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Effects of the cationic protein poly-L-arginine on airway epithelial cells *in vitro*

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BACKGROUND: Allergic asthma is associated with an increased number of eosinophils in the airway wall. Eosinophils secrete cationic proteins, particularly major basic protein (MBP).
***Aim*: To investigate the effect of synthetic cationic polypeptides such as poly-L-arginine, which can mimic the effect of MBP, on airway epithelial cells.**
***Methods*: Cultured airway epithelial cells were exposed to poly-L-arginine, and effects were determined by light and electron microscopy.**
***Results*: Poly-L-arginine induced apoptosis and necrosis. Transmission electron microscopy showed mitochondrial damage and changes in the nucleus. The tight junctions were damaged, as evidenced by penetration of lanthanum. Scanning electron microscopy showed a damaged cell membrane with many pores. Microanalysis showed a significant decrease in the cellular content of magnesium, phosphorus, sodium, potassium and chlorine, and an increase in calcium. Plakoglobin immunoreactivity in the cell membrane was decreased, indicating a decrease in the number of desmosomes**
***Conclusions*: The results point to poly-L-arginine induced membrane damage, resulting in increased permeability, loss of cell–cell contacts and generalized cell damage.**

Key words: Allergic asthma, Eosinophils, Major basic protein, Poly-L-arginine, Epithelial damage

Introduction

Asthma is a chronic inflammatory disease characterized clinically by repeated episodes of reversible airway narrowing, airway hyperresponsiveness to a variety of stimuli, and chronic inflammation of the airway wall, associated with infiltration of eosinophils in peripheral blood, bronchial tissue and sputum. There has been considerable interest in the role of eosinophils and, in particular, eosinophil-derived cationic proteins in the pathogenesis of bronchial asthma[1,2] In allergic asthma, the eosinophil is the dominating leukocyte in the airway wall, and a correlation between the number of eosinophils and the extent of damage shown by the airway epithelium has been established.[3] The best studied among the eosinophil-derived cationic proteins is major basic protein (MBP), a highly charged protein with 117 amino acid residues, 17 of which are arginine and seven of which are lysine.[1,4] These proteins are localized at the core of the granule of the eosinophil. Although a relationship between epithelial damage, bronchial hyperresponsiveness and MBP in allergic asthma has already been established, the mechanism by which this occurs has not yet been clarified.

High MBP levels in serum and bronchoalveolar lavage fluid have been correlated with bronchial hyperreactivity in asthmatics.[1,5] It has also been demonstrated that MBP induces damage to the airway epithelium and reduces ciliary motility,[6,7] increases ion fluxes and prostaglandin synthesis in the airway epithelium,[8] induces histamine release from human basophils and rat mast cells,[9] mediates airway smooth muscle contraction,[10] promotes edema, increases airway responsiveness to spasmogens,[11] and causes smooth muscle contraction via an epithelium-dependent mechanism.[12]

It has been demonstrated that synthetic cationic polypeptides such as poly-L-arginine and poly-L-lysine can mimic the effects of MBP. They can induce airway hyperresponsiveness,[11,13] can cause smooth muscle contraction by an epithelium-independent mechanism,[13,14] can increase the permeability of the airway epithelium,[15] and can promote pulmonary edema.[16] High concentrations of these polypeptides have been used to obtain destruction and exfoliation of epithelial cells.[17,18] In the *in vivo* or *in situ* studies the trachea was exposed to concentrations of about 1 mg/ml of the polycations,[11,13–15] and cells in the *in vitro* studies were exposed to 0.4–1 mg/ml of poly-L-arginine.[17,18]

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The aim of the present investigation was to obtain more information on the process by which the damage occurs. Therefore, lower concentrations than in previously published studies were used.

Materials and methods

Poly-L-arginine (molecular weight, 8500–13,000) and low-molecular weight heparin were obtained from Sigma Chemicals (St Louis, MO, USA).

Cell culture

The 16HBE14o– cell line, a kind gift from Dr D.C. Gruenert (University of California, San Francisco, CA, USA) was cultured in Eagle's minimal essential medium (EMEM) (National Veterinary Institute, Uppsala, Sweden) supplemented with 10% fetal bovine serum (Gibco BRL/Life Technologies, Paisley, UK), 100 U/ml of penicillin and 100 of µg/ml streptomycin sulfate. The culture flasks were coated with fibronectin-coating solution containing 0.01 mg/ml of fibronectin, 0.029 mg/ml of collagen and 0.1 mg/ml of bovine serum albumin for at least 1 h before culturing the cells.

Normal human bronchial epithelial (NHBE) cells (Clonetics, San Diego, CA, USA) were used. Culture of these cells was established at Clonetics cell culture facility from normal human tissue taken from a 16-year-old female. The cells were cultured in plastic culture flasks (Corning Costar Corporation, Cambridge, MA, USA) in bronchial epithelial basal medium (BEGM) (Clonetics) supplemented according to the manufacturer's instruction.

Both cell lines were cultured in a humidified atmosphere of 5% CO_2/95% air at 37°C and the culture medium was changed every 48 h. Desmosome formation in NHBE cells required a different medium, namely Dulbecco's modified Eagle's medium (DMEM):F12 (1 : 1) (Gibco BRL/Life Technologies) supplemented with 5% fetal bovine serum, 100 U/ml of penicillin, 100 µg/ml of streptomycin, and non-essential amino acids (Sigma). The cells were cultured on Lab-Tek chamber slides (Nalge Nunc International, Rochester, NY, USA) or in Petri dishes (Becton Dickinson, Plymouth, UK).

Cell induction

Cells were cultured to about 90% confluence and then incubated with poly-L-arginine. In initial experiments, cell viability was assessed using poly-L-arginine in the concentration range of 2.5 to 20 µM. Based on the data obtained in those experiments, concentrations of 15 µM for 16HBE14o– cells and 2.5 µM for NHBE cells was chosen. Unexposed cells served as controls. The cells were incubated for 24 h with poly-L-arginine, except for the desmosome experiments where an incubation period of 48 h was used, since this was the time required for desmosome formation.

Cell viability

The viability of both types of cells as a function of the concentration of poly-L-arginine (in the presence or absence of heparin) was determined by the fluorescein di-acetate (FDA) dye exclusion test. Intact viable cells take up FDA, which is cleaved by intracellular esterases to fluorescein showing green fluorescence. The cells were grown in a 96-well microplate and were treated with poly-L-arginine. After exposure to poly-L-arginine, 100 µl of a 10 µg/ml FDA solution in phosphate-buffered saline was added to each well, and the cells were incubated for 20 min at 37°C. Then the number of viable luminating cells in each well was measured using a luminometer.

Analysis of apoptotic and necrotic cells

Both cell types were cultured in 24-well plates and exposed to poly-L-arginine. Control cells were not exposed to poly-L-arginine, but were otherwise treated in the same way. The cells were then stained with bisbenzimide H 33342 (20 µg/ml) and propidium iodide (10 µg/ml) (Sigma) for 10 min at 37°C, according to the manufacturer's instructions. The cells were analyzed using a Leica DMR fluorescent microscope (Leica, Wetzlar, Germany).

Transmission electron microscopy

16HBE14o– cells grown in Petri dishes in EMEM media, and NHBE cells in either BEGM or DMEM:F12 medium were fixed in 2.5% glutaraldehyde (Sigma) in 0.1 M cacodylate buffer (Agar Scientific, Stansted, UK) for 1 day. After being washed in 0.1 M cacodylate buffer, the cells were post-fixed in 1% OsO_4 in cacodylate buffer for 20 min. After another wash in buffer, cells were dehydrated in graded ethanol solutions and finally embedded in Agar 100 epoxy resin (Agar Scientific). The embedded cells were removed from the bottom of the Petri dish with a saw. Sections 50 nm thick were cut on an ultramicrotome (LKB, Bromma, Sweden), contrasted with 5% uranyl acetate/3% lead citrate and examined in a Hitachi H7100 transmission electron microscope (Hitachi, Tokyo, Japan) at 75 kV.

The paracellular permeability barrier function of both the 16HBE14o– cells and the NHBE cells was investigated by visualizing the tight junctions by means of a lanthanum nitrate tracer. Lanthanum nitrate (1%) was added to the fixative.

Scanning electron microscopy

Both cell lines were grown and fixed with glutaraldehyde as already described for transmission electron microscopy. After washing in 0.1 M cacodylate buffer, post-fixation in 1% OsO_4in the same buffer for about 1 h was carried out. Then the cells were washed again in 0.1 M cacodylate buffer and dehydrated in a graded series of acetone. Subsequently, the cells were dried on cover slips by means of the critical point drying method. Finally, the cover slips were mounted on the holders and sputter-coated with gold. The specimens were examined in a LEO 1530 field emission scanning electron microscope (LEO, Cambridge, UK) at an accelerating voltage of 1 kV.

X-Ray microanalysis

Cells were cultured on 75-mesh titanium grids (Agar Scientific) covered with a carbon-coated Formvar film (Merck, Darmstadt, Germany), sterilized by ultraviolet light and coated with fibronectin-coating solution (see Cell culture) for 16HBE14o- cells. Both types of cells were then incubated in a 5% CO_2/95% air atmosphere at 37°C. About 48–72 h later, cells were exposed to poly-L-arginine. Unexposed cells served as controls. After 24 h exposure, cells were rinsed for a few seconds with cold distilled water and frozen in liquid propane cooled by liquid nitrogen (–180°C) and freeze dried overnight under vacuum at –130°C.[19,20] Before analysis, the specimens were coated with a conductive carbon layer. Analysis was performed in a Hitachi H7100 electron microscope in the scanning transmission electron microscopy mode at 100 kV with an Oxford Instruments (Oxford, UK) ISIS energy-dispersive spectrometer system. Quantitative analysis was carried out based on the peak-to-continuum method after correction for extraneous background and by comparing the spectra from the cells with those from a standard.[21] Spectra were acquired for 100 sec and each cell was analyzed only once.

Immunocytochemistry

Confluent 16HBE14o- and NHBE cells grown on glass slides were exposed to poly-L-arginine for 48 h. The cells were fixed in methanol for 3 min at –20°C, rinsed with tris-hydroxymethyl-amino methane (Tris)-buffered saline (TBS) (0.05 M Tris–HCl, 0.15 M NaCl; pH 7.6) for 5 min and then blocked with 5% normal rabbit serum (NRS) (Dako, Glostrup, Denmark) in TBS for 15 min. Following primary incubation with monoclonal anti-plakoglobin (Sigma) at a dilution of 1 : 6000 for 16HBE14o- cells and 1 : 10,000 for NHBE in TBS for 1 h at 37°C, the cells were rinsed twice with TBS. The cells were again blocked with 5% NRS for 15 min and incubated with a fluorescein isothiocyanate-conjugated secondary antibody (Dako) at a dilution of 1 : 40 for 1 h. After rinsing with TBS, the slides were mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA) and examined with a Leica DMR fluorescent microscope (Leica).

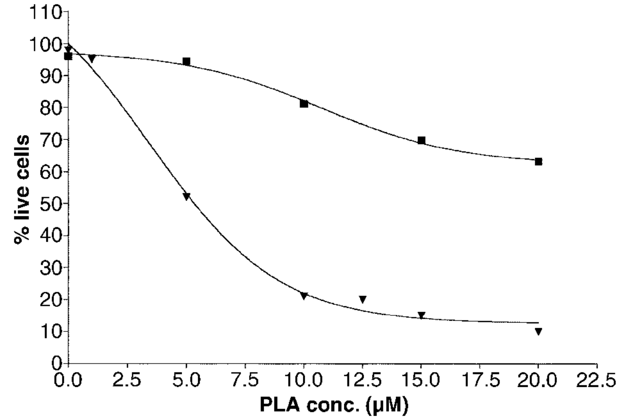

FIG. 1. Concentration dependence of the effect of poly-L-arginine (PLA conc.) on the viability of 16HBE14o– (■) and NHBE (▼) cells. Data (expressed as percentage of live cells) represent the mean of 12 experiments, standard errors too small to show.

Statistical analysis

Data are presented as the mean±standard error of the mean, and statistical analysis was performed using an unpaired Student's *t*-test, or a Mann–Whitney test when the distribution of data was not normal. Significance was attributed to probability values <0.05.

Results

Cell viability

16HBE14o- cultures exposed to 5–20 μM poly-L-arginine for 24 h showed a concentration-related increase in the number of dead cells (Fig. 1). Also, NHBE cells exposed to 1–15 μM poly-L-arginine for 24 h showed a concentration-dependent increase in the number of dead cells and appeared more sensitive than 16HBE14o- cells (Fig. 1). Heparin (100 μg/ml) inhibits the toxic effect of 15 μM poly-L-arginine completely.

Analysis of apoptotic and necrotic cells

After treatment of 16HBE14o- cells with 15 μM poly-L-arginine, the number of necrotic cells was increased from 6% to 29% and the number of apoptotic cells was increased from 1% to 4%. In the case of NHBE cells, poly-L-arginine increased the number of necrotic cells from 4% to 22% and the number of apoptotic cells to about 5% (Table 1).

Table 1. Effect of poly-L-arginine on necrosis and apoptosis of airway epithelial cells

Cells		Viable	Necrotic	Apoptotic
16HBE14o–	Control	93 ± 8	6 ± 1	1 ± 0.3
	PLA treated	67 ± 5**	29 ± 3**	4 ± 0.5**
NHBE	Control	96 ± 3	4 ± 1	0.2 ± 0.2
	PLA treated	73 ± 4**	22 ± 3*	5 ± 0.5**

16HBE14o– cells were exposed to 15 μM poly-L-arginine (PLA), NHBE cells were exposed to 2.5 μM poly-L-arginine. Data are given as percentage of the total number of cells, and represent mean (standard error (n = 6). Significant differences between treated cells and controls are indicated by asterisks, * $p < 0.01$, ** $p < 0.001$.

Transmission electron microscopy

Control 16HBE14o- cells were flat, with a central dome-shaped elevation containing the nucleus, and the mitochondria were normal and compact (Fig. 2a). In most of the poly-L-arginine-exposed cells, the mitochondria showed fewer cristae (Fig. 2b).

Control NHBE cells were flatter than the 16HBE14o- cells and also had a dome-shaped elevation containing the nucleus, which had a regular shape and size (Fig. 3a). After 24 h treatment with poly-L-arginine, most of the cells showed an irregularly shaped nucleus where the nuclear membrane started to invaginate and the nucleus appeared to be divided into small lobes. Mitochondria had a normal appearance (Fig. 3b).

In both cell lines, no lanthanum was found in the intercellular spaces in the controls. After 24 h exposure to poly-L-arginine, lanthanum penetrated the tight junctions of most of the cells in both cell lines (Fig. 4).

Scanning electron microscopy

Control 16HBE14o- cells grown in EMEM were flat and were evenly covered with cell processes (Fig. 5a). Poly-L-arginine-exposed cells (15 μM, 24 h) showed

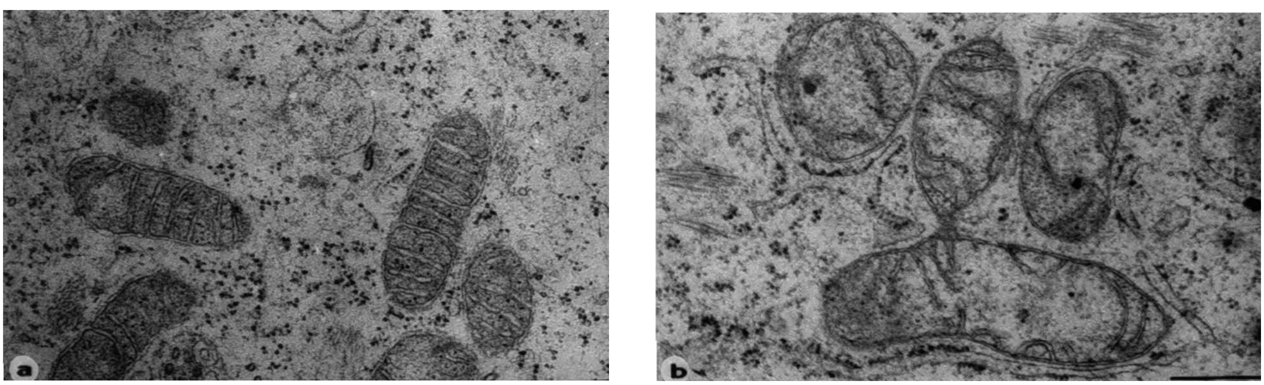

FIG. 2. Transmission electron micrographs of 16HBE14o– cells: (a) control, (b) exposed to 15 μM poly-L-arginine. Note changes in mitochondria. Bar = 0.5 μm.

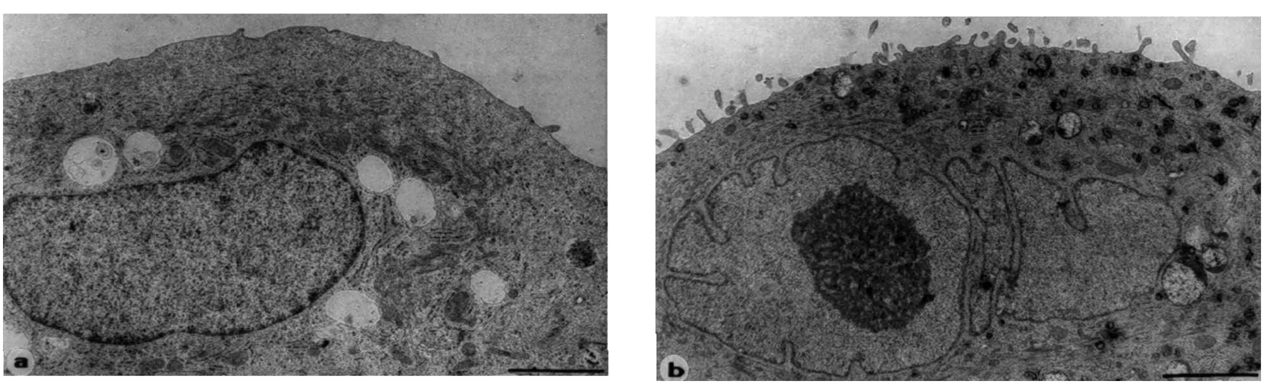

FIG. 3. Transmission electron micrographs of NHBE cells: (a) control, (b) exposed to 2.5 μM poly-L-arginine. Note changes in nucleus. Bar = 2 μm.

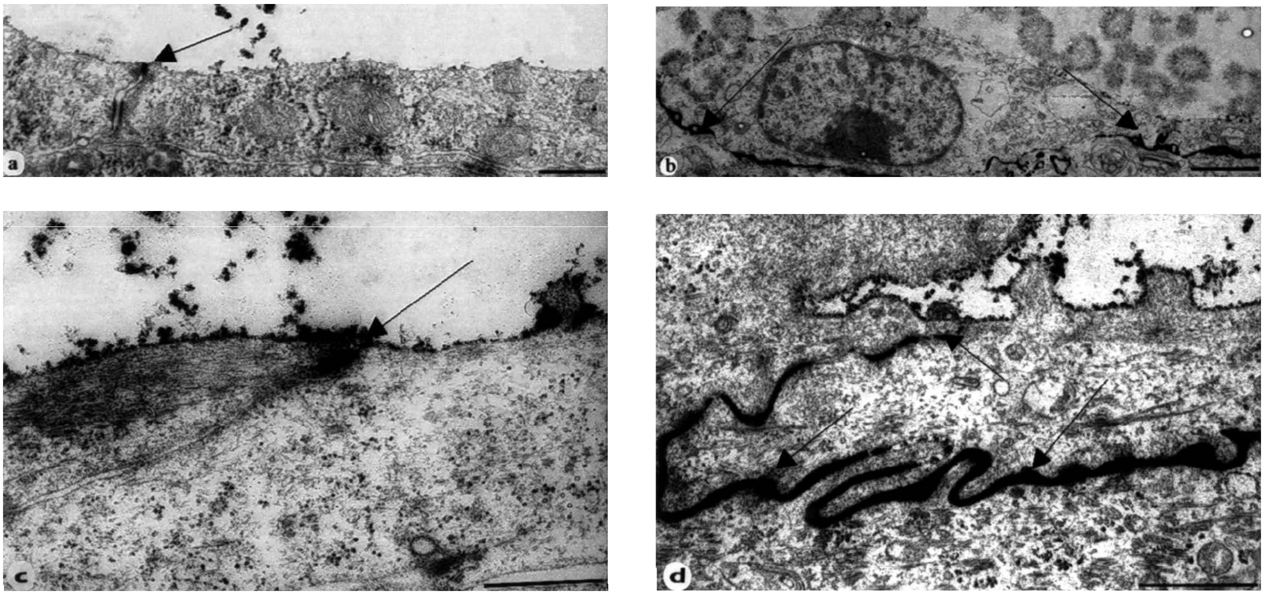

FIG. 4. (a, b) Transmission electron micrographs of 16HBE14o– cells fixed in the presence of lanthanum: (a) control, (b) exposed to 15 μM poly-L-arginine. Bar = 2 μm. (c,d) Transmission electron micrographs of NHBE cells fixed in the presence of lanthanum: (c) control, (d) exposed to 2.5 μM poly-L-arginine. Bar = 0.5 μm. Note that lanthanum does not penetrate the tight junctions in the controls (arrow) but penetrates into the intercellular space after treatment with poly-L-arginine (arrow).

shorter and fewer cell processes that were evenly distributed (Fig. 5b).

NHBE control cells grown in BEGM also had a flat shape and were evenly covered with cell processes (Fig. 6a). After treatment with 2.5 μM poly-L-arginine, the shape of all the cells became rounded and cell processes were present mainly on the top of the cell. These processes were unevenly distributed and longer than in the control (Fig. 6b). After poly-L-arginine exposure, the cell membrane appeared damaged with many pores (Fig. 6b).

X-Ray microanalysis

In 16HBE14o– cells, there was a significant decrease in the cellular content of potassium, phosphorus, sulfur, magnesium and chloride after exposure to 15 μM poly-L-arginine for 24 h. The intracellular sodium content was not significantly altered in the treated cells compared with the controls. However, the total intracellular content of calcium was raised significantly in the treated cells (Fig. 7a).

When NHBE cells were treated with 2.5 μM poly-L-arginine for 24 h, there was a significant reduction in the intracellular content of potassium, phosphorus and magnesium. No significant difference was observed in the cellular content of sodium, chloride and sulfur between the control and treated cells. As in 16HBE14o– cells, the total intracellular content of calcium was raised significantly in the NHBE cells (Fig. 7b).

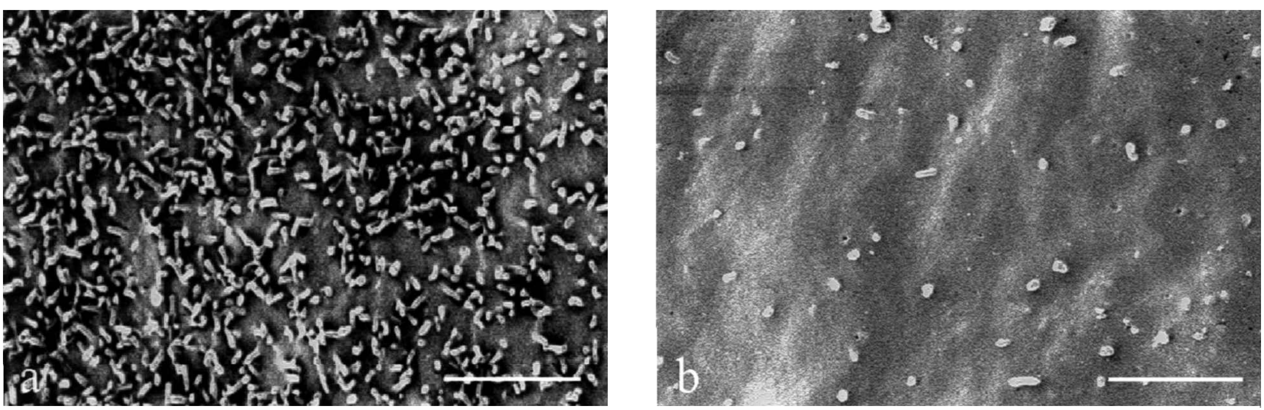

FIG. 5. Scanning electron micrographs of 16HBE14o– cells: (a) control, (b) exposed to poly-L-arginine. Note changes in cell processes. Bar = 2 μM.

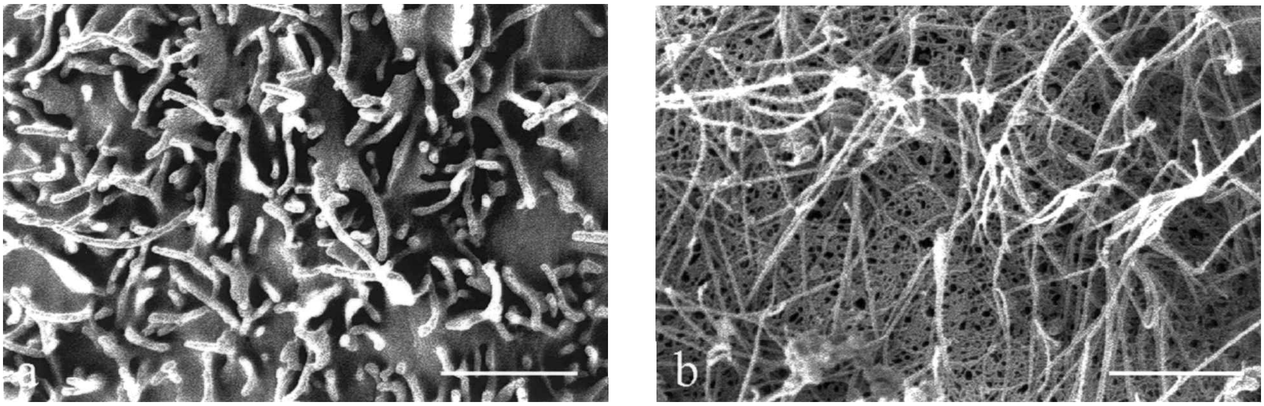

FIG. 6. Scanning electron micrographs of NHBE cells: (a) control, (b) exposed to 2.5 μM poly-L-arginine. Note small holes in cell membrane after treatment with poly-L-arginine. Bar = 2 μm.

Immunocytochemistry

Confluent monolayers of 16HBE14o− cells were grown in EMEM. In the control cells there was a continuous immunofluorescent band of desmosomes lining the cell membrane. Treatment of the cells with poly-L-arginine (15 μM for 48 h) caused a marked reduction in the number of desmosomes (Fig. 8a,b).

NHBE cells were cultured in a medium supplemented with fetal calf serum to be confluent. When the NHBE cells were treated with 2.5 μM poly-L-arginine for 48 h, the number of desmosomes was markedly reduced compared with the controls (Fig. 8c,d).

Discussion

The present study demonstrated that the exposure of the bronchial epithelial cell to the cationic protein poly-L-arginine results in several types of damage to the cells. We found changes in the ultrastructure of mitochondria indicating damage to these organelles, which could lead to disturbance of cell metabolism and cell death. We also found increased leakage of diffusible ions from the cells. Cell–cell contacts (tight junctions and desmosomes) seem to be disrupted. Although there was a generalized damage to both kinds of cells, NHBE cells (representative of basal cells) were more sensitive to poly-L-arginine than 16HBE14o− cells (representative of columnar cells). Zhang *et al.*[18] have shown that myofibroblasts are much less sensitive to poly-L-arginine than epithelial cells. For cell types, divergent results have been obtained. It has been claimed that poly-L-arginine and poly-L-lysine already at concentrations of 10^{-8} to 10^{-7} M could induce relaxation of vascular smooth muscle via a nitric oxide (NO)-dependent mechanism,[22] but other workers showed that poly-L-arginine was not effective in inducing NO formation at concentrations below 100 μM.[23] Moreover, it has been shown that poly-L-arginine can inhibit L-arginine uptake in tracheal epithelial cells, and that this can limit NO synthesis.[24] It has been speculated that this deficiency of endogenous NO could contribute to airway hyperreactivity induced by polycations.[25]

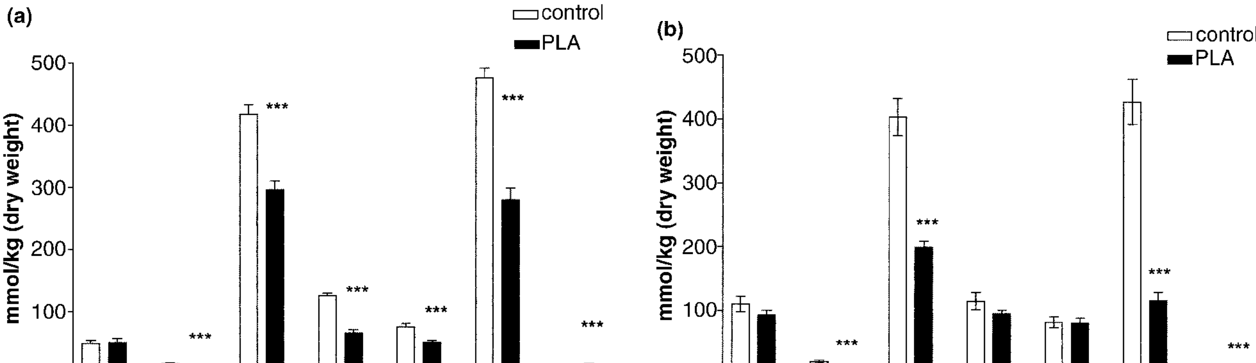

FIG. 7. X-ray microanalysis of (a) 16HBE14o− cells and (b) NHBE cells. Open bars, control; solid bars, exposed to poly-L-arginine (PLA). Significant differences between exposed cells and controls are indicated by asterisks, *** $p < 0.001$. Between 30 and 80 cells in each group were analyzed. Thin lines indicate standard errors.

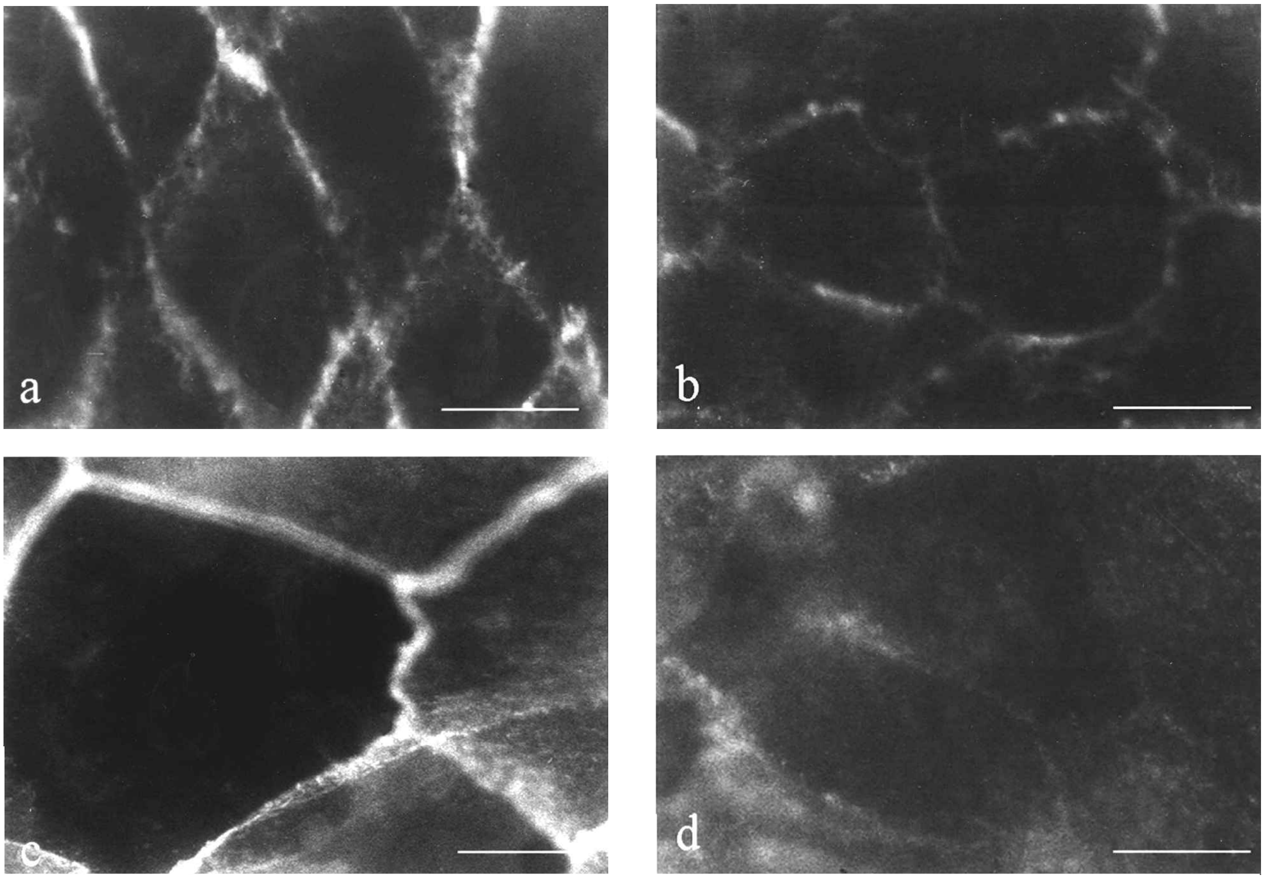

FIG. 8. Immunocytochemistry of plakoglobin (desmosomes) in (a) 16HBE14o– control cells and (b) 16HBE14o– cells exposed to poly-L-arginine, and in (c) NHBE control cells and (d) NHBE cells exposed to poly-L-arginine. Note decreased staining for desmosomal protein after exposure to poly-L-arginine. Bar = 40 μm.

It has been claimed that MBP interacts with the negatively charged plasma membrane lipids due to hydrostatic interactions.[26] Another eosinophil protein, eosinophilic cationic protein, was found to cause pores in the cell membrane.[27] Our high-resolution scanning electron micrographs show small pores in the membrane. Many, if not all, of the other observed changes in the epithelial cells could be due to this effect of poly-L-arginine. The holes in the membranes would result in leakage of low-molecular weight, water-soluble substances from the cells and/or make the cells more sensitive to the procedure of rinsing with distilled water, which is used in preparing the cultured cells for X-ray microanalysis. Normally, cells withstand this procedure well and retain the high potassium/sodium ratio typical for intact living cells.[20,28] The X-ray microanalysis data show a loss of potassium, but at the same time an increase in calcium, which is the hallmark of damaged cells.[29] The increase in calcium is due to influx from the medium, in which the concentration of (free) Ca^{2+} ions is higher than in the cells. Increased cellular calcium concentrations result in damage to mitochondria,[30,31] as well as damage to the tight junctions resulting in increased paracellular permeability.[32] Whether an increase in intracellular calcium concentration would affect desmosomes is unknown. It is well known that desmosomes only form in the presence of extracellular Ca^{2+}, but much less is known about the role of intracellular Ca^{2+} ions. However, Stuart *et al.*[33] have claimed that there are Ca^{2+}-sensitive intracellular mechanisms involved in the sorting and the cytoskeletal stabilization of desmosomes. Loss of K^+ ions from the cell is not only a sign of cell damage, but can in itself induce cell death.[34] The effect of polycations is assumed to be due to their charge,[35] as the effects can be inhibited by polyanions such as heparin or by reducing the charge of the cations by acetylation.[1] In accordance with this, we found that heparin inhibited the toxic effect of poly-L-arginine on 16HBE14o– cells.

The effects of polycations on epithelial cells are well in accordance with the changes in the airway epithelium observed in patients with asthma. Poly-L-arginine induces a marked decrease in the number of desmosomes, which could be a cause of exfoliation of epithelial cells in the airway of asthmatic patients where MBP levels are increased.[36,37] Already the increased permeability of the epithelium due to damage to the tight junctions would allow allergens and other noxious substances to penetrate deeply into the airway wall, and would disrupt the delicate balance in fluid transport for which an intact epithelium is necessary. Hyperresponsiveness of the airway could thus be a consequence of both increased paracellular permeability and of exfoliation of epithelial cells. Increased responsiveness of airway smooth muscle to constrictor mediators *in vitro* has been demonstrated after removal of the airway epithelium.[36] Apart from increased permeability of substances that can activate the smooth muscle cells, the reduced amount of epithelium-derived relaxing factor may important in the development of hyperresponsiveness.[1] Eosinophils can, however, induce damage to airway epithelial cells by other mechanisms as well. One possible mechanism is by the production of hypochlorite by lysosomal peroxidases (e.g. EPO) that can oxidize halides to generate reactive hypohalous species. Hypochlorite is also known to induce the generation of other free radicals that could damage the epithelium.[38] Also, hypochlorite has been shown to decrease the expression of desmosomal proteins in cultured airway epithelial cells.[39]

The results described herein suggest that MBP produced by eosinophils could be an important, although not the only, factor in the epithelial damage in the respiratory tract, which is a common feature of bronchial asthma.

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