correspond to those of other studies in allergic asthma [19, 20], and indicate that BDP inhalation seemed to suppress cellular infiltration in airway tissues and also improve airway hypersensitivity in nonatopic asthma.

Four hundred micrograms of inhaled BDP is known to have antiasthma effects comparable to 5–7.5 mg of oral prednisone [26]. SVENDSEN *et al*. [27] reported that FEV_1, PEF, forced vital capacity (FVC) and airway hypersensitivity improved significantly after 8 weeks of treatment of atopic asthma with BDP inhalation at 400 $\mu g \cdot day^{-1}$. In addition, we found improvements in symptom score, PEF%, FEV_1, and D_{min} in nonatopic asthma after the same dose of BDP.

LUNDGREN *et al*. [21] also demonstrated symptomatic improvement in nonatopic asthma; however, they observed the total number of inflammatory cells and not their subtypes. EG2 immunopositive eosinophils are thought to represent activated eosinophils [28]: an increase in their number is thought to lead to the release of tissue-damaging granular proteins, which damage the airway epithelium and results in airway hypersensitivity [29–31]. The fact that the number of EG2-positive eosinophils in the bronchial mucosa reported here significantly decreased after treatment, is consistent with the report that steroid inhalation decreases eosinophil cationic protein (ECP) levels in BAL [32], and reports [19, 20] that the number of eosinophils in bronchial mucosa decreases after treatment with inhaled steroid. Regarding the mechanism of action of steroids on eosinophils, there have been suggestions that steroids act directly on eosinophils to suppress their infiltration in airway tissues [33], that interleukin (IL)-5 plays an important role in the chemotaxis of eosinophils in airway tissues [34], and that steroids suppress gene expression by IL-5 [35].

AA1 antibody, which does not stain basophils in peripheral blood, seems to be specific for mast cells [36]. Several studies have demonstrated that there is no difference between asthmatic subjects and normal subjects with regard to the number of mast cells in the airway wall [37, 38]. However, there have been other reports that asthmatic patients have a greater number of mast cells in bronchial mucosal epithelium than normal subjects [39, 40]. A recent report suggested that stem cell factor (SCF) is a differentiation factor of human mast cells [41]. If so, the decrease in the number of mast cells after BDP inhalation may be as a result of the suppression of mast cell differentiation by BDP.

In a biopsy study on bronchial mucosa in atopic asthma and nonatopic asthma, the numbers of CD3-, CD4- and CD25-positive T-cells were found to be significantly increased in patients with nonatopic asthma, compared to normal subjects, but there was no significant difference in the number of CD8-positive cells, and, in atopic asthma, only CD25-positive cells increased in number [10]. CD25-positivity is considered to be a marker of activated T-cells, although it may also be expressed on macrophages. WALKER *et al*. [42] reported that T-cell activation and the cytokine pattern in BAL differed between atopic asthma and nonatopic asthma. Unlike CD3-, CD4- and CD25-positive T-cells, which decreased significantly in number after treatment with BDP

inhalation, CD8-positive cells did not. A possible reason is that steroids may decrease the number of lymphocytes - mainly T-cells [43], especially CD4-positive cells [44, 45], in peripheral blood. ROBINSON *et al*. [46] reported that the number of cells expressing messenger ribonucleic acid (mRNA) for IL-4 and IL-5 in BAL from asthmatic patients decreased significantly after steroid therapy. The reduction in the number of T-cells after treatment with BDP inhalation in our study may be a result of the inhibition of cytokine production by BDP.

The suggestion that the number of neutrophils increases in airway tissues of asthmatic patients has not been confirmed in BAL [3, 47], or biopsy of the bronchial mucosa [5, 38]. This does not suggest that neutrophils play an important role in chronic asthma, although they take part in the immediate reaction to allergen. Usually, the number of neutrophils increases after the administration of steroids [48]. However, in our study, the number of NP57-positive neutrophils did not significantly increase after BDP.

Theophylline has been known to have an anti-inflammatory action [49, 50]. However, in our study, the serum level of theophylline was almost unchanged before and after BDP. Consequently, it is possible to ignore its effect on asthmatic symptoms or improvement of inflammation which, we believe, is directly due to BDP.

In conclusion, we have demonstrated that the treatment of nonatopic asthmatics with BDP inhalation at 400 $\mu g \cdot day^{-1}$ for 8 weeks results in a significant improvement in asthmatic symptoms, diurnal variation in PEF, $FEV_1\%$ and airway hypersensitivity. The present histopathological findings on bronchial mucosa demonstrate that the number of eosinophils, mast cells and CD3-, CD4- and CD25-positive T-cells decrease significantly after BDP treatment. In this regard, the inflammation both of atopic and nonatopic asthma is responsive to corticosteroid treatment given by the inhaled route.

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