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Morphological studies of bronchial mucosal biopsies from asthmatics before and after ten years of treatment with inhaled steroids

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Morphological studies of bronchial mucosal biopsies from asthmatics before and after ten years of treatment with inhaled steroids. R. Lundgren, M. Söderberg, P. Hörstedt, R. Stenling.

ABSTRACT: To examine the influence of inhaled steroids on the bronchial mucosa, biopsies from six patients with severe bronchial asthma were studied before and after ten years of daily treatment with inhaled steroids. Biopsies from six healthy subjects were also examined. In the biopsies taken from the asthmatic patients before treatment there was a significant increase in inflammatory cell numbers compared with the biopsies from the control subjects. In all patients scanning electron microscopy showed a reduced coverage by cilia. Squamous cell metaplasia was seen in two patients. After ten years of treatment the number of inflammatory cells was significantly reduced compared to that before treatment and was not different from the control biopsies. Most of the epithelial cells showed a ciliated surface. Small focal areas with non-ciliated cells could still be seen in four patients. In the two patients with squamous cell metaplasia before treatment, small areas of metaplasia could still be seen. Despite the absence of inflammation and reduced epithelial damage during treatment all patients still had bronchial hyperresponsiveness.

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Bronchial mucosal inflammation and damage to the airway epithelium is considered to play an important role in the development and maintenance of bronchial asthma [1–3]. Inhaled steroids have been shown to control the symptoms in many patients with bronchial asthma, probably by their anti-inflammatory effect. Histopathological studies of bronchial mucosal biopsies from patients, treated for up to three years with inhaled steroids, have not shown bronchial mucosal damage associated with such therapy [4–7].

In a previous study [6], bronchial mucosal biopsies from eight patients with advanced bronchial asthma were examined by scanning electron microscopy (SEM). They were examined before and after six months of daily therapy with up to 400 μg beclomethasone dipropionate inhalations.

The present study focuses on long-term treatment and examines the efficacy of steroid therapy on inflammation and epithelial damage. Six of the eight patients have been re-examined after more than ten years of daily treatment with inhaled steroids.

Patients and methods

Patients

Eight non-smoking patients, six men and two women (aged 32–56 yrs, median age 50 yrs), entered the study [6]. All patients had severe intrinsic bronchial asthma. They had all been treated with oral steroids for some months during the year before treatment but only one patient was treated with oral steroids at the time of the first examination [6]. One patient died from his asthma six years after the first examination and was thus not re-examined. One patient was unwilling to participate in the ten year re-examination. The remaining six patients are included in this study (table 1). During treatment with inhaled steroids, they improved clinically with fewer attacks of asthma. Two patients, however, needed a higher dosage after some years to remain stable (table 1). All examinations were performed with the patients in a stable phase and without respiratory tract infections.

Control subjects

Six healthy non-smoking volunteers without asthma, bronchitis or other bronchial disease served as normal controls. One of the volunteers was a woman and five were men (age 34–40 yrs, median age 37 yrs).

The patients and volunteers were examined after giving their informed consent. The study was approved by the Ethical Committee of the University of Umeå.

Prior to bronchoscopy the patients underwent clinical examination and spirometry. Vital capacity (VC) and forced expiratory volume in one second (FEV_1) was measured on a Vitalograph (Vitalograph Ltd, Buckingham, UK).

Table 1. – Vital capacity and forced expiratory volume before and after ten years of treatment with inhaled steroids

Patient	Sex	Age	Before treatment		After 10 yrs of treatment			Treatment (at last examination)
			VC	FEV_1	VC	FEV_1	PC_{20}	
		yrs	*l*	*l*	*l*	*l*	$mg \cdot ml^{-1}$	
ER	M	45	4.80 (5.24)	1.55 (4.14)	3.85 (5.02)	1.95 (3.78)	0.06	T 7.5 mgx2; S inh as req. Bud inh 400 µgx2 last 4 yrs (BD 200 µgx2 first 6 years)
SS	M	58	4.40 (5.06)	2.45 (3.75)	3.30 (4.84)	2.00 (3.38)	0.13	S 4 mgx2; The 300 mgx2; S inh as req BD 200 µgx2
EH	F	42	3.75 (3.83)	2.30 (3.18)	3.15 (3.59)	2.00 (2.87)	0.30	T 7.5 mgx2; The 300 mgx2; S inh as req BD 100–200 µgx2
IH	F	63	2.45 (2.85)	1.70 (2.21)	2.20 (2.60)	1.50 (1.97)	0.19	S 4 mgx2; S inh as req BD100 µgx2
BM	M	63	2.65 (5.85)	2.00 (4.48)	4.70 (5.63)	3.20 (3.85)	0.30	T 7.5 mgx2; The 270 mgx2; S inh as req Pred 10 mg every other day; Bud inh 400–800 µgx2 last 6 yrs (BD100 µgx2–4 before)
BO	M	42	5.40 (5.20)	3.00 (4.48)	4.46 (4.98)	3.00 (3.81)	1.50	S 2 mgx2; The 270 mgx2; S inh as req BD 100 µgx2

Numbers within brackets: predicted normal values; VC: vital capacity; FEV_1: forced expiratory volume in one second; PC_{20}: concentration of methacholine chloride causing a 20% fall in FEV_1; T: terbutaline tablets (slow release); S: salbutamol tablets; The: theophylline tablets (slow release); Pred: prednisolone tablets; S inh as req: salbutamol inhalations as required; BD: beclomethasone dipropionate inhalations; Bud inh: budesonide inhalations; PC_{20}<0.25 $mg \cdot ml^{-1}$: severely increased irritability; <2.0 $mg \cdot ml^{-1}$: moderately increased irritability.

Methacholine test

Bronchial responsiveness was tested by inhalation of increasing dosages of methacholine chloride. A Wright nebulizer (Roxon Medi-Tech Ltd, Quebec, Canada) with an output of about 0.13 $ml \cdot min^{-1}$ was used to nebulize methacholine. The aerosol was inhaled by tidal breathing over two minutes and methacholine was used in concentrations from 0.03 $mg \cdot ml^{-1}$ in doubling dosages to a maximum of 16 $mg \cdot ml^{-1}$ [8]. The provocation concentration causing a fall in FEV_1 of 20% was expressed as the PC_{20}-value. A PC_{20}-value of more than 8 $mg \cdot ml^{-1}$ was considered to be normal.

Flexible fibreoptic bronchoscopy (FFB)

Pre-medication with 0.5–0.75 ml of morphine-scopolamine (10 $mg \cdot ml^{-1}$+0.4 $mg \cdot ml^{-1}$) was used and all examinations were performed under topical anaesthesia using about 250 mg of lignocaine [9]. Before bronchoscopy, all patients were pre-treated with 4 mg disodium betamethasone intravenously two hours before examination; 230 mg theophylline intravenously and 0.5 mg terbutaline sulphate subcutaneously 30 min before examination. The patients also had a continuous infusion of theophylline and supplementary oxygen during the examination. The healthy volunteers were only pre-medicated with morphine-scopolamine or 0.5 mg of atropine. Heart rate was monitored with a cardioscope. FFB was performed with the patients in the supine position and 3–5 forceps biopsies were taken from the proximal part of the right upper lobe bronchus and another 3–5 biopsies from the proximal part of the right lower lobe bronchus using an Olympus BF-20C forceps through an Olympus BF-5B2 or BF-1T fibreoptic bronchoscope (Olympus Tokyo, Japan). To avoid mechanical damage the bronchial mucosa was not touched by the bronchoscope before the biopsies were taken.

Light microscopy (LM)

Routine histopathological preparative techniques were used for light microscopical analysis. The biopsies were fixed in phosphate buffered 4% formaldehyde, and embedded in paraffin. Four µm thick sections were cut and stained with haematoxylin-eosin and Giemsa.

The amount of lymphocytes and plasma cells (% of mucosal volume) within the subepithelial region were quantitatively estimated stereologically [10]. The results were obtained from measurements of 4–5 regions from each biopsy using a square grid applied within the eye piece of a light microscope. Measurements were made at an objective lens magnification of x40. No correction for shape or section thickness was made. Morphometric analyses were also performed concerning the thickness of both the epithelial layer and the basement membrane. From each biopsy five measurements were made in sections roughly perpendicular to the luminal surface.

Scanning electron microscopy (SEM)

After gentle washing in normal saline to remove mucus, the biopsies were fixed in 2.5% glutaraldehyde, critical point dried, mounted on holders and vacuum coated with gold [11]. The biopsies were examined in a Cambridge S-4 scanning electron microscope (Cambridge Instruments Ltd, Cambridge, England). The biopsies were studied at four different magnifications: x60 for an overview of the biopsies; x600 for an overview of the structure of the epithelial surface and for estimation of the proportion between ciliated and non-ciliated cells. Cell surfaces and cilia were studied at a magnification of x2,400 and details of cilia and microvilli were studied at a magnification of x12,000.

Statistics

For comparison between groups Wilcoxon's non-parametric rank sum test was used. A value of $p<0.05$ was considered to be statistically significant.

Results

During bronchoscopy the bronchial mucosa appeared macroscopically normal in all patients and volunteers. In two patients a slight bronchospasm, which did not need treatment, was noted after bronchoscopy, otherwise there were no complications.

VC and FEV_1 as well as bronchial responsiveness expressed as PC_{20}-values are shown in table 1.

Control subjects

Light microscopic examination of the biopsies from the healthy control subjects showed a pseudostratified airway epithelium with ciliated cells but also secretory cells and basal cells. Only a few inflammatory cells, mainly lymphocytes, could be seen in the subepithelial tissue (table 2). Ciliated cells with a high density of cilia

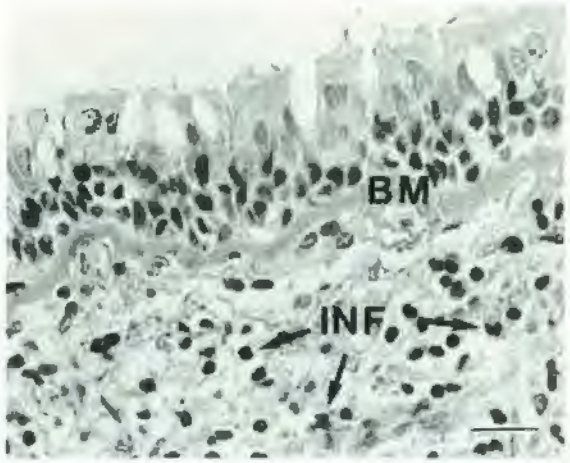

Fig. 1. – Light microscopy of bronchial mucosal biopsy from patient with bronchial asthma (IH) before treatment with inhaled steroids. There are large numbers of inflammatory cells, mainly lymphocytes (INF) in the lamina propria. BM: basement membrane. Stained with haematoxylin-eosin. Bar: 20 μm.

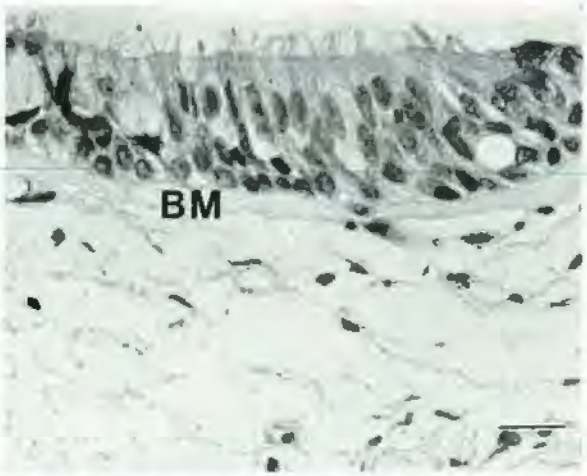

Fig. 2. – Bronchial mucosal biopsy from a patient with bronchial asthma (ER) after ten years of treatment with inhaled steroids. By light microscopy only a few inflammatory cells can be seen in the lamina propria. BM: basement membrane. Haematoxylin-eosin x600. Bar: 20 μm.

Table 2. – Inflammatory cells, thickness of epithelium and thickness of basement membrane in control subjects and in asthmatic patients before and after ten years of treatment with inhaled steroids expressed as median values and range (within brackets)

Control subjects	Inflammatory cells (% of mucosal volume)	Thickness of epithelium (μm)	Thickness of basement membrane (μm)
Control subjects (n=6)	0.73 (0.30–1.10)	33.1 (31.2–43.2)	4.8 (2.9–6.7)
Asthmatic patients (n=6)	x	NS	NS
Before treatment with inhaled steroids	3.46 (1.09–6.49)	34.7 (31.9–36.9)	7.0 (3.8–9.6)
	†	NS	NS
After treatment with inhaled steroids	0.41 (0.30–0.79)	36.0 (32.6–42.5)	5.8 (3.8–7.2)

n: number of patients. Statistical significance. N.S.: not significant; x: $p<0.05$, asthmatics before treatment compared with control subjects; †: $p<0.05$, asthmatics after treatment compared with before treatment.

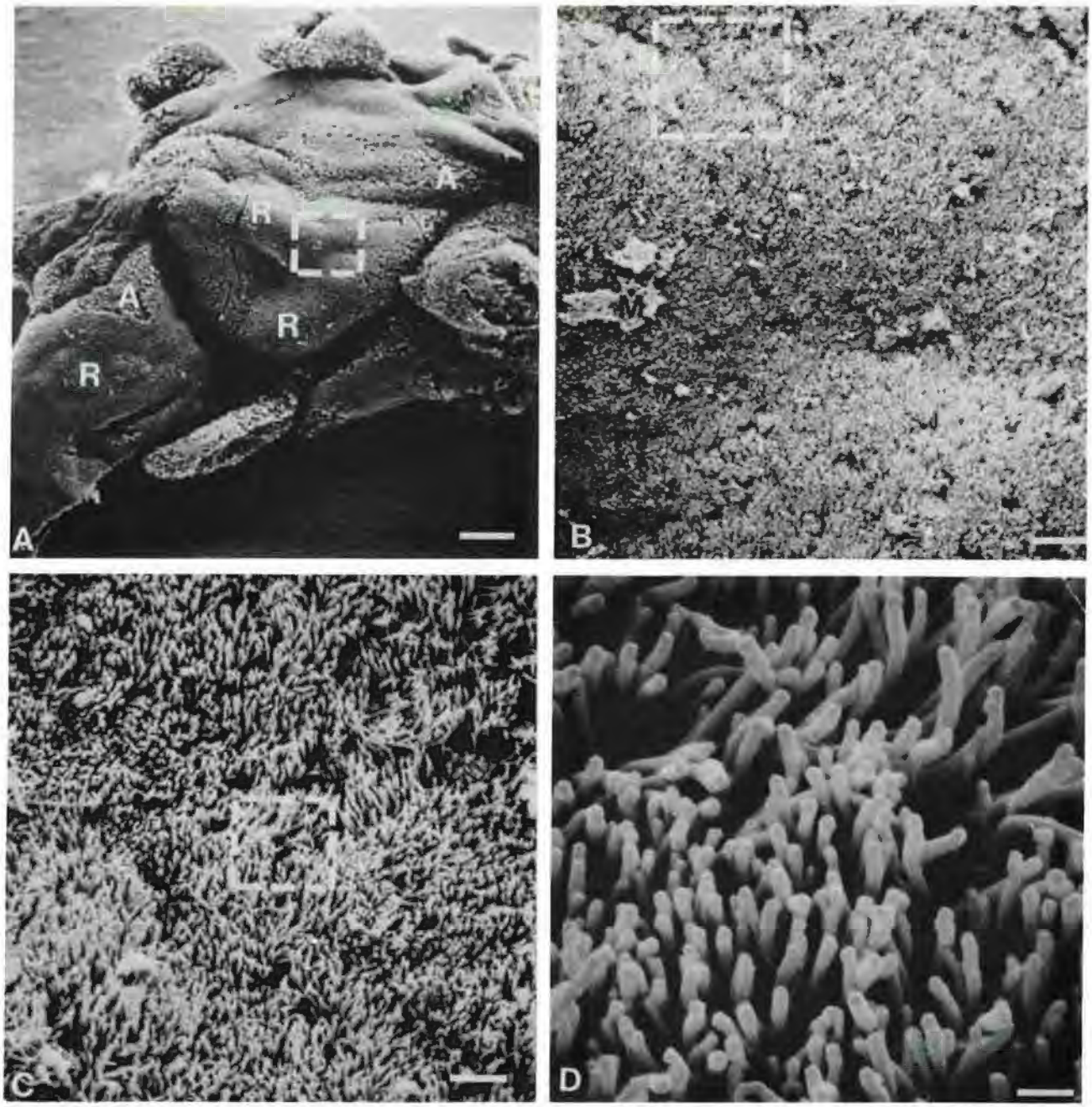

Fig. 3a. – An overview of a bronchial mucosal biopsy from an asthmatic patient (BO) treated for ten years with inhaled steroids. R: respiratory surface epithelium. A: artefact where epithelial cells have been torn off. SEM x60. Bar: 200 µm. Fig. 3b. – Area within the square in figure 3a showing normal ciliated respiratory epithelium. The mucous layer has been washed away. M: fragment of mucus. SEM x600. Bar: 20 µm. Fig. 3c. – Area within the square in figure 3b showing normal ciliated epithelium. SEM x2,400. Bar: 5 µm.Fig. 3d. – Area within the square in figure 3c showing normal cilia. SEM x12,000. Bar: 1 µm.

dominated the field of view as seen by SEM. Secretory cells could, only occasionally, be seen through the ciliated carpet. No cilial abnormality was observed.

Asthma patients before treatment with inhaled steroids

There was a significant ($p<0.05$) increase in inflammatory cell numbers in the biopsies taken from asthmatic patients before treatment compared with the biopsies from the control subjects (fig. 1, table 2). The cells consisted mainly of lymphocytes, some plasma cells and eosinophils while neutrophils were observed only occasionally. There was no difference in the thickness of the basement membrane or the thickness of the epithelium between asthmatic patients and control subjects (table 2). In two patients a small focal area with squamous metaplasia of the epithelium was observed.

SEM showed reduced coverage by cilia in the biopsies from all six patients (table 3). In three patients there were large areas with non-ciliated cells. In the remaining three patients the normal ciliated epithelium was mixed

with areas showing both ciliated and non-ciliated cells or areas covered with only non-ciliated cells. In all patients, small areas with abnormal, usually short, cilia were found.

Table 3. – Proportion of non-ciliated epithelium as viewed by scanning electron microscopy in bronchial mucosal biopsies taken before and after ten years of treatment with inhaled steroids

Patient	Before treatment	After 10 yrs of treatment
ER	4/5	1/4
SS	1/3	1/10
EH	1/4	1/5
IH	4/5	<1/10
BM	4/5	<1/10
BO	1/5	1/10

Asthma patients after ten years of treatment with inhaled steroids

In the biopsies taken after ten years of treatment with inhaled steroids there were few inflammatory cells, and only isolated eosinophils could be seen in the subepithelial tissue (fig. 2). There was a significantly reduced number of inflammatory cells after treatment (table 2). There was no significant difference in inflammatory cell numbers between the biopsies taken from the asthmatic patients after treatment and the biopsies taken from the control subjects. There was no significant difference in the thickness of the epithelium or the thickness of the basement membrane between control subjects and asthmatics treated with inhaled steroids (table 2). Small focal areas of squamous cell metaplasia were seen in the same two patients as before treatment.

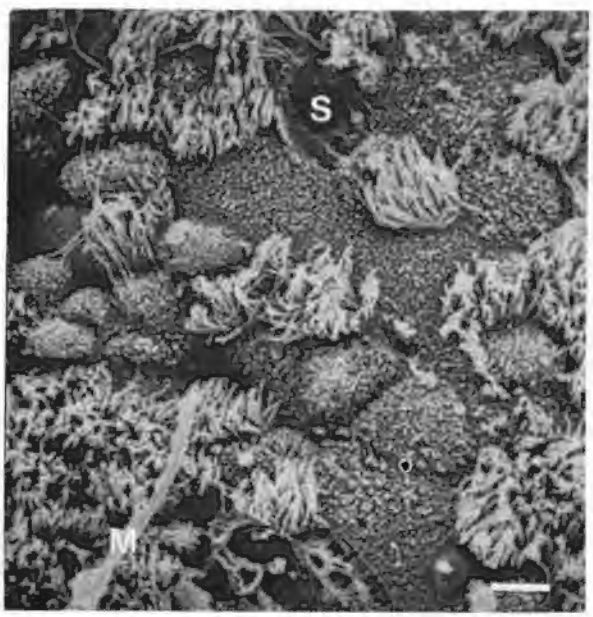

Fig. 4. – Biopsy from an asthmatic patient (BO) treated for ten years with inhaled steroids showing bronchial epithelium with a mixture of ciliated and non-ciliated cells. S: secretory cell; M: mucus. SEM x2,400. Bar: 5 μm.

Fig. 5. – Bronchial mucosal biopsy from an asthmatic patient (SS) treated for ten years with inhaled steroids showing epithelium with normal ciliated cells (to the right) and a focal area without ciliated cells (to the left). SEM x600. Bar: 20 μm.

In all patients the non-ciliated areas had decreased in the biopsies after treatment (table 3). As viewed by SEM an almost normal ciliated epithelium (fig. 3) was seen in two patients. In three of the six patients the biopsies showed small focal areas with a mixture of non-ciliated and ciliated cells among otherwise normal ciliated epithelium (fig. 4). In four patients small focal areas with non-ciliated columnar or flattened cells could also be seen (fig. 5). Abnormal cilia were seen in four patients. In one of the patients a focal area with irregularly arranged elongated cilia was observed.

Discussion

The possibility of performing FFB on patients with bronchial asthma has been discussed because of the potential danger. SAHN and SCOGGIN [12] reported three severe complications among asthmatics examined with FFB. The National Heart, Lung and Blood Institute workshop summaries [13] concluded that bronchoalveolar lavage performed *via* FFB on volunteer subjects with asymptomatic asthma receiving minimal medication can be performed safely. The patients examined in the present study had rather severe asthma, but were in a stable stage at the time of examination, and they were all carefully pre-treated with steroids and bronchodilators.

In two of the patients, a slight bronchospasm was noted after bronchoscopy, but otherwise there were no complications.

Bronchial epithelial damage has been observed in patients with bronchial asthma and, in recent years, the role of epithelial damage and bronchial mucosal inflammation has been discussed as a possible pathogenetic

factor in bronchial asthma [2, 3, 14–16]. DUNNILL [1] noted mucosal oedema and separation of mucosal cells in patients dying from asthma. CUTZ *et al.* [17] showed changes in the lungs of children with severe asthma even when they were asymptomatic. They noted goblet cell hyperplasia, "thickening of the basement membrane", peribronchial smooth muscle hypertrophy and eosinophilic infiltration. The luminal surfaces of ciliated cells showed cytoplasmic blebs and abnormal cilia. We could not measure any thickening of the basement membrane but eosinophils were noted before treatment and abnormal cilia were seen before as well as after treatment.

BARNES [16] has discussed possible mechanisms whereby epithelial damage could cause bronchial hyperresponsiveness. LAITINEN *et al.* [2] observed epithelial changes in eight patients with mild to severe bronchial asthma. Major epithelial damage appeared as focal lesions and different stages of damage could be seen in the same biopsy specimens. In our study all patients had focal areas of epithelial changes in the biopsies taken before treatment with inhaled steroids. Even after ten years of treatment epithelial changes with reduced numbers of ciliated cells could be seen in biopsies from all but two patients. The changes were, however, less pronounced in the biopsies taken after ten years of treatment compared with biopsies taken before treatment.

It has been suggested that inflammation plays an important role in the pathogenesis of bronchial hyperresponsiveness. HOLTZMAN *et al.* [19] noted an increase in the number of neutrophils in the epithelium in biopsies from dogs that became hyperresponsive after ozone inhalation. LAITINEN *et al.* [2], although observing epithelial changes in all the asthmatic patients examined, rarely noted mast cells, leucocytes were found only occasionally and eosinophils were not reported. In our study an increase in inflammatory cells above normal was seen in the biopsies taken from asthmatics before treatment. Lymphocytes and plasma cells were common, eosinophils could also be seen and neutrophils were rare. The number of attacks of asthma decreased during the years of treatment compared with the year before treatment with beclomethasone dipropionate inhalation had commenced. It is tempting to suggest that the decreased number of inflammatory cells following treatment is the result of the anti-inflammatory effect of inhaled steroids, and that there is a relationship between bronchial hyperreactivity and bronchial inflammation [18, 19]. On the other hand, following treatment all patients were still hyperresponsive to methacholine, despite the absence of histological evidence of inflammation in the biopsies. Unfortunately, no examinations of bronchial reactivity were performed before treatment for comparison. All patients were examined when their asthma was stable. It is possible that the degree of inflammation is increased during an acute attack of asthma but assessment by biopsy is difficult to take during such attacks.

In two patients small areas of metaplasia were seen in the biopsies taken after ten years of treatment, in contrast to the majority of the biopsies from these patients which had a normal ciliated epithelium. During the last years these two patients had increased their daily dosage of inhaled steroids (table 1). The fact that the biopsies taken from these two patients before treatment also showed metaplasia of the epithelium suggests that the metaplasia was not caused by the treatment with inhaled steroids.

The biopsies taken from the bronchial mucosa were small and it is difficult to be sure that they were representative of the whole bronchial mucosa. During bronchoscopy there were, however, no macroscopic signs of change in the epithelium and several biopsies were taken to obtain material as representative as possible.

In conclusion the present study showed bronchial mucosal inflammation and epithelial damage in asthmatics. Long-term treatment with inhaled steroids significantly decreased inflammation and also reduced epithelial damage, but the patients still had bronchial hyperresponsiveness.

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Etudes morphologics des biopsies de la muqueuse bronchique chez les patients asthmatiques avant et après dix ans de traitement avec l'inhalation de stéroïdes. R. Lundgren, M. Söderberg, P. Hörstedt, R. Stenling.

RÉSUMÉ: Pour examiner l'influence de l'inhalation de stéroïdes sur la muqueuse bronchique, nous avons examiné des biopsies de six patients atteints d'asthme bronchique sévère, avant et après dix ans de traitement quotidien par stéroïdes. Les biopsies de six sujets normaux ont également été examinées. Dans les biopsies prélevées chez les patients asthmatiques avant traitement, on note une augmentation significative du nombre de cellules inflammatoires, par comparaison avec les biopsies des sujets contrôles. Chez tous les patients, la microscopie électonique à balayage montre une couverture ciliare réduite. Chez chaucun de deux patients, une métaplasie épidermoïde a été observée. Après dix ans de traitment, le nombre de cellules inflammatoires était réduit de façon significative, par comparaison avec la situation avant traitement, et il ne diffère plus de celui des biopsies contrôles. La plupart des cellules épithéliales ont une surface ciliée. De petites zones focales recouvertes de cellules non ciliées existent encore chez quatre patients. Chez chacun des deux patients atteints d'une métaplasie épidermoïde avant traitement, on pouvait toujours voir de petites zones de métaplasie. Malgré l'absence d'inflammation et la diminution des lésions épithéliales au cours du traitement, tous les patients avaient encore une hyperréactivité bronchique.
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