

ORIGINAL ARTICLE

# Dysfunctional Interaction of C/EBP $\alpha$ and the Glucocorticoid Receptor in Asthmatic Bronchial Smooth-Muscle Cells

Michael Roth, Ph.D., Peter R.A. Johnson, Ph.D., Peter Borger, Ph.D., Michel P. Bihl, Ph.D., Jochen J. Rüdiger, M.D., Gregory G. King, M.D., Qi Ge, M.Sc., Katrin Hostettler, M.D., Janette K. Burgess, Ph.D., Judith L. Black, M.B., B.S., Ph.D., and Michael Tamm, M.D.

## ABSTRACT

### BACKGROUND

From the Department of Pharmacology and the Woolcock Institute of Medical Research, University of Sydney, Sydney, Australia (M.R., P.R.A.J., P.B., G.G.K., Q.G., K.H., J.K.B., J.L.B.); and the Departments of Research and Internal Medicine, Pulmonary Cell Research, University Hospitals Basel, Basel, Switzerland (M.R., M.P.B., J.J.R., M.T.).

Drs. Johnson, Borger, Black, and Tamm contributed equally to this article.

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Increased proliferation of bronchial smooth-muscle cells may lead to increased muscle mass in the airways of patients with asthma. The antiproliferative effect of glucocorticoids in bronchial smooth-muscle cells in subjects without asthma is mediated by a complex of the glucocorticoid receptor and the CCAAT/enhancer binding protein  $\alpha$  (C/EBP $\alpha$ ). We examined the signaling pathway controlling the inhibitory effect of glucocorticoids on cell proliferation and interleukin-6 synthesis in bronchial smooth-muscle cells of subjects with asthma and those without asthma.

### METHODS

Lines of bronchial smooth-muscle cells were established from cells from 20 subjects with asthma, 8 subjects with emphysema, and 26 control subjects. Cell proliferation was determined by means of cell counts and [ $^3$ H]thymidine incorporation. Signal transduction was studied by means of an electrophoretic DNA mobility-shift assay, a super-shift electrophoretic-mobility assay, immunoblotting, use of C/EBP $\alpha$  antisense oligonucleotides, and use of a human C/EBP $\alpha$  expression vector. Interleukin-6 release was determined by means of an enzyme-linked immunosorbent assay.

### RESULTS

Glucocorticoids activated the glucocorticoid receptor and inhibited serum-induced secretion of interleukin-6 in bronchial smooth-muscle cells from both subjects with asthma and those without asthma; however, glucocorticoids inhibited proliferation only in bronchial smooth-muscle cells from subjects without asthma. C/EBP $\alpha$  protein was detected by immunoblotting in all bronchial smooth-muscle cells from subjects without asthma but not in those with asthma, whereas the protein was expressed in lymphocytes from both groups of subjects. C/EBP $\alpha$  antisense oligonucleotides or the glucocorticoid-receptor inhibitor mifepristone reversed the antiproliferative effect of glucocorticoids in bronchial smooth-muscle cells from subjects without asthma. When bronchial smooth-muscle cells from subjects with asthma were transiently transfected with an expression vector for human C/EBP $\alpha$ , two forms of the protein were expressed, and subsequent administration of glucocorticoids inhibited cell proliferation.

### CONCLUSIONS

We hypothesize that a cell-type-specific absence of C/EBP $\alpha$  is responsible for the enhanced proliferation of bronchial smooth-muscle cells derived from subjects with asthma and that it explains the failure of glucocorticoids to inhibit proliferation in vitro.

**A**STHMA IS CHARACTERIZED PATHOLOGICALLY by an increased mass of bronchial smooth muscle,<sup>1,2</sup> apparently as a result of the enhanced proliferation of bronchial smooth-muscle cells.<sup>3,4</sup> Bronchial smooth-muscle cells are thus considered a major target for antiasthma treatments such as glucocorticoids.<sup>5</sup>

Glucocorticoids down-regulate the secretion of several inflammatory cytokines by various types of cells resident in the human lung,<sup>6-9</sup> and this process is thought to be mediated by the ligand-activated glucocorticoid receptor.<sup>9-11</sup> On activation, the glucocorticoid receptor migrates to the nucleus, where it binds to its specific DNA consensus sequence, the glucocorticoid-responsive element,<sup>9-11</sup> or interacts with other transcription factors.<sup>10,12,13</sup>

CCAAT/enhancer-binding proteins (C/EBPs) are involved in cell proliferation. The activation of C/EBP $\alpha$  and C/EBP $\epsilon$ , which are antiproliferative transcription factors, leads to cell differentiation.<sup>14-17</sup> In contrast, C/EBP $\beta$  and C/EBP $\delta$  seem to drive cell-cycle progression.<sup>16-19</sup> C/EBP $\gamma$  forms complexes with C/EBP $\alpha$  and C/EBP $\beta$ , thereby modulating their function.<sup>17</sup> C/EBP $\zeta$  is a relatively recently identified isoform with various synonyms (e.g., Ig/EBP and Gadd153) and may play a specific role in the function of the endoplasmic reticulum.<sup>17,20</sup>

Glucocorticoids down-regulate the proliferation of airway smooth-muscle cells<sup>21</sup> by decreasing the expression of cyclin D1 and the phosphorylation of retinoblastoma protein<sup>22</sup> and by activating p21<sup>(Waf1/Cip1)</sup>.<sup>23</sup> In human-lung fibroblasts and pulmonary vascular and bronchial smooth-muscle cells from patients without asthma, glucocorticoid treatment rapidly induces the activation of glucocorticoid receptors and C/EBP $\alpha$ , and subsequently the expression of the p21<sup>(Waf1/Cip1)</sup> gene is faster than predicted on the basis of animal models.<sup>23-28</sup> Furthermore, transcriptional activation of the p21<sup>(Waf1/Cip1)</sup> gene by glucocorticoids does not require the glucocorticoid-receptor-mediated synthesis of C/EBP $\alpha$  in lung fibroblasts, pulmonary vascular smooth-muscle cells, or bronchial smooth-muscle cells.<sup>25,26</sup> Because of the importance of glucocorticoid treatment in asthma, we investigated whether the pathways outlined above are operative in bronchial smooth-muscle cells obtained from subjects with asthma. Furthermore, we assessed whether the modulation of cytokine release by glucocorticoids involves the same signaling cas-

cade in bronchial smooth-muscle cells from subjects with asthma as in those from subjects without asthma.

## METHODS

### CELL CULTURE

Primary cultures of bronchial smooth-muscle cells were established from airway-muscle bundles obtained from 26 control subjects (14 who were undergoing lung resection for a tumor, 4 who were undergoing transplantation for cystic fibrosis, and 8 who were healthy), 8 subjects with emphysema, and 20 subjects with asthma. Cells were grown as described previously in Dulbecco's minimal essential medium containing 10 percent fetal-calf serum, a 1 percent minimal essential medium-vitamin mix, and 20 mM HEPES.<sup>3</sup> The characteristics of the subjects are summarized in Table 1. All experiments were performed between passages 3 and 6. In addition, peripheral-blood lymphocytes were isolated from 10 ml of blood from four subjects with asthma and four controls according to standard procedures with the use of a Ficoll gradient.<sup>25</sup>

Approval for the experiments was provided by the human ethics committee of the University of Sydney and by the Central Sydney Area Health Service. All patients provided written informed consent.

### CELL COUNTING AND DNA SYNTHESIS

Cells were seeded at a density of 10<sup>4</sup> cells per square centimeter in 24-well cell-culture plates in growth medium (Dulbecco's minimal essential medium containing 5 percent fetal-calf serum) for 24 hours to allow adherence. The medium was replaced by low-serum medium (Dulbecco's minimal essential medium containing 0.1 percent fetal-calf serum) for one day and then exchanged with growth medium, with or without a single or repeated doses of budesonide, dexamethasone, prednisolone, or hydrocortisone (10<sup>-12</sup> to 10<sup>-8</sup> M). Cell proliferation was assessed after three and five days of treatment with 5 percent fetal-calf serum; cells were washed twice with ice-cold phosphate-buffered saline and then treated with trypsin and EDTA (1 mM and 0.05 percent, respectively) for one minute. The trypsin solution was removed, and the cells were resuspended in 0.5 ml of phosphate-buffered saline. Cells contained in 0.01 ml of phosphate-buffered saline were counted in duplicate for each well with the use of a Neubauer improved hemocytometer.<sup>3,25</sup>

The incorporation of [<sup>3</sup>H]thymidine was mea-

**Table 1. Characteristics of the 20 Subjects with Asthma, the 8 Subjects with Emphysema, and the 26 Controls.\***

Group and Sex	Diagnosis or Health Status	Age yr	FEV <sub>1</sub> percent	Drugs†
<b>Subjects with asthma</b>				
Male	Asthma	33	76	Glucocorticoid, β <sub>2</sub> -agonist
Male	Status asthmaticus	15	NA	NA
Male	Asthma	50	NA	NA
Male	Asthma	52	58	NA
Female	Asthma	37	67	Glucocorticoid, β <sub>2</sub> -agonist
Male	Asthma	78	75	Glucocorticoid
Male	Asthma	80	NA	Glucocorticoid, β <sub>2</sub> -agonist
Female	Asthma	44	109	Glucocorticoid, β <sub>2</sub> -agonist
Male	Asthma	33	NA	NA
Male	Asthma	58	92	Glucocorticoid, β <sub>2</sub> -agonist
Male	Asthma	46	60	Glucocorticoid, β <sub>2</sub> -agonist
Female	Asthma	30	64	Glucocorticoid, β <sub>2</sub> -agonist
Female	Asthma	42	58	Glucocorticoid, β <sub>2</sub> -agonist
Male	Asthma	25	79	Glucocorticoid, β <sub>2</sub> -agonist
Female	Asthma	49	84	Glucocorticoid, β <sub>2</sub> -agonist
Female	Asthma	33	NA	NA
Female	Asthma	43	85	None
Male	Asthma	40	47	Glucocorticoid, β <sub>2</sub> -agonist
Female	Asthma	20	75	Glucocorticoid, β <sub>2</sub> -agonist
Female	Asthma	18	82	None
Mean ±SD		41.3±17.3	74.1±15.7	
<b>Subjects with emphysema</b>				
Female	Emphysema	52	NA	NA
Male	Emphysema	57	NA	NA
Female	Emphysema	56	NA	NA
Male	Emphysema	55	NA	NA
Male	Emphysema	44	NA	NA
Female	Emphysema	52	NA	NA
Male	Emphysema	61	100	NA
Male	Emphysema	48	NA	NA
Mean ±SD		53.1±5.4		

sured according to standard protocols. Cells were prepared as they were for cell counts but were cultured for 24 hours only after stimulation and the addition of 5 μCi of [<sup>3</sup>H]thymidine per milliliter during the last 6 hours of culture. Thymidine incorporation was determined by liquid scintillation, with counting performed in triplicate.<sup>25,26,28</sup>

**TREATMENT**

Mifepristone (10<sup>-7</sup> or 10<sup>-6</sup> M) was used to inhibit glucocorticoid-receptor-dependent cell responses.

Sulfonated antisense C/EBPα oligonucleotide (5'-AAGGCCGCGGCGCTGCTGGGCGCGT3', MWG Biotech) was used to down-regulate C/EBPα protein,<sup>17,18,25</sup> and a scrambled sulfonated oligonucleotide (5'-AGCTCGGATGCATGGAGGAG3', MWG Biotech) was used as a negative control.

**C/EBPα EXPRESSION VECTOR**

The C/EBPα expression vector was constructed from the total RNA of human peripheral-blood lymphocytes with the use of RNeasy (Qiagen). One mi-

**Table 1. (Continued.)**

Group and Sex	Diagnosis or Health Status	Age yr	FEV <sub>1</sub> percent	Drugs†
<b>Controls</b>				
Male	Tumor	59	90	NA
Female	Tumor	47	NA	NA
Female	Tumor	73	66	NA
Male	Healthy	28	100	None
Male	Healthy	52	NA	None
Female	Healthy	61	98	None
Female	Cystic fibrosis	24	NA	NA
Male	Healthy	43	NA	None
Male	Tumor	62	94	NA
Male	Healthy	36	87	None
Male	Healthy	57	NA	None
Male	Cystic fibrosis	34	NA	NA
Female	Tumor	56	NA	None
Male	Tumor	66	60	None
Male	Cystic fibrosis	50	NA	NA
Female	Tumor	76	NA	None
Male	Tumor	58	95	None
Male	Healthy	53	100	None
Female	Healthy	21	NA	None
Male	Cystic fibrosis	25	NA	NA
Male	Tumor	73	NA	None
Male	Tumor	66	NA	None
Male	Tumor	74	130	None
Male	Tumor	57	79	Glucocorticoid, $\beta_2$ -agonist
Male	Tumor	69	90	None
Male	Tumor	78	90	None
Mean $\pm$ SE		53.8 $\pm$ 17.8	92.1 $\pm$ 16.8	

\* FEV<sub>1</sub> denotes forced expiratory volume in one second as a percentage of the predicted value, and NA not available.

† The drugs are those that were being taken within six weeks before tissue sampling.

rogram of total RNA was reverse-transcribed by means of SuperScript II (Invitrogen), and the entire coding sequence of C/EBP $\alpha$  was amplified with Pfx polymerase enzyme (Invitrogen) by means of the polymerase chain reaction (PCR) under the following conditions: primary denaturation at 98°C for 2 minutes was followed by 55 cycles of denaturation at 98°C for 30 seconds, annealing at 61°C for 30 seconds, elongation at 68°C for 100 seconds, and elongation at 68°C for 7 minutes. The C/EBP $\alpha$  primers were 5'CACCATGGAGTCGGCCGACT-TCT3' and 3'TCACGCGCAGTTGCCCATG5'. After electrophoresis on a 5 percent agarose gel, the C/EBP $\alpha$  DNA band was isolated with the use of Nu-

cleoSpin extract (Macherey-Nagel) and was cloned into a pVDNA3.1D/V5-His6-TOPO vector (Invitrogen) by mixing 4  $\mu$ l of CEBP $\alpha$  DNA extract with 1  $\mu$ l of salt solution (1.2 M sodium chloride and 0.06 M magnesium chloride) and 1  $\mu$ l of the TOPO vector. The mixture was incubated for five minutes at room temperature, and 2  $\mu$ l of C/EBP $\alpha$  vector was transfected into *Escherichia coli*. The success of transfection was confirmed on the basis of the response to ampicillin. Transfected cells were assessed for C/EBP $\alpha$  DNA, which was sequenced with the use of an automated sequencer (MWG Biotech).

Cells were transiently transfected with the C/EBP $\alpha$  expression vector or a green fluorescent

protein vector for one hour in serum-free medium containing 1.0 ng of either vector per microliter in Tfx50 reagent (Promega) (ratio of Tfx50 to DNA, 3:1). Transfected cells were then incubated in growth medium for 24 hours in the presence or absence of drugs. The transfection rate was measured in transiently transfected bronchial smooth-muscle cells with the use of a green fluorescent protein vector — that is, a cytomegalovirus-epidermal growth factor promoter-driven vector (p-d2EGFP-N1, Clontech). Transfection was carried out with the use of 1  $\mu$ g of DNA per milliliter and Tfx50 (ratio, 1:2 to 1:4) for one hour in serum-free medium. Five percent fetal-calf serum was then added to the medium for 24 hours before confocal microscopy of the living cells was performed (model LSM 510, Zeiss). The laser excitation wavelength was 488 nm; fluorescence was detected between 505 and 550 nm with the use of a pinhole aperture of 106  $\mu$ m. Autofluorescence was not detected between 560 and 615 nm with the use of a wavelength of 543 nm.

#### NUCLEAR AND CYTOSOLIC EXTRACTS AND ELECTROPHORETIC MOBILITY-SHIFT ASSAY

Nuclear and cytosolic extracts were prepared from cultures that were 80 percent confluent, as described previously.<sup>25</sup> Bronchial smooth-muscle cells were scraped off 175-cm<sup>2</sup> flasks and centrifuged at 5000 $\times$ g for 2 minutes at 4°C, and the pellet was resuspended in 80  $\mu$ l of low-salt buffer (20 mM HEPES [pH 7.9], 10 mM potassium chloride, 0.1 mM sodium vanadate, 1 mM EDTA, 1 mM EGTA, 0.2 percent NP-40, 10 percent glycerol, and complete protease inhibitor [Roche Diagnostics]) and incubated for 10 minutes at 4°C. After centrifugation at 13,000 $\times$ g for one minute at 4°C, the supernatant (the cytosolic fraction) was collected. The remaining pellet was dissolved in 50  $\mu$ l of high-salt buffer (420 mM sodium chloride, 20 mM HEPES at pH 7.9, 10 mM potassium chloride, 0.1 mM sodium vanadate, 1 mM EDTA, 1 mM EGTA, 20 percent glycerol, and complete protease inhibitor), kept on ice for 30 minutes, and centrifuged at 13,000 $\times$ g for 5 minutes at 4°C. The supernatant collected was considered the nuclear fraction.<sup>25,26,28</sup> The protein level was determined with the use of the Bradford assay.

#### IMMUNOBLOTTING

Proteins were fractionated according to size, as follows: 5  $\mu$ g of total protein was applied to a polyacrylamide-sodium dodecyl sulfate gel with a gra-

dient of 4 to 15 percent and transferred to polyvinylidene fluoride membranes (Millipore) according to standard protocols; transfer was confirmed by Ponceau's staining.<sup>17,25</sup> Membranes were blocked by the addition of 5 percent skim milk in 10 mM TRIS, 150 mM sodium chloride, and 0.05 percent Tween 20 for 1 hour; incubated for 12 hours at 4°C with a primary monoclonal antibody specific for the glucocorticoid receptor (sc-100X), C/EBP $\alpha$  (sc-61X), or c-Fos/activator protein-1 (AP-1) (sc-253X) (all Santa Cruz Biotechnology); washed three times in blocking solution; and then incubated with a secondary antirabbit antibody conjugated with horseradish peroxidase (sc-2054, Santa Cruz Biotechnology) for 1 hour. Membranes were washed three times with blocking buffer and once with phosphate-buffered saline, and protein bands were visualized by means of chemiluminescence (ECL, Pierce).

DNA-binding proteins were characterized by means of an electrophoretic mobility-shift assay with the use of a <sup>32</sup>P-labeled glucocorticoid-responsive element or CCAAT-binding oligonucleotide (sc-2545 and sc-2525, respectively; Santa Cruz Biotechnology) as described previously.<sup>25</sup> We also used a <sup>32</sup>P-labeled p21<sup>(Waf1/Cip1)</sup> promoter fragment containing the C/EBP-binding sites (a gift from Dr. B. Vogelstein, Johns Hopkins University, Baltimore),<sup>29</sup> which has been used by others to characterize the glucocorticoid receptor-C/EBP $\alpha$  DNA consensus sequence of the p21<sup>(Waf1/Cip1)</sup> promoter,<sup>23,24,27</sup> and which we have previously used to show the formation of the glucocorticoid receptor-C/EBP $\alpha$  complex.<sup>25</sup> Supershift assays (defined by a mobility shift of the p21 promoter fragment) were performed by incubating nuclear extracts with one of the above antibodies (1 mg per milliliter) specific for the glucocorticoid receptor, C/EBP $\alpha$ , or the transcription factor AP-1 overnight at 4°C; DNA-protein complexes and supershifts were analyzed on a nondenaturing 4 percent polyacrylamide gel exposed to x-ray films. The specificity of the observed DNA-protein complexes was characterized by preincubating the extracts with 10 times the normal amount of competitive, unlabeled glucocorticoid-responsive element or CCAAT oligonucleotides.<sup>25,26,28</sup>

#### COIMMUNOPRECIPITATION

**OF THE GLUCOCORTICOID RECEPTOR OR C/EBP- $\alpha$**  Coimmunoprecipitations were performed as described previously.<sup>25</sup> Extracts of nuclear protein

(20  $\mu$ l) were incubated overnight at 4°C with antibody against C/EBP $\alpha$  (1  $\mu$ g per milliliter) or against glucocorticoid receptor (1  $\mu$ g per milliliter, Santa Cruz) and then incubated for one hour with protein A Sepharose (1 mg per milliliter) in phosphate-buffered saline at 37°C. After centrifugation at 5000 $\times$ g for five minutes, the precipitate was washed twice with 2 ml of phosphate-buffered saline and then resuspended in Laemmli buffer and analyzed by means of immunoblotting.<sup>25</sup>

#### INTERLEUKIN-6 ENZYME-LINKED IMMUNOSORBENT ASSAY

Cell-culture medium was collected 0, 6, 12, and 24 hours after stimulation of cells with 5 percent fetal-calf serum in the presence or absence of drugs or antisense oligonucleotides. The release of interleukin-6 was detected by means of an enzyme-linked immunosorbent assay (Quantikine, R&D Systems).

#### STATISTICAL ANALYSIS

Analysis of variance and an unpaired Student's t-test were used to assess the data for cell counts and [<sup>3</sup>H]thymidine incorporation. Results were considered significant if the two-sided P value was less than 0.05.

### RESULTS

The clinical characteristics of all 54 study subjects from whom we obtained cells for bronchial smooth-muscle cell cultures are shown in Table 1. There were 35 males and 19 females. Owing to the wide range of ages among subjects with asthma, the mean age of this group did not differ significantly from that of the other two groups (Table 1).

Figure 1A shows a representative time course of budesonide-induced ( $10^{-8}$  M) translocation of glucocorticoid receptor from the cytosol to the nucleus in bronchial smooth-muscle cell lines from subjects with asthma and those with emphysema. Budesonide activated the glucocorticoid receptor within 30 minutes, with levels peaking at 1 hour and declining thereafter. Similar time courses were observed for dexamethasone, prednisolone, and hydrocortisone at concentrations ranging from  $10^{-9}$  to  $10^{-6}$  M. As summarized in Figure 1B, the time course of the activation of the glucocorticoid receptor by budesonide was similar among bronchial smooth-muscle cell lines from 10 controls, 8 subjects with emphysema, and 12 subjects with asthma, and the time course of the accumulation of

glucocorticoid receptors in the nucleus was similar for all four glucocorticoids, with no significant differences in efficacy (data not shown). A second, minor band appeared soon after glucocorticoid treatment (Fig. 1A). This band may represent the  $\beta$  isoform of the glucocorticoid receptor but represents only a small proportion of the total.

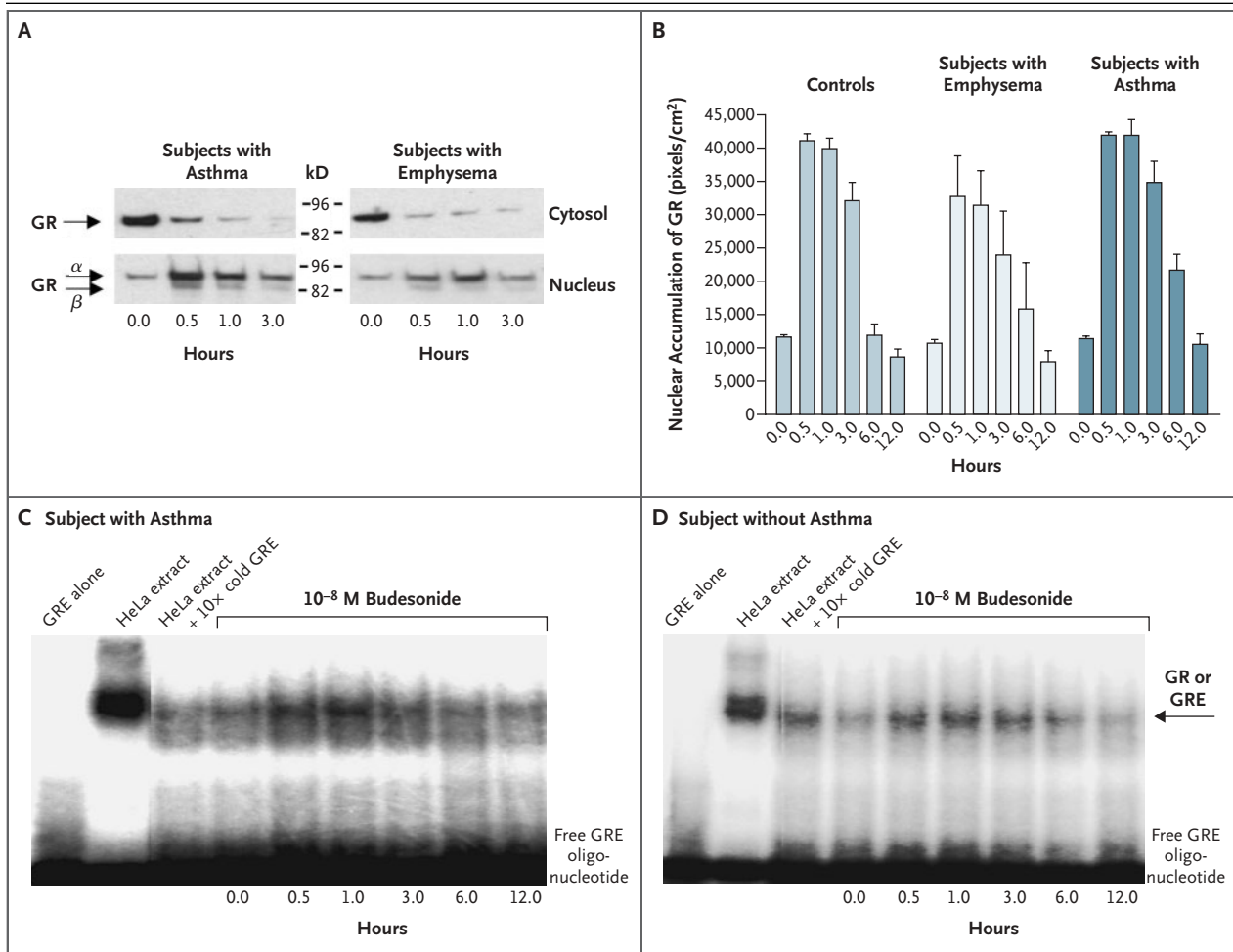
The activation of the glucocorticoid receptor by glucocorticoids and its binding to a glucocorticoid-responsive element oligonucleotide were confirmed by electrophoretic mobility-shift assays in bronchial smooth-muscle cells from subjects with asthma (Fig. 1C) and those without asthma (Fig. 1D). Similar results were obtained in all other lines of bronchial smooth-muscle cells (six asthma cell lines and six nonasthma cell lines).

To determine the antiinflammatory effect of glucocorticoids, we analyzed the cell-culture medium of the above experiments for the presence of interleukin-6. After adjustment for the cell numbers, the level of secretion of interleukin-6 induced by the addition of 5 percent fetal-calf serum after 24 and 48 hours was similar between bronchial smooth-muscle cells from 10 subjects with asthma and bronchial smooth-muscle cells from 10 subjects without asthma (Fig. 2A). All glucocorticoids significantly inhibited the serum-induced secretion of interleukin-6 ( $P < 0.001$ ).

To characterize the effect of the inhibitory signaling pathway of glucocorticoids on the secretion of interleukin-6, we treated cells with either the glucocorticoid-receptor inhibitor mifepristone ( $10^{-7}$  M) or a C/EBP $\alpha$  antisense or sense oligonucleotide. The addition of 1.0  $\mu$ M C/EBP $\alpha$  antisense oligonucleotide resulted within 24 hours in the down-regulation of serum-induced C/EBP $\alpha$  protein expression in total cell-protein extracts in bronchial smooth-muscle cells from subjects without asthma (Fig. 2B).

When cells were pretreated for one hour with  $10^{-7}$  M mifepristone, the inhibitory effect of the glucocorticoids on the secretion of interleukin-6 was substantially reduced (Fig. 2C). However, preincubation for 24 hours with 1  $\mu$ M C/EBP $\alpha$  antisense oligonucleotides did not significantly alter the inhibitory effect of the glucocorticoids on the serum-induced secretion of interleukin-6.

Previously, we reported that a single dose of  $10^{-8}$  M budesonide led to a mean ( $\pm$ SE) maximal decrease of  $21 \pm 4$  percent in the serum-induced proliferation of bronchial smooth-muscle cells from subjects without asthma.<sup>26</sup> In the present study, we



**Figure 1. Immunoblotting, Densitometric Analysis, and Electrophoretic Mobility-Shift Assays in Bronchial Smooth-Muscle Cells from Subjects with Asthma and Those without Asthma.**

Panel A shows a representative immunoblot of the distribution of the glucocorticoid receptor (GR) in the cytosol and the nucleus of bronchial smooth-muscle cells from a subject with asthma and a subject with emphysema in the presence of 10<sup>-8</sup> M budesonide.  $\alpha$  and  $\beta$  denote the  $\alpha$  and  $\beta$  isoforms of the GR. Panel B shows a densitometric analysis of the nuclear location of the GR over time in the presence of 10<sup>-8</sup> M budesonide in bronchial smooth-muscle cells from 10 controls, 8 subjects with emphysema, and 12 subjects with asthma. Bars represent the mean  $\pm$  SE. Panels C and D show the time course of budesonide-induced binding of the GR to the glucocorticoid-responsive element (GRE) in a representative electrophoretic mobility-shift assay of bronchial smooth-muscle cells from one subject with asthma and one without asthma, respectively.

observed a similar inhibition of fetal-calf serum-stimulated cell proliferation after three days of culture in the presence of any one of the four glucocorticoids studied after a single treatment on day 1 (Fig. 3A). In bronchial smooth-muscle cells from 10 subjects, 10<sup>-8</sup> M budesonide reduced the serum-dependent proliferation of cells by 28 $\pm$ 4 percent ( $P < 0.01$  by an unpaired Student's *t*-test), 10<sup>-8</sup> M dexamethasone reduced proliferation by 21 $\pm$ 4 percent ( $P < 0.01$ ), and 10<sup>-8</sup> M prednisolone reduced proliferation by 24 $\pm$ 3 percent ( $P < 0.01$ ),

whereas 10<sup>-8</sup> M hydrocortisone did not inhibit proliferation significantly (Fig. 3A). A comparison of Figures 3A and 3B shows that bronchial smooth-muscle cells from 15 subjects with asthma grew about 1.5 times as fast as bronchial smooth-muscle cells from subjects without asthma, a finding that is similar to our previous findings.<sup>3</sup> Surprisingly, none of the glucocorticoids inhibited the proliferation of bronchial smooth-muscle cells from subjects with asthma (Fig. 3B).

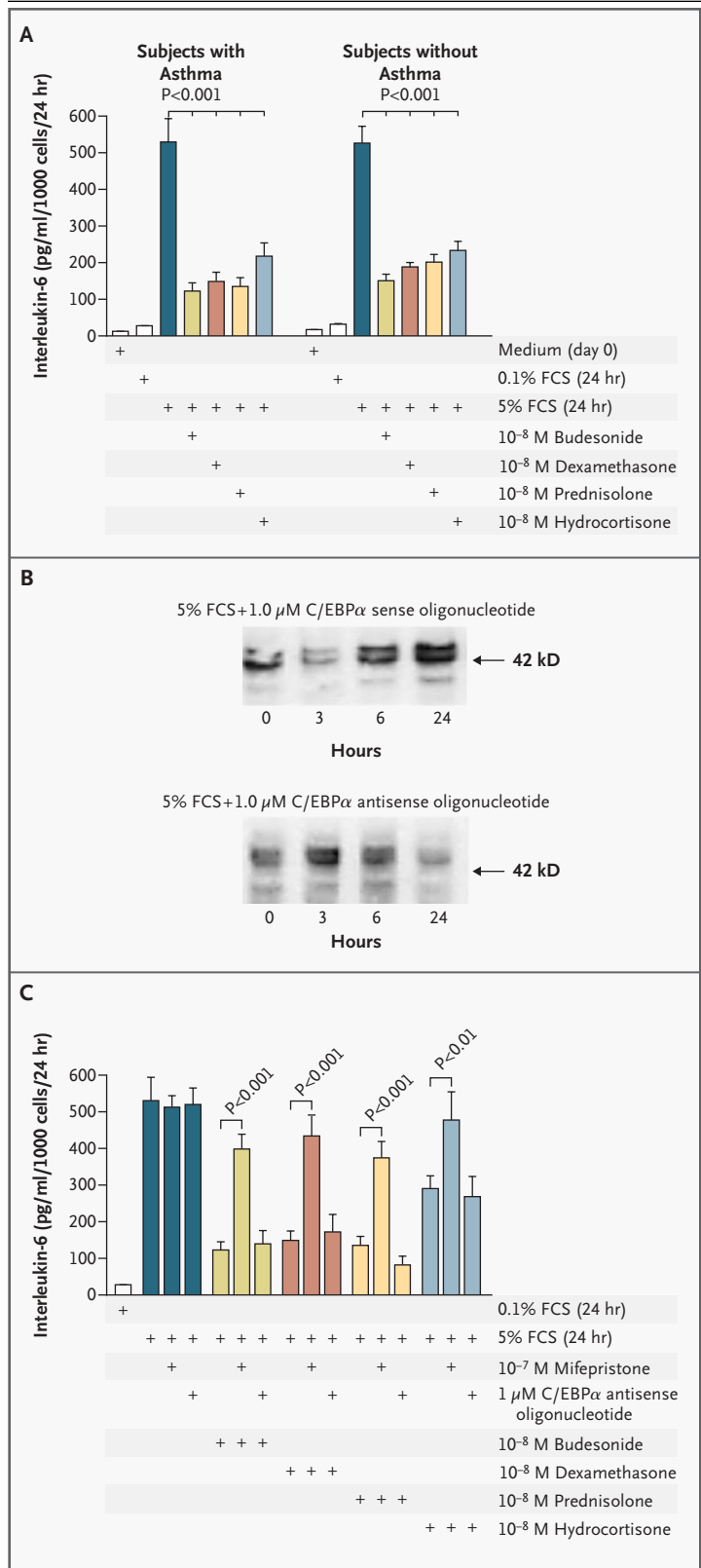
To investigate whether the absence of antipro-

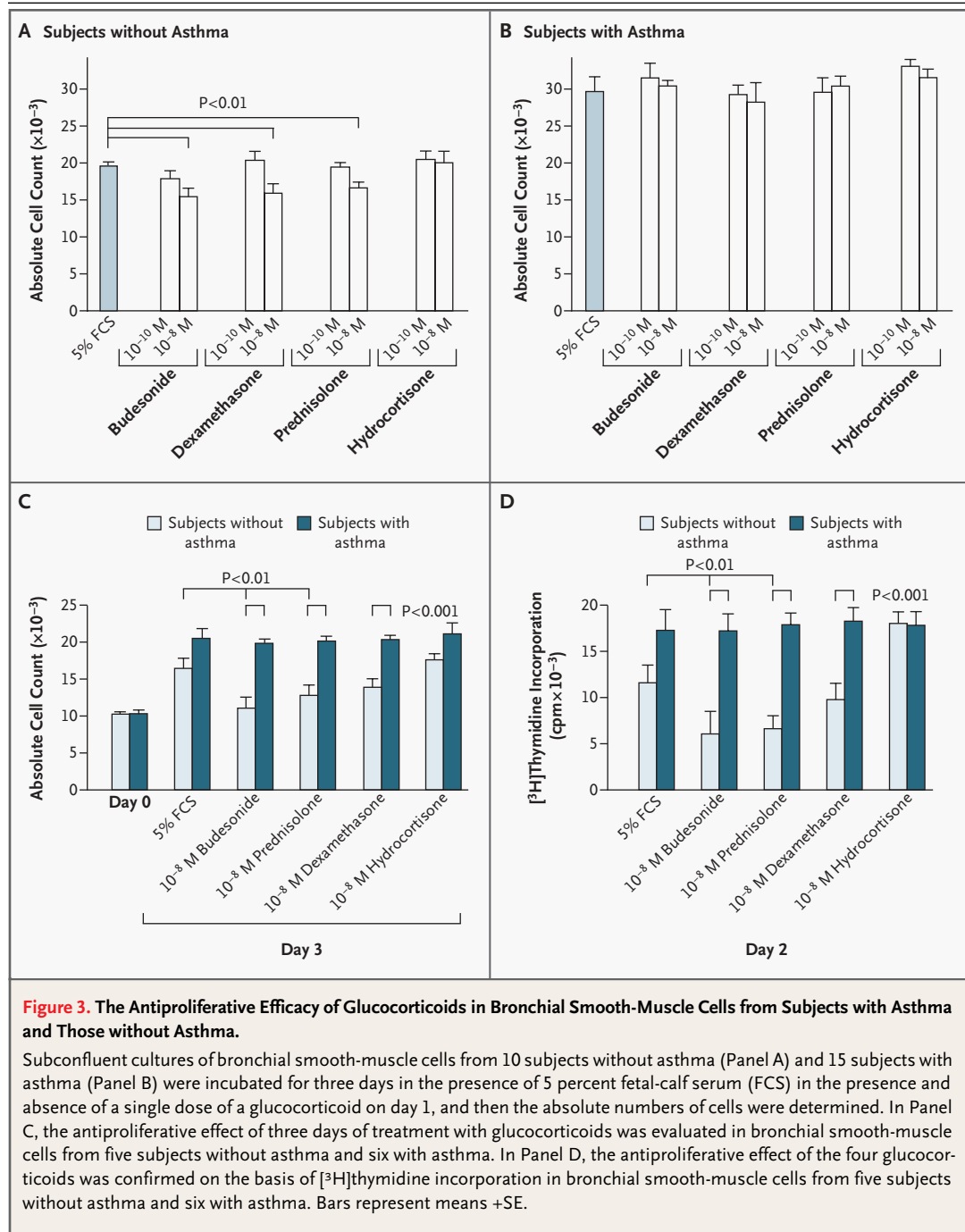
**Figure 2. Effect of Glucocorticoids on Serum-Induced Secretion of Interleukin-6.**

In Panel A, bronchial smooth-muscle cells were stimulated with 5 percent fetal-calf serum (FCS) for 24 hours alone and in combination with a glucocorticoid, and interleukin-6 levels in the cell-culture medium were determined by ELISA. Bars represent the mean (+SE) values in bronchial smooth-muscle cells from 10 subjects with asthma and 10 without asthma. Panel B shows a representative immunoblot of the time course of serum-induced expression of C/EBP $\alpha$  protein in bronchial smooth-muscle cells from a subject without asthma and its inhibition by transient transfection with 1.0  $\mu$ M C/EBP $\alpha$  antisense oligonucleotide. In Panel C, pretreatment of the cells for 1 hour with 10<sup>-7</sup> M mifepristone substantially reduced the inhibitory effect of the glucocorticoids on serum-induced interleukin-6 secretion, whereas pretreatment with 1  $\mu$ M C/EBP $\alpha$  antisense oligonucleotide for 24 hours had no significant effect. Bars represent the mean (+SE) values in bronchial smooth-muscle cells from 10 subjects with asthma and 10 controls.

liferative action of glucocorticoids on bronchial smooth-muscle cells from subjects with asthma could be overcome by long-term treatment, we exchanged the culture medium and drugs daily over a period of three days. In bronchial smooth-muscle cells from 8 subjects without asthma, long-term treatment resulted in a stronger antiproliferative effect of the glucocorticoids than did a single treatment, but it had no significant effect on bronchial smooth-muscle cells from 10 subjects with asthma (Fig. 3C). The absence of an antiproliferative effect of glucocorticoids was confirmed by assessing [<sup>3</sup>H]thymidine incorporation in the two groups of smooth-muscle cells (Fig. 3D).

Given our previous results,<sup>25,26</sup> we assessed the role of C/EBP $\alpha$  in glucocorticoid-inhibited proliferation of bronchial smooth-muscle cells. As shown in a set of representative immunoblots (Fig. 4A), bronchial smooth-muscle cells from 20 subjects with asthma did not express C/EBP $\alpha$ , whereas serum-starved bronchial smooth-muscle cells from 8 subjects with emphysema and 15 controls expressed the 46-kD C/EBP $\alpha$  protein. The expression of C/EBP $\alpha$  could be induced by 5 percent fetal-calf serum within 24 hours in all bronchial smooth-muscle cells from subjects without asthma (Fig. 2B and 4A). However, bronchial smooth-muscle cells from 4 of the 20 subjects with asthma expressed a smaller protein that reacted with the anti-





body against C/EBP $\alpha$  antibody (Fig. 4A). However, to visualize these smaller protein bands, we had to double the duration of exposure of the immunoblots.

To determine whether the absence of C/EBP $\alpha$  is a pathophysiological characteristic present in cell types other than bronchial smooth-muscle

cells, we isolated peripheral-blood lymphocytes from four subjects with asthma and four without asthma. We detected C/EBP $\alpha$  protein in all the samples (Fig. 4B).

To overcome the absence of C/EBP $\alpha$  in bronchial smooth-muscle cells from subjects with asthma, we transiently transfected three such cell lines

with an expression vector for the human C/EBP $\alpha$  isolated from bronchial smooth-muscle cells from subjects without asthma. Immunoblotting revealed a time-dependent increase in the expression of C/EBP $\alpha$  within 24 and 48 hours after transfection (Fig. 4C). The smooth-muscle cells expressed two proteins that reacted with the antibody against C/EBP $\alpha$  (Fig. 4C). According to the molecular-weight markers, the upper band appeared at the expected size for C/EBP $\alpha$ , whereas the smaller band was similar to the second C/EBP $\alpha$  band that was observed in nontransfected bronchial smooth-muscle cells from subjects with asthma (Fig. 4A and 4C). The rate of transfection was controlled with the use of a cytomegalovirus epidermal growth factor promoter-driven vector; the level of expression of epidermal growth factor by bronchial smooth-muscle cells was 62 percent 24 hours after transfection (Fig. 4D).

The absence of C/EBP $\alpha$  in bronchial smooth-muscle cells from subjects with asthma was confirmed with the use of a supershift electrophoretic mobility-shift assay with a p21<sup>(Waf1/Cip1)</sup> promoter fragment containing the glucocorticoid receptor-C/EBP $\alpha$  complex binding site<sup>23-25,27,29</sup> as the target for C/EBP $\alpha$  proteins, in the presence and absence of antibodies specific for C/EBP $\alpha$  or the glucocorticoid receptor; antibodies against c-Fos/AP-1 protein served as a negative control. As shown in Figure 4E, incubation of the p21<sup>(Waf1/Cip1)</sup> promoter fragment with nuclear-protein extracts that were obtained from bronchial smooth-muscle cells from subjects without asthma in the presence of 10<sup>-8</sup> M budesonide for three hours resulted in a defined signal that could be abrogated by incubation with 10 times the normal amount of cold CCAAT oligonucleotide overnight at 4°C. When the complex formed by the p21<sup>(Waf1/Cip1)</sup> promoter fragment and the nuclear-protein extracts of budesonide-treated bronchial smooth-muscle cells was further incubated with antibodies specific for C/EBP $\alpha$ , the level of the complex increased and its migration was further delayed (Fig. 4E, lane 4). A similar supershift was observed when the p21<sup>(Waf1/Cip1)</sup> promoter fragment formed a complex with the nuclear-protein extract of budesonide-treated bronchial smooth-muscle cells from subjects without asthma and was incubated with antibodies specific for the glucocorticoid receptor (Fig. 4E, lane 6), whereas no such supershift was detected when we used antibodies against c-Fos/AP-1 (lane 8). In contrast, the nuclear-protein fraction obtained from

budesonide-treated bronchial smooth-muscle cells from subjects with asthma did not produce any clear signal when they were incubated with the p21<sup>(Waf1/Cip1)</sup> promoter fragment (Fig. 4E, lanes 5, 7, and 9).

To further assess the role of C/EBP $\alpha$  in bronchial smooth-muscle cells from subjects with asthma, we transiently transfected subconfluent cultures with an expression vector for human C/EBP $\alpha$  or the empty vector as a control (Fig. 5A). The level of the C/EBP $\alpha$  protein in nuclear extracts of bronchial smooth-muscle cells from subjects with asthma increased within 48 hours in the presence of budesonide (Fig. 5A, lanes 1 and 2), preincubation with 10 times the normal level of unlabeled CCAAT oligonucleotide diminished the signal (Fig. 5A, lane 3), and no signal was observed when the cells were transfected with the empty control vector for 48 hours (Fig. 5A, lane 4). When the extract was subsequently incubated with antibodies specific for C/EBP $\alpha$ , migration of the DNA-protein band was retarded (Fig. 5A, lane 5), whereas there was no signal when the cells were transfected with the empty control vector (Fig. 5A, lane 6). We observed no supershifts when the same extracts were incubated in the presence of antibodies against c-Fos/AP-1 (Fig. 5A, lane 7), whereas a supershift was observed in the presence of antibodies against glucocorticoid receptor (Fig. 5A, lane 8). Bronchial smooth-muscle cells transfected with an empty vector did not show a supershift in the presence of antibodies against the glucocorticoid receptor (Fig. 5A, lane 9).

To investigate whether, in asthmatic bronchial smooth-muscle cells from subjects with asthma, transient transfection with the human C/EBP $\alpha$  expression vector reestablished the formation of the glucocorticoid receptor-C/EBP $\alpha$  complex previously noted in bronchial smooth-muscle cells from subjects without asthma,<sup>25,26</sup> we performed coimmunoprecipitation experiments with the use of nuclear extracts of bronchial smooth-muscle cells that had been incubated with 10<sup>-8</sup> M budesonide for three hours (Fig. 5B). Western blot analysis of the coimmunoprecipitates showed a clear band for C/EBP $\alpha$  when we used an antibody against the glucocorticoid receptor for immunoprecipitation and for the glucocorticoid receptor when an antibody against C/EBP $\alpha$  was used. In contrast, nontransfected bronchial smooth-muscle cells from subjects with asthma did not show any signal for either C/EBP $\alpha$  or the glucocorticoid receptor. However, when these bronchial smooth-muscle cells were

transfected with a human C/EBP $\alpha$  expression vector 48 hours before stimulation with budesonide and nuclear extracts were prepared 3 hours after the addition of the drug and subsequent coimmunoprecipitation, we observed a band for C/EBP $\alpha$  when an antibody against the glucocorticoid receptor was used, as well as a band for the receptor when an antibody against C/EBP $\alpha$  was used (Fig. 5B).

We obtained further evidence of the function of the transfected C/EBP $\alpha$  expression vector in bronchial smooth-muscle cells from subjects with asthma by counting cells. The numbers of cells in five such nontransfected cell lines doubled within three days in the presence of 5 percent fetal-calf serum, and daily treatment with budesonide ( $10^{-8}$  M) did not significantly alter cell growth (Fig. 5C). Transient transfection with an expression vector for human C/EBP $\alpha$  significantly slowed the proliferation of smooth-muscle cells in the presence of fetal-calf serum (by  $30 \pm 4.9$  percent,  $P < 0.01$  by an unpaired Student's *t*-test), on the basis of the increase in the numbers of cells from day 1 to day 3 in the presence of 5 percent fetal-calf serum. Transfection with the empty vector did not inhibit proliferation. In contrast, transfected cells treated daily with  $10^{-8}$  M budesonide had a further reduction of proliferation, as compared with transfected cells that were not treated with budesonide (Fig. 5C). The additional effect of the glucocorticoid on the inhibitory effect of the C/EBP $\alpha$  expression vector was significant ( $P < 0.01$ ) in bronchial smooth-muscle cells from five subjects with asthma and was significantly different ( $P < 0.01$ ) from the effect achieved with the C/EBP $\alpha$  vector alone. The presence of the empty vector together with budesonide did not inhibit the proliferation of bronchial smooth-muscle cells from subjects with asthma.

#### DISCUSSION

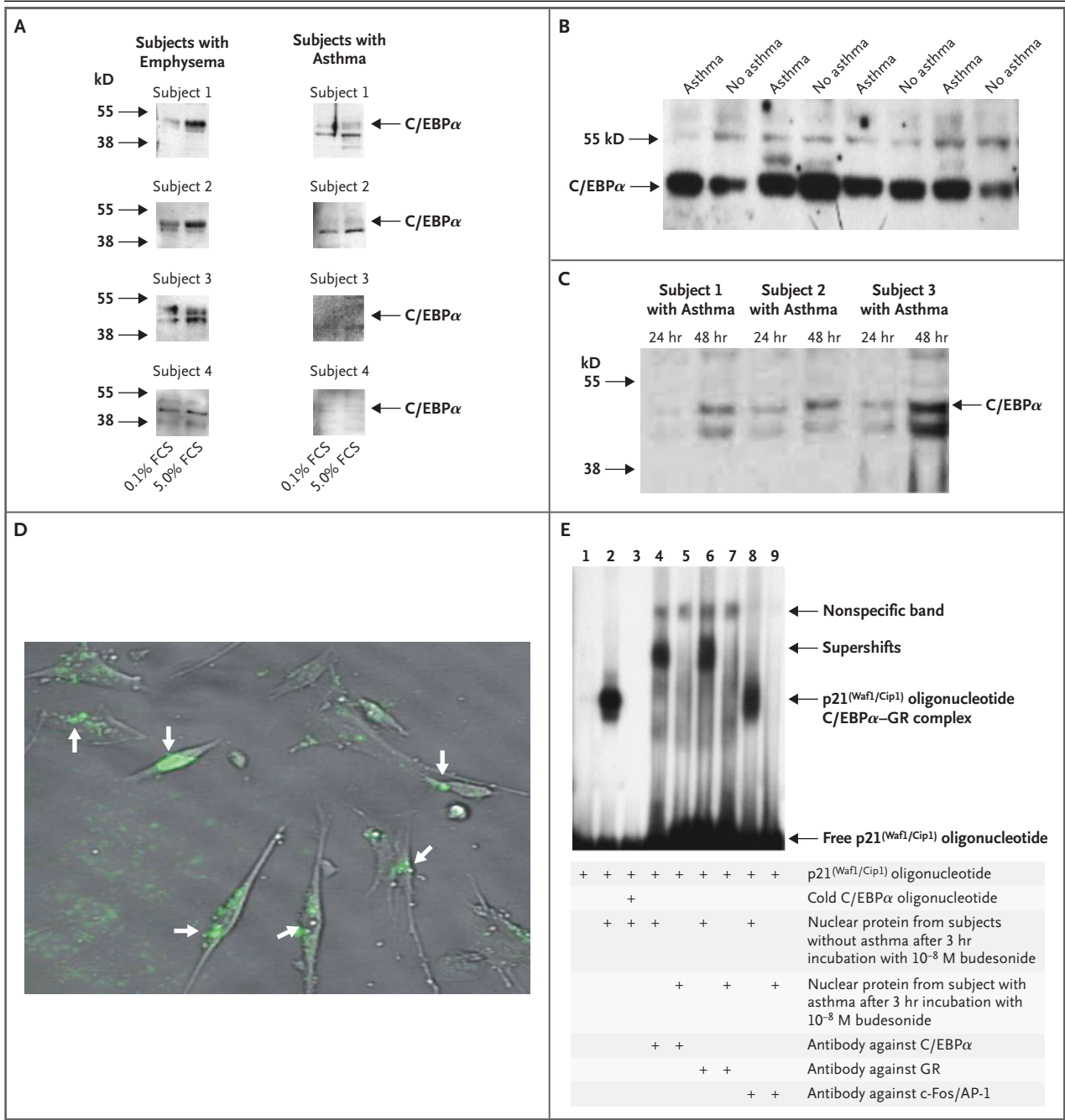
Our data indicate that the proliferation of airway smooth-muscle cells in patients with asthma may result from an absence of C/EBP $\alpha$ . The absence of C/EBP $\alpha$  also explains why the antiproliferative action of glucocorticoids on these cells was absent, while the glucocorticoid-induced suppression of interleukin-6 was not affected. Since peripheral-blood lymphocytes had normal levels of C/EBP $\alpha$ , we propose that the absence of C/EBP $\alpha$  is cell-type-specific.

Hypertrophy and hyperplasia are characteristic of the bronchial smooth-muscle bundles in asth-

#### Figure 4 (facing page). Expression of C/EBP $\alpha$ in Bronchial Smooth-Muscle Cells from Subjects with Asthma and Those without Asthma.

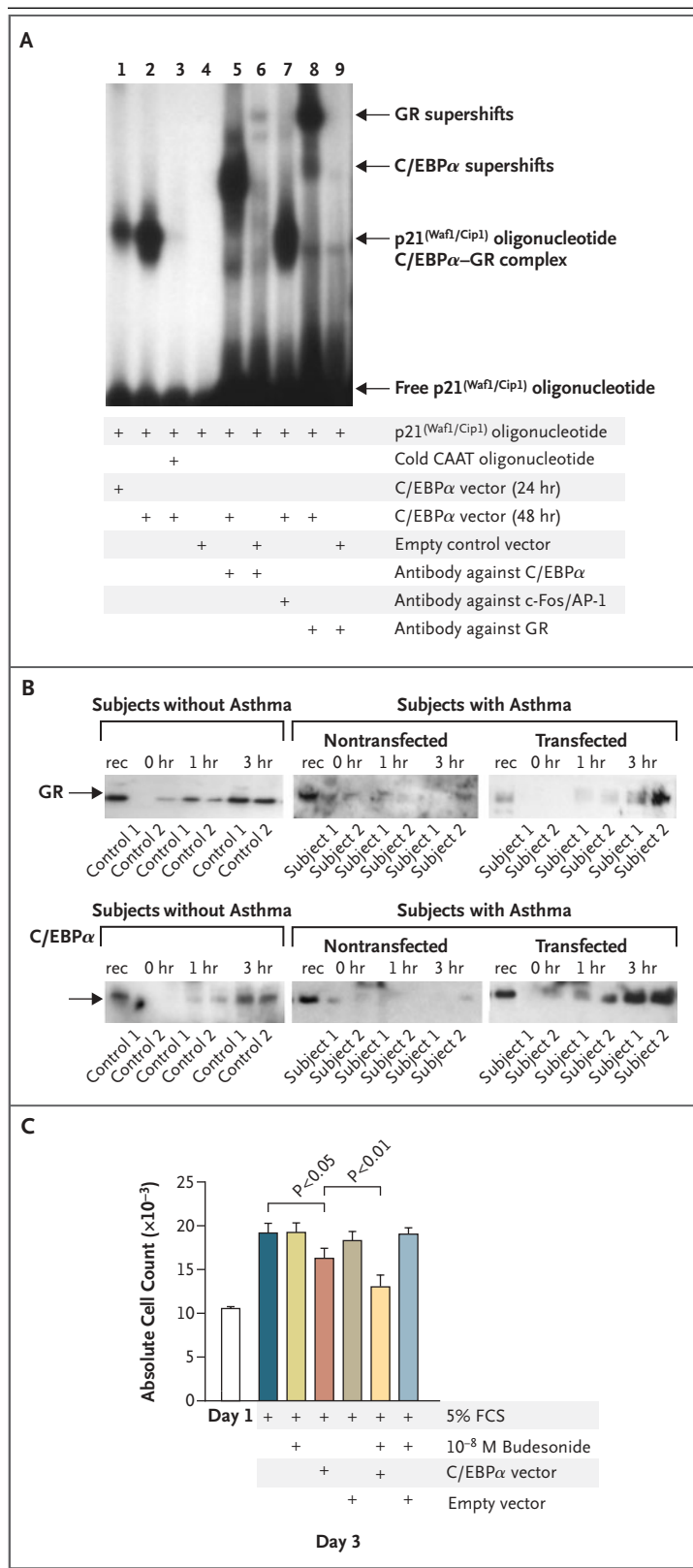
Panel A shows four immunoblots of bronchial smooth-muscle cells representative of the findings in 8 subjects with emphysema and 20 subjects with asthma. Subconfluent cultures of bronchial smooth-muscle cells were incubated with 0.1 percent fetal-calf serum (FCS) for 24 hours and then stimulated with 5 percent FCS. C/EBP $\alpha$  expression was determined in total cell lysates. Panel B shows representative immunoblots of the expression of C/EBP $\alpha$  in total-protein extract isolated from peripheral-blood lymphocytes in four subjects with asthma and four without asthma. Panel C shows representative immunoblots of C/EBP $\alpha$  expression in bronchial smooth-muscle cells from three subjects with asthma that were transiently transfected with an expression vector containing the complementary DNA encoding human peripheral-blood lymphocyte-derived C/EBP $\alpha$ . Cells were transiently transfected in the presence of 5 percent FCS with 1 ng of C/EBP $\alpha$  expression vector DNA per microliter for 24 and 48 hours. Panel D shows the efficacy of transient transfection on immunofluorescence. The arrows indicate the cells that express the green fluorescent protein and that therefore have been successfully transfected. Panel E shows a representative electrophoretic mobility-shift assay of p21<sup>(Waf1/Cip1)</sup> promoter-binding proteins with the use of nuclear extracts from bronchial smooth-muscle cells treated for three hours with  $10^{-8}$  M budesonide. Lane 1 shows the p21<sup>(Waf1/Cip1)</sup> promoter fragment alone, lane 2 the promoter with nuclear extracts of budesonide-treated bronchial smooth-muscle cells from subjects without asthma, lane 3 the same extract as in lane 2 but with 10 times the normal amount of cold CCAAT oligonucleotides, lane 4 antibody against C/EBP $\alpha$  and nuclear extracts of bronchial smooth-muscle cells from subjects without asthma, lane 5 antibody against C/EBP $\alpha$  and nuclear extracts of bronchial smooth-muscle cells from subjects with asthma, lane 6 antibody against the glucocorticoid receptor (GR) and nuclear extracts from bronchial smooth-muscle cells from subjects without asthma, lane 7 antibody against GR and nuclear extracts of bronchial smooth-muscle cells from subjects with asthma, lane 8 antibody against c-Fos/AP-1 and nuclear extracts of bronchial smooth-muscle cells from subjects without asthma, and lane 9 antibody against c-Fos/AP-1 and nuclear extracts of bronchial smooth-muscle cells from subjects with asthma.

matic airways, and this increased layer of bronchial smooth muscle may explain the hyperresponsiveness of asthmatic bronchi.<sup>1,2,4</sup> It may also explain the local recruitment of immunocompetent cells into the smooth muscle, leading to chronic inflammation.<sup>1,2,4</sup> Glucocorticoids are potent suppressors of this chronic inflammation and are the mainstay of therapy for moderate and severe asthma.<sup>5-11</sup>



Impaired expression of the glucocorticoid receptor or a mutation in it has been linked to asthma but has never been proved to play a causative role.<sup>30-32</sup> The expression of the receptor has been assumed to be faulty in patients with asthma, but no significant differences between patients with asthma and those without asthma have been found in the tissue distribution of the glucocorticoid re-

ceptor in lung-biopsy specimens.<sup>32</sup> Some data have suggested that the receptor has two isoforms,  $\alpha$  and  $\beta$ , resulting from differential splicing of the same messenger RNA.<sup>33</sup> Both isoforms bind to the same DNA consensus sequence. Because the consensus sequence has a higher affinity for the  $\beta$  isoform, it has been assumed that this isoform inhibits binding of the  $\alpha$  isoform,<sup>33</sup> but a link between overex-



**Figure 5. The Glucocorticoid Receptor (GR)–C/EBPα Complex and Its Antiproliferative Function in Bronchial Smooth-Muscle Cells from Subjects with Asthma.**

Panel A shows a representative electrophoretic mobility-shift assay of nuclear extracts of bronchial smooth-muscle cells from subjects with asthma that were transiently transfected for 24 hours (lane 1) or 48 hours (lane 2) with C/EBPα expression vector and incubated for the last 3 hours with 10<sup>-8</sup> M budesonide. Lane 3 shows the C/EBPα vector with 10 times the normal amount of unlabeled CCAAT oligonucleotide, lane 4 empty control vector, lane 5 C/EBPα vector with antibody against C/EBPα, lane 6 empty control vector with antibody against C/EBPα, lane 7 C/EBPα vector with antibody against c-Fos/AP-1, lane 8 C/EBPα vector with antibody against GR, and lane 9 empty control vector with antibody against GR. Panel B shows representative immunoblot analysis of the GR and C/EBPα protein in bronchial smooth-muscle cells stimulated with budesonide for 0, 1, or 3 hours from two subjects without asthma and two with asthma in the presence and absence of transient (24-hour) transfection with the C/EBPα expression vector (1 ng per microliter); rec denotes recombinant. Panel C shows the effect of transfection with the C/EBPα expression vector on the proliferation of bronchial smooth-muscle cells from four subjects with asthma. Cells were transfected with C/EBPα expression vector (1 ng per microliter) for 24 hours in the presence of either 5 percent fetal-calf serum (FCS) or 10<sup>-8</sup> M budesonide, and the empty vector was used as a control. Bars represent means + SE, and each cell line was tested in duplicate.

pression of the β isoform and the diagnosis of asthma has not been established.<sup>34</sup> In our study, glucocorticoids activated the glucocorticoid receptor in all lines of bronchial smooth-muscle cells, irrespective of the disease state, and we did not observe a stronger signal for the β isoform in bronchial smooth-muscle cells from subjects with asthma than in those without asthma. Furthermore, we did not detect a double band for the receptor in the cytosolic protein fractions, whereas a second protein band that reacted with the antibody against the glucocorticoid receptor became visible only in the nuclear protein fractions early after treatment with a glucocorticoid.

The activation of the glucocorticoid receptor by various glucocorticoids suppressed serum-stimulated secretion of interleukin-6 in all lines of bronchial smooth-muscle cells, and mifepristone reversed this effect. Our data indicate that the inhibition of interleukin-6 secretion by glucocorticoids does not involve C/EBPα in human bronchial

smooth-muscle cells. Therefore, distinct signaling pathways mediate the antiproliferative and the interleukin-6–blocking effects of glucocorticoids. However, the latter effect may include the interaction of the receptor with other transcription factors, including nuclear factor- $\kappa$ B, AP-1, and signal transducers and activators of transcription (STATs).<sup>10,12,13,35-38</sup>

The observed failure of glucocorticoids to inhibit cell proliferation in bronchial smooth-muscle cells from subjects with asthma could not be explained by an absent or defective glucocorticoid receptor. Several studies have shown that the antiproliferative effect of glucocorticoids is mediated by the receptor and C/EBP $\alpha$ ,<sup>14,23-25</sup> and both active transcription factors are required to induce the synthesis of p21<sup>(Waf1/Cip1)</sup>.<sup>24,25,27</sup> Moreover, in human cells, including lung fibroblasts, pulmonary and bronchial smooth-muscle cells, and peripheral-blood lymphocytes, the glucocorticoid receptor forms a complex with C/EBP $\alpha$ , which then binds to the CCAAT DNA consensus sequence in the p21<sup>(Waf1/Cip1)</sup> promoter.<sup>25,27,28</sup> We found that this complex was absent in budesonide-treated bronchial smooth-muscle cells from subjects with asthma, but that it could be reestablished by transfecting the cells with a human C/EBP $\alpha$  expression vector. Furthermore, transfected cells had a slower rate of proliferation than nontransfected cells, and their proliferation could be inhibited by glucocorticoids. These findings indicate that the absence of C/EBP $\alpha$  in bronchial smooth-muscle cells from patients with asthma may account for their increased rate of proliferation.<sup>3</sup> In addition, direct protein–protein interaction with the glucocor-

ticoid receptor and other transcription factors has been demonstrated only for members of the STAT family<sup>13,37,38</sup> for C/EBP $\alpha$ <sup>23,25,27</sup> and C/EBP $\beta$ .<sup>36</sup> Therefore, the receptor may regulate cell proliferation and cell differentiation or apoptosis through its interaction with various members of the C/EBP family. We did not observe any interaction between the glucocorticoid receptor and AP-1, which indicates that this transcription factor is not involved in the p21<sup>(Waf1/Cip1)</sup> regulating complex consisting of the receptor and C/EBP $\alpha$ .

Moreover, the antiproliferative action of glucocorticoids was preserved in bronchial smooth-muscle cells from eight subjects with emphysema, and C/EBP $\alpha$  was also normally expressed in these cells. These observations suggest a difference in the pathogenesis of asthma and emphysema, even though the latter is sometimes regarded as a subtype of asthma.<sup>39,40</sup>

In conclusion, we have demonstrated that the antiproliferative and the cytokine-inhibitory effects of glucocorticoids are mediated by different signaling pathways. Both pathways involve the activation of the glucocorticoid receptor, but the pathways subsequently diverge. The antiproliferative pathway does not function in patients with asthma because bronchial smooth-muscle cells in these patients lack C/EBP $\alpha$  protein required to form a complex with the glucocorticoid receptor. The pathological implications of this absence of C/EBP $\alpha$  require evaluation.

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