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Quantification and localization of HLA-DR and intercellular adhesion molecule-1 (ICAM-1) molecules on bronchial epithelial cells of asthmatics using confocal microscopy

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SUMMARY

An increased expression of HLA-DR and ICAM-1 molecules on bronchial epithelial cells has been observed in asthmatic patients. The aim of this study was to evaluate the localization and to quantify the spontaneous expression of HLA-DR and ICAM-1 on bronchial epithelial cells recovered by bronchial brushing of nine asthmatics and nine controls. Epithelial cells constituted over 95% of cells recovered as shown using an anti-cytokeratin MoAb. Expression of HLA-DR and ICAM-1 was studied using indirect immunofluorescence and confocal microscopy. The intensity of fluorescence of epithelial cells expressing HLA-DR and ICAM-1 was significantly ($P < 0.003$) increased in asthmatics. In asthmatics, but not in controls, the expression of both molecules was localized in the cytoplasm on the apicolateral portions of the cells. This study shows an up-regulation in the expression of HLA-DR and ICAM-1 molecules by bronchial epithelial cells from asthmatics and a localization of these molecules within the cell.

Keywords asthma bronchial epithelium HLA-DR confocal microscopy intercellular adhesion molecule-1

INTRODUCTION

Epithelial cells appear to play an active role in asthma. They are spontaneously activated and release increased amounts of inflammatory mediators [1–3] and express higher levels of HLA-DR and ICAM-1 molecules [4], both of which have been implicated in the cellular network underlying airways inflammation in asthma. Moreover, epithelial cells contribute to mucous secretion in the airways and are involved in the removal of noxious agents by ciliary action. These cells have been shown to control ion transport [5] as well as the traffic of other molecules, including inflammatory mediators [6], between the submucosa and the microenvironment [7]. This latter function may be attributed to the polarized organization which characterizes all epithelia separating compartments of the body from the external milieu [7]. In particular, the membrane surface of polarized epithelial cells can be divided into apical and basolateral domains which differ in molecular composition and function. Certain components of the cytoskeleton are critically involved in both the generation and maintenance of cell polarity. Generation of polarity is controlled by microtubules which serve as uniformly aligned polarized cytoplasmic guiding structures for the selective vectorial transport of carrier vesicles from the intracellular compartments to the apical cell surface [8]. Since surface antigens play a crucial role in the regulation of the cellular physiology, a similar mechanism may be envisaged for surface molecules which could be selectively expressed on the apical or basal portion of the cells in order to accomplish specific biological functions, especially during inflammation.

Several immunochemical methods can be used to visualize membrane or intracytoplasmic markers using specific MoAbs against these markers. A relatively new method for detecting these molecules involves the use of a confocal microscope. The advantage of this microscope is that cells can be viewed in sections along the z-axis. In addition, cell types can be more easily identified using the phase contrast or interference contrast modes [9–11], and the expression of molecules, marked using antibodies conjugated to a fluorescent marker, can be more precisely localized. Furthermore, the confocal microscope has a zoom facility which allows the operator to concentrate on a particular area in order to examine the cellular structure and any associated staining. Since we have previously demonstrated that the percentage of bronchial epithelial cells expressing HLA-DR and ICAM-1 molecules is greater in asthmatics than control subjects [4], we attempted to evaluate whether the intensity of

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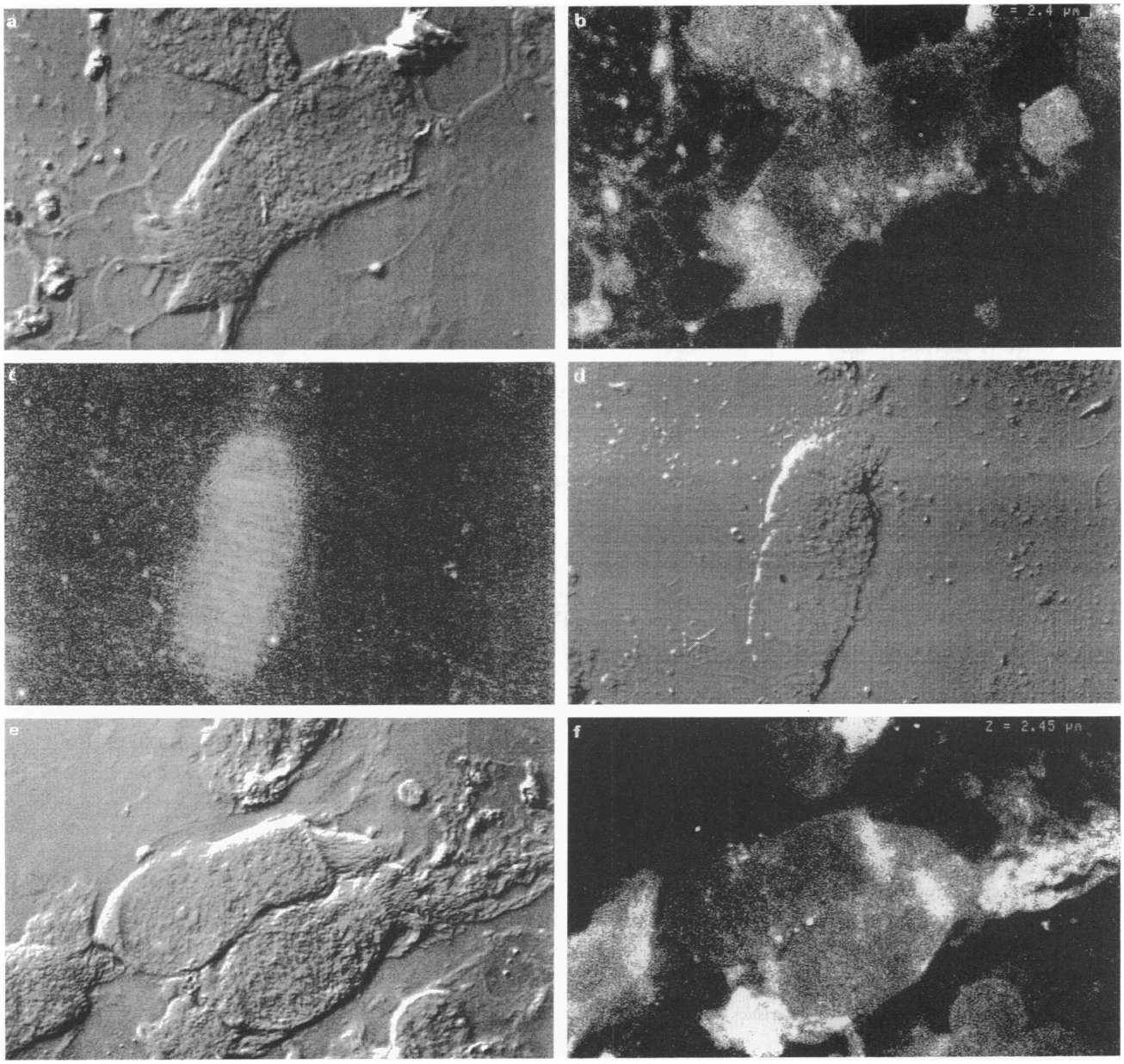

Fig. 1. (a) Ciliated bronchial epithelial cell from an asthmatic identified by interference contrast. (b) Same ciliated cell stained with anti-HLA-DR monoclonal antibody. The fluorescence is mainly located on the apical surface of the cell. (c) Ciliated epithelial cell from a control subject stained with a monoclonal antibody against HLA-DR. The fluorescence is spread over the cell. (d) Same ciliated bronchial epithelial cells of c identified by interference contrast. (e) Ciliated bronchial epithelial cells from an asthmatic identified by interference contrast. (f) Same ciliated bronchial epithelial cells of e stained with anti-ICAM MoAb. (g) Cellular scanning of the same ciliated epithelial cell shown in e. The cells are sectioned from their surface by a laser beam in nine sections of 350 nm along the *z*-axis. The apicolateral localization of the expression of ICAM-1 molecule is present in all the cellular sections. (× 3780) (Continued on next page.)

expression of HLA-DR and ICAM-1 molecules or their intracellular location differed between the two groups. Bronchial epithelial cells were recovered by bronchial brushing and the expression of HLA-DR and ICAM-1 molecules was studied by an indirect immunofluorescence technique using confocal microscopy.

PATIENTS AND METHODS

Subjects

Nine asthmatics (20–60 years old) were selected according to a study previously published [12] and the criteria of the American Thoracic Society [13]. None of the subjects was a smoker. Nine

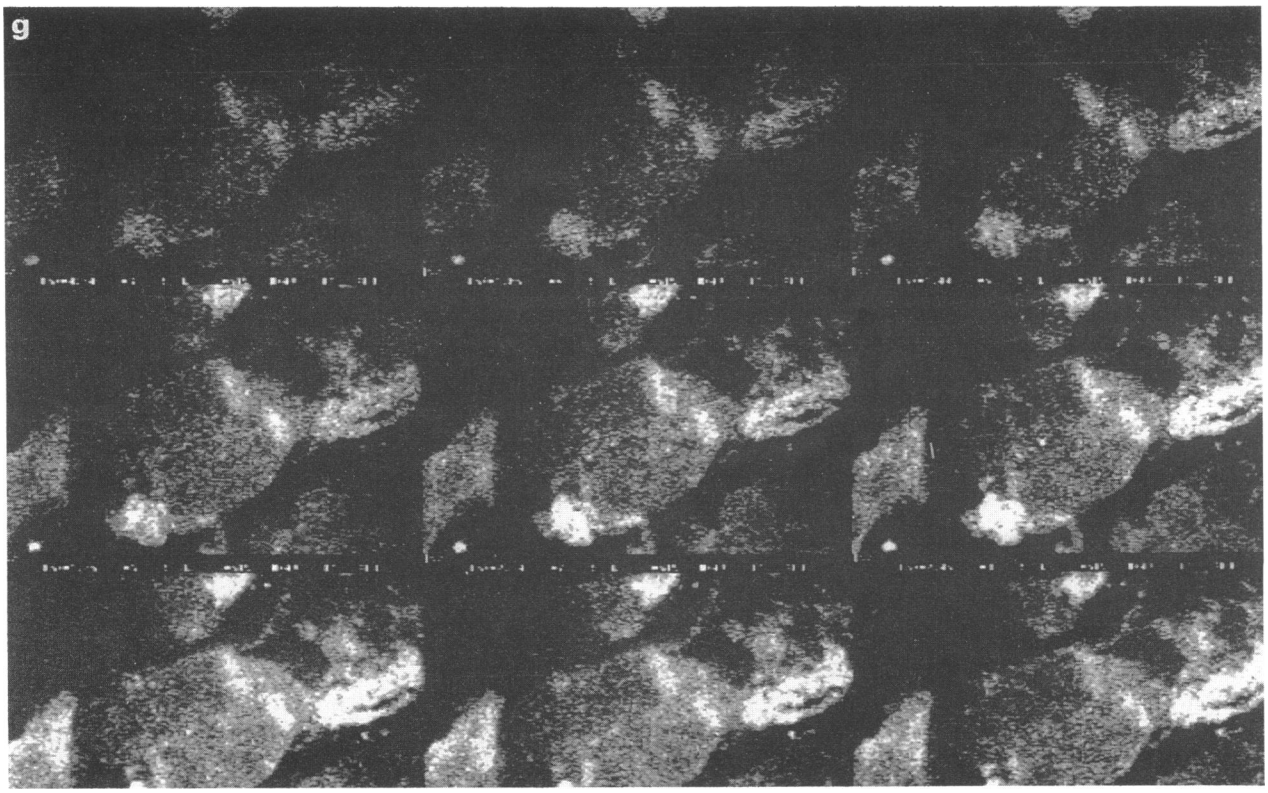

Fig. 1 (*cont.*)

normal healthy volunteers (20–50 years old) were used as a control group. None of them had asthma and their pulmonary functions were within the normal range. No subject had any respiratory tract infection during the month preceding the test. Patients were excluded from the study if they had taken drugs that may have affected the interpretation of the results. Demographic characteristics of subjects are presented in Table 1.

The study was performed after informed consent by the patients and it fulfilled all the criteria set by the Ethical Committee of the Hospital.

Table 1. Demographic characteristics of the patients

Subject	Age (years)	AAS	FEV_1	EOS1
1	39	2	100	0
2	49	3	64	2
3	30	1	102	0
4	23	1	96	0
5	25	3	94	19
6	47	2	98	27
7	21	3	66	10
8	39	1	100	0
9	49	3	52	0
Mean	36	2	86	6
s.d.	11	1	19	10

AAS, Score of severity of asthma; FEV_1, forced expiratory volume in 1 s; EOS1, percentage of eosinophils in the first bronchoalveolar lavage (BAL) recovery.

Bronchial brushing and recovery of epithelial cells

Bronchial brushings were always performed in the same manner by the same investigator [3,4] using fibreoptic bronchoscopy. The brush was introduced via the fibroscope into the subsegmental bronchi of the left lower lobe. The brushing was performed at two different sites in the lobe and at each site 10 gentle upwards and downwards strokes were made.

In this study, human bronchial epithelial cells from normal and asthmatic subjects were recovered using the same sampling procedure as previously described [3,4,14] by shaking the brush in RPMI and further centrifugation. After washing, epithelial cells were resuspended at a final density of 1×10^5 cells/ml. Viability of cells was assessed by trypan blue exclusion. Slides were prepared by cytocentrifugation (800 ***g***, 10 min) of 200 μl of the cell suspension.

Characterization of the origin of the cells

Slides were stained with May–Grünwald–Giemsa to allow identification of cell types, and with a MoAb against cytokeratin using an alkaline phosphatase anti-alkaline phosphatase (APAAP) system (Dako, Versailles, France) to assess their epithelial origin [15]. Negative controls were prepared using a mouse IgG1 MOPC cell line antibody (Organon Laboratories, West Chester, PA) as the primary antibody.

Immunostaining of epithelial cells

Cytospins were prepared immediately after fibreoptic bronchoscopy and stored at $-20°C$. The slides from both groups

were removed from the freezer exactly 5 min before staining was performed. The slides were then fixed immediately in acetone:methanol:formaldehyde (19:19:2) before performing the immunohistochemistry. The staining technique used for both groups was identical both in terms of concentration of the antibody and incubation time. The primary MoAbs were obtained from Immunotec (Luminy, France) in the case of ICAM-1 [16] and Dako [17] for HLA-DR. These precautions were taken to avoid significant redistribution of the surface molecules during the experiments or other artefacts. The specificity and the optimal concentration of MoAbs against ICAM-1 and class II antigens had been previously characterized using a positive control represented by peripheral blood mononuclear cells (PBMC) stimulated for 36 h with phytohaemagglutinin (PHA; 1 μg/ml) and interferon-gamma (IFN-γ; 300 U/ml) and fixed after cytocentrifugation.

An FITC-conjugated rabbit anti-mouse (Cappel, West Chester, PA) was used as a second antibody. Immunofluorescence was evaluated using a Zeiss laser confocal scanning laser microscope system (S.L.M., Carl Zeiss, Oberkochen, Germany), working with an argon laser at a wavelength of 488 nm, which allows identification of epithelial cells by their typical morphology either in phase contrast or in interference contrast [9–11]. In the confocal fluorescence mode, defined focus series were recorded with a $\times 63$ magnification plan neofluor objective, using the programmed operation mode with 1 μm steps along the z-axis. Each individual image was averaged and finally stored on a hard disc. We have quantified fluorescence using a photometer (MPM 200 Microscope Photometer, Zeiss) connected with a high performance system processor (MSP 20 Microscope System Processor, Zeiss). Each ciliated bronchial epithelial cell was first identified by interference contrast (Fig. 1a) and the intensity of fluorescence (Fig. 1b, c) was then evaluated by microphotometry. Three hundred cells per patient were studied. This technique limits any subjective interpretation and makes it possible to quantify the expression of molecules on a single cell. In addition, by using special software (Zeiss) capable of rapid interaction during image acquisition, image processing and final three-dimensional reconstructions from serial sections were made. Briefly, from a specimen various optical sections were obtained and automatically recorded. A single section or 3-D reconstruction of all sections recorded in the hardware can be observed on a display [9]. Another important advantage of confocal microscopy is the ability to zoom in on parts of the cell, allowing the observer to discriminate cellular structures and staining. The intensity of fluorescence was measured by the MPM 200 microphotometer on Axioplan (Zeiss) universal microscope, with allows quantification of the fluorescence of markers of specimens with sizes ranging from mm to μm. The MPM 200 microphotometer was connected to a measuring, control and data processing unit MSP 20 microscope photometer system, which assures highest measuring accuracy and a highly flexible control of all measuring processes. For each experiment, ICAM-1, HLA-DR and a negative control were examined. Results are expressed in Zeiss Units which is a light intensity measurement, related to a standard value. The software displays the results as a histogram with the number of positive cells on the abcissa and the intensity of fluorescence on the ordinate. For each experiment, ICAM-1, HLA-DR and negative control were examined. The intensity of fluorescence due to background staining was assessed by measuring the fluorescence on 300 cells of the negative control. The mean value was then subtracted from the readings obtained for the same sample stained with antibodies against HLA-DR or ICAM-1, and cells which had an intensity of fluorescence greater than the background value were considered to be positive, allowing us to compare the results obtained using both the APAAP and the immunofluorescence technique.

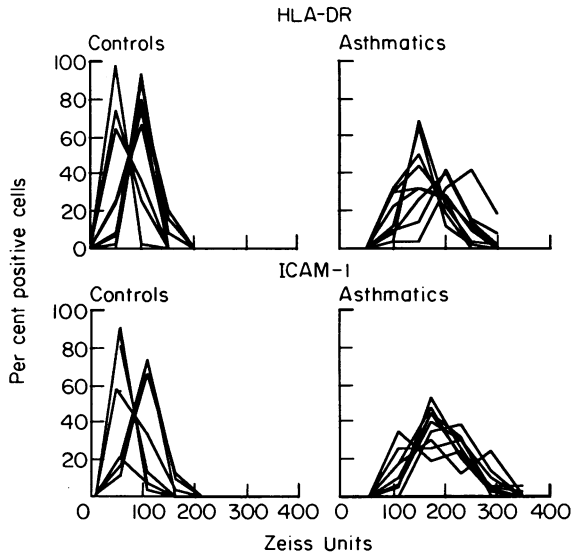

Fig. 2. Fluorescence intensity (Zeiss Units) of 300 bronchial epithelial cells stained with anti-HLA-DR and anti-ICAM-1 antibodies. Data are shown for the nine asthmatic and nine healthy subjects. Results are expressed as per cent cells at each fluorescence intensity studied.

Finally, as it has been shown that HLA-DR molecules migrate inside the cells from endoplasmic structures, at the beginning of the synthesis, to the external cytoplasmic membrane [18], we studied the localization of the HLA-DR and ICAM-1 molecules by examining single 2·4-μm sections of epithelial cells using a confocal microscope.

Statistical analysis

Results are expressed as mean or medians. Statistical differences were evaluated using non-parametric tests.

RESULTS

Characterization of epithelial cells

Epithelial cells comprised over 94% (means $\pm$ s.d. 95·3 $\pm$ 2·1%) of the cell population as shown by using an anticytokeratin antibody. The viability of these cells was always over 40% (43·7 $\pm$ 4·2%). Mononuclear cells (lymphocytes and macrophages) (3·6 $\pm$ 1·0%) and eosinophils (1·4 $\pm$ 0·9%) were the other cells recovered.

HLA-DR and ICAM-1 expression

The different morphological characteristics of epithelial cells such as the columnar shape and the ciliary structure were visualized using high-contrast resolution (Fig. 1a). In some samples, many ciliated bronchial epithelial cells were still adherent to each other and basal cells, forming cellular clusters which reproduced the *in vivo* organization of the bronchial epithelium. Fluorescence was localized principally on ciliated epithelial cells and in some cases on cellular clusters which,

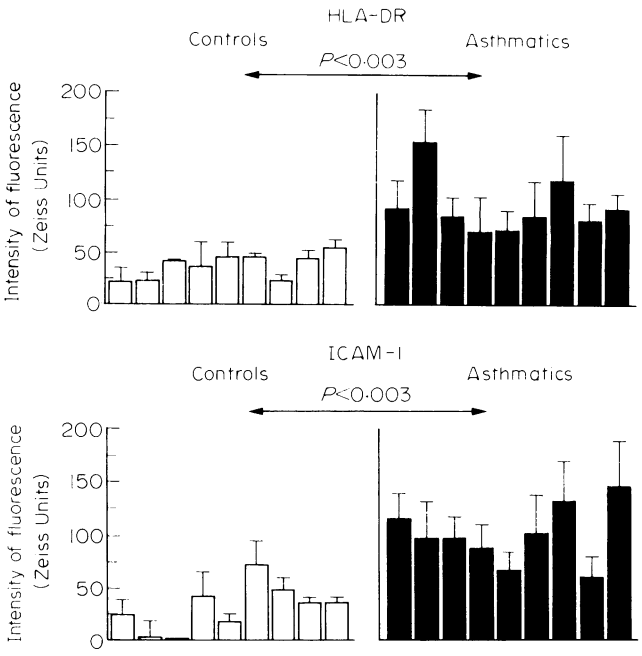

Fig. 3. Mean fluorescence intensity (Zeiss Units) of 300 bronchial epithelial cells stained with anti-HLA-DR and anti-ICAM-1 antibodies. Data are shown for the nine asthmatic and nine healthy subjects. Results are expressed as means of fluorescence intensity for each subject.

because of their morphology, might be considered as basal cells. Almost all cells from asthmatics or normal subjects expressed HLA-DR or ICAM-1.

Microphotometric studies revealed that the intensity of fluorescence due to background staining assessed by measuring the fluorescence on 300 cells of the negative control was 52 ± 4 Zeiss Units. For further analyses, the background was substracted. The intensity of expression of HLA-DR and ICAM-1 in the cells of the asthmatics was significantly ($P < 0{\cdot}003$, Mann–Whitney U-test) increased by comparison with those of controls (Figs 2 and 3). In controls a high percentage of epithelial cells displayed an intensity of fluorescence ranging between 0 and 100 Zeiss Units, whilst in asthmatics the intensity of fluorescence ranged between 50 and 200 Zeiss Units. In asthmatics there was a larger scatter of fluorescence than in controls. The percentage of epithelial cells with an intensity of fluorescence greater than the background value was significantly ($P < 0{\cdot}001$, Mann–Whitney U-test for HLA-DR and ICAM-1) increased in asthmatics compared with controls.

Fluorescence was localized all over the cells in controls, and principally on the apicolateral surfaces in asthmatics (Fig. 1b–g). This preferential localization was observed in different cellular sections of 350 nm along the z-axis, using the confocal microscope. In addition, the detection of fluorescence at a distance of 2·4 μm from the cell surface along the z-axis indicated that both HLA-DR and ICAM-1 were present in the cytoplasm in asthmatics but not in controls.

DISCUSSION

This study shows that the intensity of the expression of ICAM-1 and HLA-DR is significantly increased on bronchial epithelial cells of asthmatics compared with controls. Studies using the scanner laser confocal microscope demonstrated that, in asthmatics, these markers were selectively expressed on the apical and lateral portions of cells and localized on the cellular surface, as well as being present in the cytoplasm surrounding the nuclei.

The aim of the study was to investigate the intensity of the staining and the cell localization of HLA-DR and ICAM-1 molecules on epithelial cells with an intact morphology and preserved phenotypic characteristics. Thus, the sampling procedure used to obtain the epithelial cells was critical. Bronchial brushing was chosen because (i) it permits the obtention of epithelial cells of greater than 95% purity; (ii) it results in a dispersed population of cells, avoiding the need for enzymatic digestion which may alter membrane markers; and (iii) cells were recovered from a larger area of the bronchi than would be obtained from a single biopsy.

The expression of HLA-DR and ICAM-1 molecules was studied using the confocal microscope as it gives better contrast, and hence more cellular detail can be observed. The use of the interference contrast mode allows spatial information to be obtained through morphological data. Using conventional microscopy, the specificity of immunofluorescence is reduced when samples are thick or have a high optical density, and when cells have an irregular shape the ability to focus is reduced. These problems are often present when working with epithelial cells, since they often have an irregular shape and the sections are usually relatively thick due to the presence of mucosal secretions. In these cases contrast and resolution can be improved using a confocal fluorescence microscope [19]. In addition, a confocal microscope can scan the sample in different parallel sections throughout its depth and store the image of each plane. Using appropriate software a series of images are reconstructed in a two- or three-dimensional model [9–11,19,20]. Furthermore, the distortions to the cells, due to the cytocentrifugation, such as the flattening of the cells, the formation of thin elongations from the cell outline and the alteration of cell organelles, do not alter the interpretation of our data since we studied only the membrane and cytoplasmic distribution of the markers [21]. Thus, the artefacts which might have been induced by cytocentrifugation are not of major importance for the interpretation of our results.

We have previously shown in a larger group of patients that the percentage of epithelial cells expressing HLA-DR and ICAM-1 molecules was significantly increased in asthmas [4]. This study using a more precise system shows that the majority of epithelial cells in both groups were positive for these two molecules, but the intensity of staining was significantly greater in asthmatics. It is likely that the increased intensity of fluorescence is related to a greater number of molecules expressed on cells of asthmatic patients.

In the present study using ciliated bronchial epithelial cells, we found a different distribution of the molecules between controls and asthmatics. In the former group, the staining was diffuse over the entire cell, whilst in asthmatics a preferential apicolateral localization of the staining was observed, and this may be related to the polarization of these cells located at the interface between the external environment and the submucosa. This redistribution of adhesion molecules to the apical surface of epithelial cells may facilitate the detachment of epithelial cells from their basal layer. In addition, in asthmatics, fluorescence of HLA-DR and ICAM-1 molecules was observed in the cytoplasm in the apical portion of the cells between the nucleus and

the apical pole of the cells. This polar organization is due to the cytoskeleton which modulates the transport pathways across airways epithelial cells [22] between the luman and the submucosa, and may also determine the localization of cell markers on a specific portion of the cellular surface.

HLA-DR and ICAM-1 molecules are involved in antigen presentation [23,24], and their increased expression may be associated with the ability of bronchial epithelial cells to act as accessory cells. The apicolateral localization of these molecules enhances this possibility, since the epithelium is the first part of the bronchi coming into contact with airborne allergens recognized through HLA-DR molecules by T-cell receptors of $CD4^+$ T cells, present in the bronchial epithelium [24–26]. ICAM-1 is a cell surface adhesion glycoprotein that mediates leucocyte adhesion through interaction with the leucocyte CD11/CD18 adhesion complex present in most inflammatory cells of the airways in asthma. Expression of ICAM-1 by epithelial cells may play a role in directing leucocyte trafficking and leucocyte–epithelial cell interactions responsible for cellular recruitment and activation in inflammation [27–29]. It is possible that cell interactions between epithelial cells and eosinophils may occur, resulting in eosinophil infiltration and destruction of the epithelium caused by the release of mediators such as the eosinophil cationic protein and the major basic protein.

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