

Expression of the potent inflammatory cytokines, granulocyte-macrophage-colony-stimulating factor and interleukin-6 and interleukin-8, in bronchial epithelial cells of patients with asthma

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We have previously demonstrated that cultured human bronchial epithelial cells produce cytokines with potent proinflammatory properties on exposure to several stimuli in vitro, and we have hypothesized that these epithelial cell-derived factors may contribute to the pathogenesis of some inflammatory diseases of the bronchial mucosa, particularly asthma, by promoting the infiltration of granulocytes and T cells and their local activation. We provide, in this study, direct evidence of an increased expression of granulocyte-macrophage-colony-stimulating factor, interleukin-6, and interleukin-8 genes and proteins in bronchial epithelium from patients with symptomatic asthma. The up regulation of the production of these cytokines in bronchial epithelial cells of patients with asthma could be abolished in vitro by corticosteroids (hydrocortisone, 10^{-7} mol/L), but the up regulation also spontaneously disappeared during a period of 6 days after the removal of the cells from the diseased tissue. (J ALLERGY CLIN IMMUNOL 1992;89:1001-9.)

Key words: Airway epithelium, cytokines, inflammation, asthma

Cultured human bronchial epithelial cells produce cytokines with potent proinflammatory properties on exposure to immunologic and nonimmunologic stimuli or during the recovery from injury,¹⁻⁵ particularly GM-CSF and IL-6. Because of this ability to release factors that affect the migration of granulocytes and lymphocytes and promote their local activation,^{1, 5-7} airway epithelial cells can be viewed as cells that signal the inflammatory and immune system that penetration or disruption of the airway mucosa has oc-

Abbreviations used

GM-CSF:	Granulocyte-macrophage-colony-stimulating factor
IL:	Interleukin
MAPPING:	Message amplification phenotyping
PCR:	Polymerase chain reaction
IMDM:	Iscove's modified Dulbecco's medium
bp:	Base pair
MAb:	Monoclonal antibody

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curred, and epithelial cell-derived cytokines may play an important role in the genesis and persistence of bronchial inflammation in some pathologic conditions, like asthma.

Indeed, we have previously demonstrated that the airway secretions from patients with symptomatic asthma contain increased amounts of GM-CSF and IL-6, and these cytokines have been localized in the cytoplasm of nonciliated bronchial epithelial cells by immunospecific labeling.⁸ In addition, we have provided direct evidence of an up regulation of the expression of GM-CSF gene and protein in the bronchial epithelial cells of these patients.⁵ The GM-CSF

TABLE I. Subjects' characteristics and profile of cytokine expression and release by immunocytochemistry and ELISA

Subject No.	FEV ₁ (% pred)	PC ₂₀ M (mg/ml)	Atopy (±)	Positive cells (%)*			Cytokine release† (ng/ml/10 ⁶ cells per 48 hr)		
				GM-CSF	IL-6	IL-8	GM-CSF	IL-6	IL-8
With asthma									
1	68.0	0.03	+	55.9	36.8	40.9	2.50	1.85	2.65
2	86.4	0.22	—	29.6	10.7	29.2	0.85	0.81	1.60
3	102.3	1.12	—	18.4	9.8	0.0	0.61	0.54	<0.35
4	93.5	0.39	—	41.5	15.4	12.6	1.52	0.96	0.87
5	84.8	0.03	+	38.4	21.9	26.8	1.91	1.12	2.11
6	65.0	0.49	—	30.6	6.3	32.4	1.28	0.41	1.86
Control subjects									
7	96.4	>32	—	0.0	0.0	0.0	<0.04	<0.16	<0.35
8	91.6	>32	+	9.8	0.0	0.0	0.15	<0.16	<0.35
9	98.5	>32	—	0.0	0.0	0.0	<0.04	<0.16	<0.35
10	105.3	>32	+	12.3	0.0	0.0	0.36	<0.16	<0.35
11	101.0	>32	—	0.0	0.0	0.0	<0.04	<0.16	<0.35
12	106.3	>32	—	0.0	0.0	0.0	<0.04	<0.16	<0.35

PC₂₀M, Provocative concentration of inhaled metacholine inducing a 20% fall in FEV₁.

*Percentage of cells labeled with the monospecific antibodies against each cytokine after subtraction of nonspecific binding.

†Results obtained during the first 48 hours of cell incubation. Triplicate samples of supernatants of bronchial epithelial cells from each donor were assayed by ELISA and the results were presented as mean.

released from bronchial epithelial cells of patients with asthma could prolong the survival of eosinophils, make them hypodense, and promote the release of mediators from those cells,⁵ thereby reproducing in vitro phenomena that are known to occur in asthma and that contribute to the pathogenesis of airway hyperresponsiveness.⁹

In the present study we have evaluated further the expression of cytokines with inflammatory properties in freshly isolated bronchial epithelial cells of patients with asthma and tested the ability of corticosteroids to down regulate their production.

SUBJECTS AND METHODS

Subjects' characteristics

Six patients with asthma (four female patients, mean age $\pm$ SD, 31.2 $\pm$ 9.8 years) and six healthy volunteers (three female volunteers, mean age $\pm$ SD, 43.0 $\pm$ 15.5 years) completed the study. All the subjects were non-smokers, and two in each group demonstrated positive responses to skin prick testing with 12 common allergen extracts¹⁰ (Table I).

The patients with asthma were receiving treatment with inhaled bronchodilators alone, on demand, but their symptoms were not controlled because they were elicited by nonspecific irritating stimuli, or required large doses of bronchodilators daily, or disturbed sleep. No patient had received corticosteroids in the last month. Patients had pul-

monary function measurements and bronchoprovocation tests with methacholine¹¹ to define their nonspecific bronchial hyperresponsiveness. Their mean baseline FEV₁ ($\pm$ SD) was 83.3% $\pm$ 14.5% of predicted value, and their geometric mean provocative concentration of inhaled methacholine inducing a 20% fall in FEV₁ was 0.187 mg/ml (natural log SD, 1.5096) (Table I), the latter indicating that airway reactivity was moderately to severely increased in those patients.

Control subjects had no past history of asthma and no evidence of bronchial hyperresponsiveness (Table I). None of the subjects had taken any medication in the last 3 months.

Subjects with asthma and control subjects underwent fiberoptic bronchoscopy according to a standard protocol.⁸ Briefly, premedication was accomplished with atropine and diazepam, 200 μ g of salbutamol was administered by inhalation, and local anesthesia was obtained with 2% lidocaine. Five to eight endobronchial biopsy specimens were obtained through the bronchoscope, in randomly selected lobar and segmental bronchi of one lung, with an Olympus FB-15C (Olympus Corp., Lake Success, N.Y.) model forcep that allows the performance of very limited stripping of the bronchial epithelium.^{5, 8}

Appropriate informed consent was obtained from all the studied subjects.

Cell isolation

Bronchial epithelial cells were isolated from the bioptic specimens as previously described.^{5, 12} Briefly, the tissue

fragments were incubated for 2 hours in Hanks' balanced salt solution containing 2% dispase (Collaborative Research, Inc., Bedford, Mass.), 5 mmol/L of dithiothreitol, 1% penicillin-streptomycin solution, and 25 mmol/L of *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (all from Sigma Chemical Co., St. Louis, Mo.), pH 7.4, at 37° C, and 5% CO₂. By this isolation procedure, semidispersed clusters, consisting of at least 30 to 80 cells, were obtained with viability >95% (trypan blue exclusion method). The cells (derived from the bioptic specimens of each donor were pooled and suspended in appropriate medium. The resulting cell suspensions were depleted of dendritic cells, granulocytes, monocytes, and lymphocytes by repeated panning¹³ with the MAb against the specific markers of these cells.^{1, 2, 3, 8, 14} One sample of cells from each donor was transferred onto glass slides (1 × 10⁵ cells per slide, 6 to 10 slides) and stored at -20° C for further immunocytochemical evaluation.

The cells were characterized for the expression of keratins by immunospecific labeling, as previously described.^{1, 2, 5} Briefly, the cells were incubated for 1 hour with the MAb against cytokeratins DAKO-CK1 (Dakopatts, Glostrup, Denmark). Control slides were incubated with an irrelevant isotype-matched antibody. Binding sites were demonstrated by the immunoalkaline-phosphatase technique (DAKO alkaline phosphatase-antialkaline phosphatase kit, Dakopatts). When the cell samples were still contaminated by 1% or more nonepithelial cells after depletion procedures, samples were discarded, and these cell donors were excluded from the investigation. The small number of patients with asthma and control subjects reported in this study is the result of this selection.

Expression of cytokines by immunocytochemistry

The identification of cells containing immunoreactive GM-CSF, IL-6 and IL-8 was obtained with immunocytochemical techniques, as previously described.⁸ Replicate slides from each donor were incubated with 0.4% Triton X-100, 50 mmol/L glycine HCl, and 5% glucose in phosphate-buffered saline, to permeabilize the cells. Then, the slides were simultaneously incubated with the DAKO-CK1 or rabbit antisera against human GM-CSF and IL-6 (Genzyme Laboratories, Boston, Mass.) or a MAb against human IL-8 (Research and Diagnostic System, Minneapolis, Minn.) for 1 hour. The binding of these antibodies was demonstrated by the immunoalkaline-phosphatase technique (DAKO alkaline phosphatase-antialkaline phosphatase kit, Dakopatts). In control slides, the relevant antibodies were replaced by preimmune serum or irrelevant isotype-matched MAbs. At least 1000 nucleated cells were evaluated on coded slides, and the percentage of positive cells was calculated after subtraction of nonspecific binding.

Expression of cytokine mRNA

To detect mRNA encoding each cytokine in the epithelial cells of subjects with asthma and control subjects, we used

the MAPPING with the PCR procedure, which allows effective amplification of cDNA corresponding to the target mRNA.^{15, 17}

Total RNA was extracted from samples of 1 × 10⁵ cells by the acid guanidinium thiocyanate-phenol-chloroform extraction procedure¹⁸ and stored in ethanol at -70° C until it was assayed. MAPPING was performed by testing simultaneously the samples from all the donors at the end of the study.

One microgram of RNA was used as the template for cDNA synthesis, with reverse transcriptase in a 20 µl reaction mixture containing 50 mmol/L of Tris HCl (pH 8.3), 100 mmol/L of MgCl₂, 3 mmol/L of dithiothreitol, 1 mmol/L (each) of deoxynucleoside triphosphate, 10 µg/ml of bovine serum albumin, 10 units of ribonuclease inhibitor, 10 pmol 3' PCR primer, and 20 units of avian myeloblastosis virus reverse transcriptase (Boehringer-Mannheim, Mannheim, Germany). This mixture was incubated at 43° C for 1 hour and then denatured at 94° C for 3 minutes. Next, for amplification by PCR, 40 pmol of the 3' primer, 50 pmol of the 5' primer, 2.5 units of Taq polymerase (Biotech, Bently, Australia), 10 µl of PCR buffer (5 mmol/L of Tris HCl, 300 mmol/L of KCl, and 10 mmol/L of MgCl₂), and distilled water were added to adjust the reaction volume to 100 µl. Pre-amplified DNA (CLONTECH Laboratories, Inc., Palo Alto, Calif.) was used as positive control (1:100 dilution). One cycle of PCR consisted of three parts: denaturation at 94° C for 1 minute, annealing of the primer at 55° C for 2 minutes, and extension at 72° C for 3 minutes. This cycle was repeated 30 times on a programmable heat block (Perkin-Elmer Cetus, Norwalk, Conn.). The target DNA is supposed to be amplified 10⁵ to 10⁶ times by this procedure.^{15, 17}

PCR-amplified products were analyzed by agarose gel (2% KILORose, CLONTECH Laboratories, Inc.) electrophoresis and ethidium bromide staining. The amplified DNA bands were visualized with an ultraviolet transilluminator.

The PCR primers for use in cytokine MAPPING analysis of mRNAs were purchased from CLONTECH Laboratories, Inc. Their sequences are provided below. The β-actin primer was used to check for quantity and quality of initially sampled RNAs.

GM-CSF 3'

5' primer 5' ATGTGGCTGCAGAGCCTGCTGC 3'
3' primer 5' CTGGCTCCCAGCAGTCAAAGGG 3'

IL-6

5' primer 5' ATGAACCTCTTCTCCACAAGCGC 3'
3' primer 5' GAAGAGCCCTCAGGCTGGACTG 3'

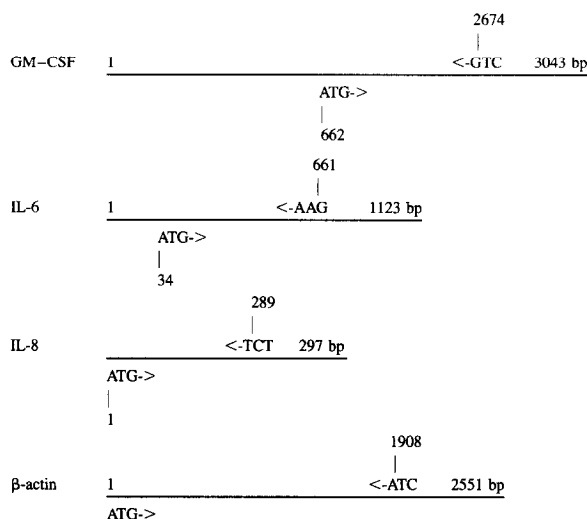
IL-8

5' primer 5' ATGACTTCCAAGCTGGCCGTGCT 3'
3' primer 5' TCTCAGCCCTCTTCAAAAACCTCTC 3'

β-actin

5' primer 5' ATGGATGATGATATCGCCGCG 3'
3' primer 5' CTAGAAGCATTTGCGGTGGGACGATGGAGGGGCC 3'

The sequence locations were



The amplified DNA sizes were 424 bp for GM-CSF, 628 bp for IL-6, 289 bp for IL-8, and 1126 bp for β -actin. The size marker used was the *fx174*/HaeIII digest.

Cytokine release and inhibitory effects of corticosteroids

To evaluate the ability of bronchial epithelial cells from patients with asthma to release GM-CSF, IL-6, and IL-8, and to test the inhibitory effect of corticosteroids, replicate samples of epithelial cells were plated at high density (10^6 cells per well) in collagen-coated 24-well culture plates and incubated for 6 days in IMDM and antibiotics without growth factors and fetal calf serum, in presence or in absence of different concentrations of hydrocortisone (Sigma Chemical Co.) (from 10^{-8} to 10^{-6} mol/L). In hormone-free medium the cells attach to the culture surface but do not grow.^{2,5} They can remain >90% viable up to 7 days of incubation if a reach medium, particularly IMDM, is used.

The supernatants were harvested at 48-hour intervals and replaced by fresh medium.

The contents of immunoreactive GM-CSF and IL-6 were measured in triplicate by ELISA with commercially available kits (Genzyme Laboratories), as previously described in detail.^{1,4,5,8} The sensitivities of these assays were 0.04 and 0.16 ng/ml, respectively, with an intra- and interassay variability <10% (coefficient of variation).

The contents of immunoreactive IL-8 were also assayed by ELISA with a double-ligand method, similar to the method published by other groups,^{19,20} with a polyclonal anti-IL-8 antiserum as capturing antibody and a biotinylated goat anti-IL-8 antibody as the revealing antibody.^{21,22} These antibodies reacted with recombinant human IL-8 in Western-blot analysis and failed to cross-react with connective tissue-activating peptide III or platelet factor 4. Recombinant human IL-8 (Genzyme Laboratories) was used as standard at one-half log dilutions. This assay consistently detected IL-8 concentrations >0.35 ng/ml in a linear fashion, with an inter- and intra-assay variability of 12.5% and 6.9%, respectively (coefficient of variations).

In all the assays reported above, fresh IMDM was used to dilute the standard GM-CSF, IL-6, and IL-8, and as blank.

Statistical analysis

Data were analyzed by the nonparametric equivalent of analysis of variance.²³ Paired results obtained within the group with asthma were analyzed by the Wilcoxon's matched-pairs test.²³ Probability values <0.05 were accepted as significant.

RESULTS

Expression of cytokines by immunocytochemistry

The isolation procedure used to purify bronchial epithelial cells allowed us to obtain a pure population of cells. The percentage of cells labeled with the anticytokeratin antibody was (mean $\pm$ SD) 99.4% $\pm$ 0.21% in the patients with asthma and 99.7% $\pm$ 0.25% in the control subjects. The proportion of ciliated and nonciliated epithelial cells was similar in the two groups, but the numbers of bioptic specimens required to obtain enough epithelial cells for each step of this study were invariably higher in the group with asthma.

The percentages of bronchial epithelial cells containing immunoreactive GM-CSF, IL-6, and IL-8 are reported in Table I. GM-CSF and IL-6 immunostaining was demonstrated in all the patients with asthma, whereas IL-8 immunostaining was observed in five patients. Few cells from the two atopic control subjects were only labeled with the antibody against GM-CSF. The cells from the other healthy control subjects did not bind any of the antibodies used to demonstrate stored cytokines.

The cellular source of cytokines was confirmed by comparing replicate slides of the cells that had been simultaneously incubated with the anticytokeratin antibody or with the monospecific antibody against each tested cytokine (Fig. 1).

Expression of cytokine mRNA

The results of cytokine MAPPING by PCR are reported in Fig. 2. The samples obtained from freshly isolated bronchial epithelial cells of patients with asthma invariably revealed amplified cDNA bands compatible with the size of PCR products for GM-CSF and IL-6 (424 and 628 bp). Products of the same size were not detected in any control sample, with the exception of the cells from the two atopic subjects (Table I) that also contained GM-CSF transcripts. PCR products of MAPPING amplimers for IL-8 appeared as bands of the appropriate size (289 bp) only in the samples of cells from five patients with asthma.

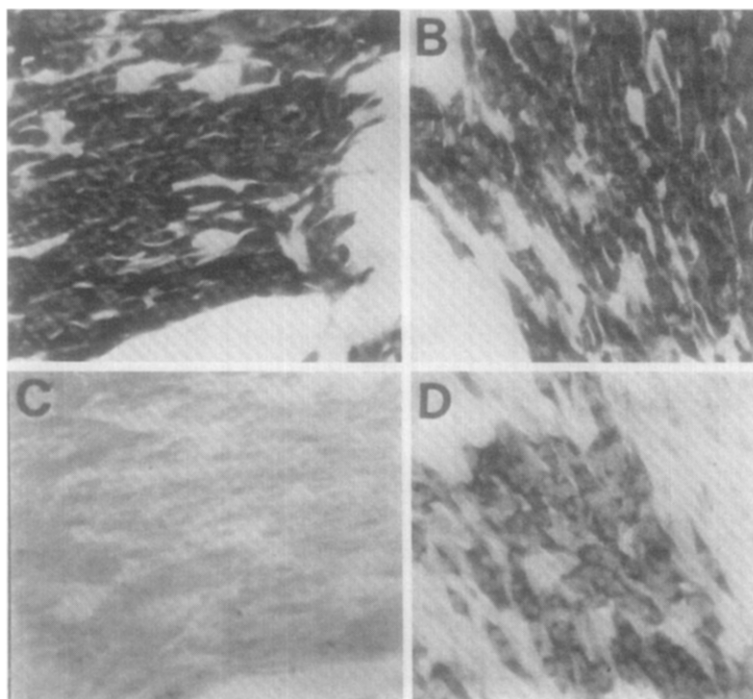


FIG. 1. Immunospecific labeling of bronchial epithelial cells isolated from one patient with asthma (**B** and **D**) and from one normal control subject (**A** and **C**). Duplicate slides from cell sample of each donor were simultaneously incubated with anticytokeratin antibody or (**A** and **B**) or with antibody against human GM-CSF (**C** and **D**). Binding of antibodies was demonstrated by the immunoalkaline-phosphatase technique. Positive cells were stained red. Counterstaining by hematoxylin. (Original magnification $\times 250$.) Most of the cells from patients with asthma that expressed keratins (true epithelial cells) also contained immunoreactive GM-CSF, whereas epithelial cells from normal subjects were negative.

MAPPING of the cells for other cytokines with proinflammatory properties, particularly IL-1 β , IL-3, IL-5, and G-CSF, revealed negative results in the patients with asthma as well as in the control subjects (data not presented).

Release of cytokines

During the first 48 hours of incubation, bronchial epithelial cells from donors with asthma released high amounts of immunoreactive GM-CSF, IL-6, and IL-8 (Table I). Only the supernatants of epithelial cells isolated from the two atopic control subjects contained measurable amounts of GM-CSF like protein. Apparently, epithelial cells from normal subjects did not produce any IL-6 or IL-8 (Table I).

The cytokine release from bronchial epithelial cells of patients with asthma spontaneously decreased during the incubation period and ceased between day 5 and day 6 (Fig. 3). For example, the contents of immunoreactive GM-CSF and IL-6 in the supernatants declined from (mean $\pm$ SD) 1.45 ± 0.69 to 0.57 ± 0.49 ng/ml/ 10^6 cells per 48 hours ($n = 6$; $p < 0.05$) and from 1.19 ± 0.46 to 0.38 ± 0.28

ng/ml/ 10^6 cells per 48 hours ($n = 4$), respectively, during a period of 4 days. In all the supernatants harvested at 6 days, the contents of immunoreactive GM-CSF, IL-6, and IL-8 were below the detection limits of the assays (Fig. 3). These results were not simply due to a significant reduction in cell viability since the percentage of viable epithelial cells (mean $\pm$ SD) was 96.5 ± 2.9 before incubation, 96.3 ± 3.3 at day 2, 95.4 ± 2.5 at day 4, and 93.4 ± 3.5 at day 6 ($p > 0.1$). Only on day 8, a progressive reduction in cell viability was observed, together with change in morphology and loss of cilia beating.

This finding indicates that the up regulation of the expression of cytokines with inflammatory properties in airway epithelium of patients with asthma is reversible and that it disappears when epithelium is removed from the diseased tissue.

Inhibitory effect of corticosteroids on cytokine release

The incubation with hydrocortisone for 48 hours of freshly isolated bronchial epithelial cells from donors

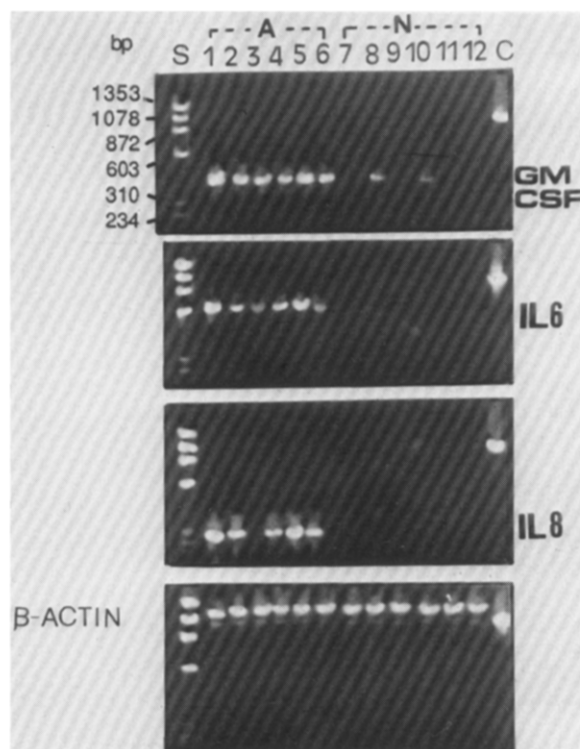


FIG. 2. MAPPING of bronchial epithelial cells from patients with asthma (*lanes 1 to 6*) and from healthy donors (*lanes 7 to 12*) with amplimer sets (CLONTECH Laboratories, Inc.) for GM-CSF, IL-6, IL-8, and β -actin (latter as control for sampling). Agarose gel (2%) electrophoresis and ethidium bromide stain. Amplified DNA bands were visualized with ultraviolet transilluminator. PCR products (cytokine MAPPING amplimers) appeared as single bands of appropriate size. S, λ 174/HaeIII digest as size marker; C, pre-amplified DNA as PCR control.

with asthma decreased the release of immunoreactive GM-CSF, IL-6, and IL-8 in a dose-dependent manner (Fig. 4). Concentrations of this compound as low as 10^{-7} mol/L completely abolished the cytokine secretion.

DISCUSSION

Our results provide direct evidence that the synthesis and release of some cytokines with inflammatory properties are up regulated in bronchial epithelium of patients with symptomatic asthma and that corticosteroids, which are the most effective drugs in inducing remission of asthmatic symptoms, also abolish this up regulation.

GM-CSF can induce the expression of adhesion molecules on endothelial and epithelial cells and can prime granulocytes to release increased amounts of mediators on stimulation,^{5,6} thereby promoting the infiltration of airway mucosa with inflammatory cells and their local activation. IL-6 induces T cell acti-

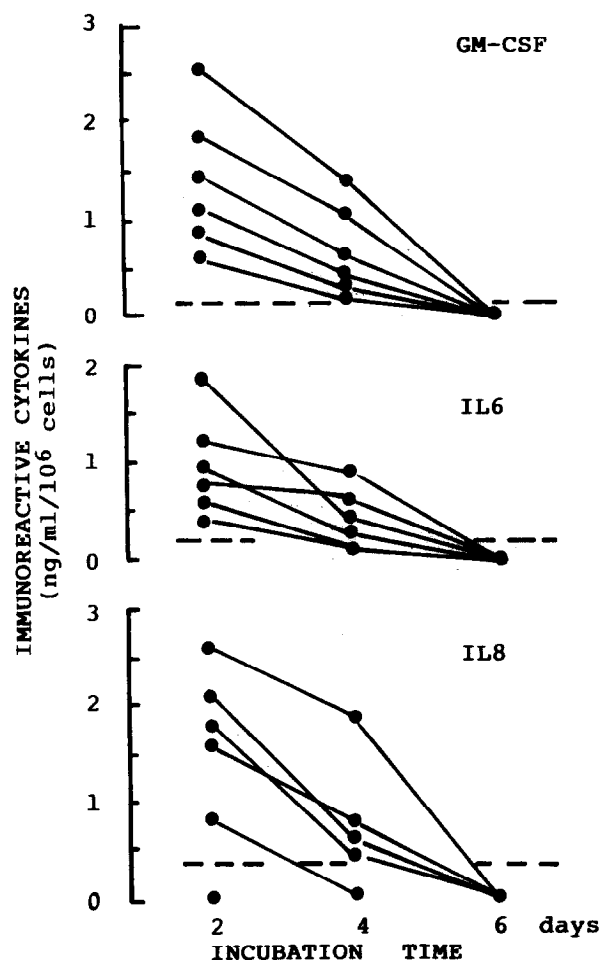


FIG. 3. Quantitation of immunoreactive GM-CSF, IL-6, and IL-8 in supernatants of bronchial epithelial cells of patients with asthma. Supernatants were harvested at days 2, 4, and 6, as indicated, and assayed by ELISA; detection limit of each assay (----).

vation and proliferation,^{1,7} and IL-8 is chemotactic for neutrophils and T cells.^{19-22,24} Since bronchial epithelium represents the first cell barrier encountered by agents known to induce asthma and airway inflammation,²⁵ we believe that the local effect of these epithelial cell-derived factors can contribute to the genesis or persistence of many pathologic alterations observed in the bronchial mucosa of patients with asthma, including eosinophil and T cell activation,^{8,26} and can make the inflammatory response in asthma different from response of any other lung disease.

More studies are needed to elucidate the mechanisms responsible for the up regulation of the expression of GM-CSF, IL-6, and IL-8 in bronchial epithelial cells from patients with asthma. We have previously demonstrated that low concentrations of

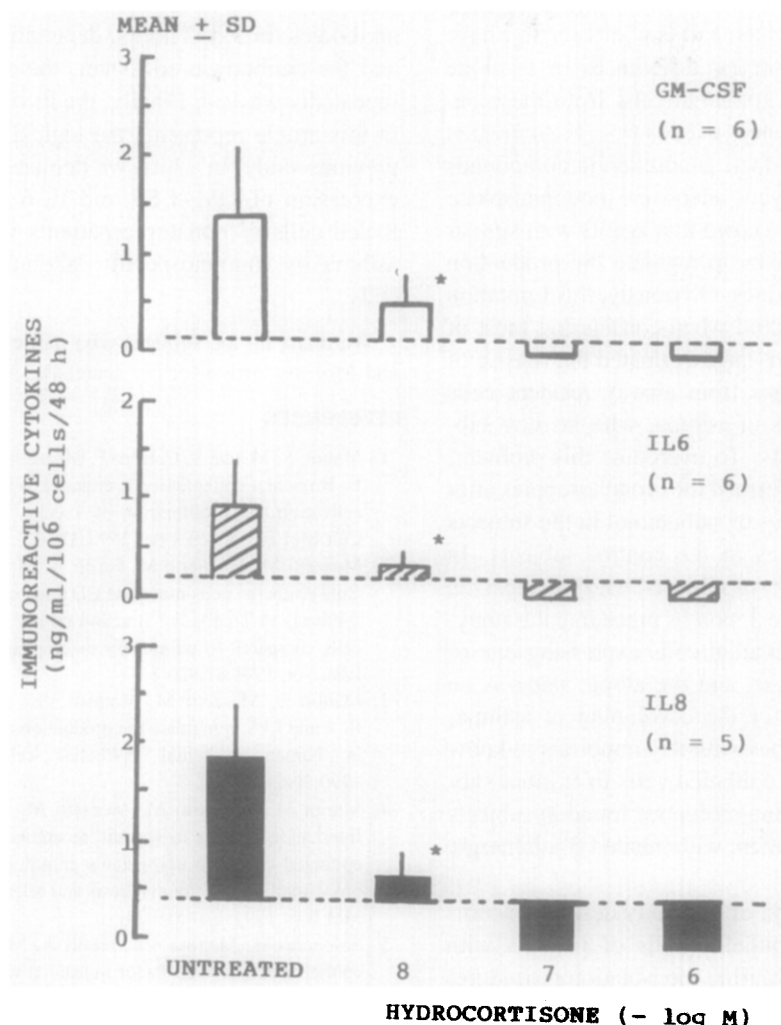


FIG. 4. Effect of different doses of hydrocortisone on release of immunoreactive GM-CSF (□), IL-6 (▨), and IL-8 (■), from bronchial epithelial cells of patients with asthma during first 48 hours of incubation after cell isolation; detection limit of each assay (----); mean amounts of immunoreactive cytokines $\pm$ SD; * $p < 0.05$ versus untreated cultures.

IL-1 α can induce a threefold to fivefold increase in the expression of GM-CSF,⁴ IL-6,³ and IL-8²² in cultured bronchial epithelial cells from normal subjects and that the loss of intercellular contacts is associated with an increase of the IL-1 receptors on those cells.⁴ Since the airway secretions of patients with symptomatic asthma do contain increased amounts of IL-1,⁸ as a result of increased local production of this cytokine by monocytes,⁸ and the integrity of airway epithelium is damaged in asthma, it is tempting to speculate that an increased release of IL-1 from airway monocytes is one of the factors that may account for the activation of bronchial epithelial cells in this disease.

Our finding that corticosteroids can inhibit the release of epithelial cell-derived cytokines is important

because these cytokines need to be released for exerting their effect, but our study does not provide any information on the mechanisms of action of hydrocortisone. In a previous study⁴ we demonstrated that this compound could reduce the expression of GM-CSF mRNA and the release of GM-CSF in normal epithelial cell cultures exposed to IL-1, suggesting that corticosteroids may have a pretranslation and a posttranslation inhibitory effect. However, the mechanism of action of corticosteroids in bronchial epithelium from patients with asthma may be different, and additional quantitative mRNA studies are required to clarify this point.

Two potential limitations of the protocol used in this study need to be mentioned.

First, the patients with asthma that we tested were

taking β -adrenergic agents, and their effects may have contributed to the observed differences in cytokine expression in isolated epithelial cells from these patients versus the differences in cells from the untreated control population. In fact, β -adrenergic compounds increase the levels of cyclic adenosine monophosphate into the cells, and it is known that agents with similar mechanism of action can up regulate the production of IL-6 and other cytokines. Obviously, this limitation must also be kept in mind when considering most of the previous studies that have evaluated the release of mediators and cytokines from airway resident cells and inflammatory cells in asthma, with healthy subjects as control subjects. To overcome this problem, at least in part, we performed the bronchoscopies after the inhalation of 200 μ g of salbutamol in the subjects with asthma as well as in the control subjects. In addition, the atopic control subjects, who had received no treatment at all in the 3 months preceding the study, did have bronchial epithelial cells expressing one of the cytokines of interest, and the atopic status is an important risk factor for the developing of asthma. Nonetheless, it would be extremely important to know whether the bronchial epithelial cells of normal subjects express the cytokine profile we found in subjects with asthma after treatment with inhaled β -adrenergic drugs.

The second limitation of our study design concerns the possibility that epithelial cells of patients with asthma were activated during the isolation procedure. It is well documented that the binding of antibodies to the surface of monocytes and T cells may result in cell activation and cytokine production.^{1, 2, 7} Since the "panning" procedure we used to eliminate nonepithelial cells involves this step¹³ and the common bioptic specimens from patients with asthma are known to contain more inflammatory cells than the specimens from normal control subjects, bronchial epithelial cells from patients with asthma may have been induced to express GM-CSF, IL-6, and IL-8 by the factors released from nonepithelial cells. However, it is unlikely that this occurred in the present investigation because the method used to perform endo-bronchial biopsies allowed us to obtain only limited strippings of bronchial epithelium, and in such specimens, nonepithelial cells were very low in number, ranging from 1.9% to 5.5% (mostly eosinophils) in patients with asthma and from 0% to 1.2% in normal control subjects. In addition, the release of cytokines from monocytes or T cells after antibody-mediated stimulation requires at least 4 hours to occur and does occur only when other factors are present,^{1, 2, 7} whereas the elimination of contaminating cells by panning involves the incubation of the cells with the relevant

antibodies for 1 or 2 hours, depending on the cell type and the antibody used. Then, the unbound cells are repeatedly washed. Finally, the in vitro study reported in this article represents the logical extension of one previous study⁸ in which we demonstrated the in vivo expression of GM-CSF and IL-6 in bronchial epithelial cells of nonatopic patients with symptomatic asthma by immunospecific labeling of unseparated cells.

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