

Systemic glucocorticoid reduces bronchial mucosal activation of activator protein 1 components in glucocorticoid-sensitive but not glucocorticoid-resistant asthmatic patients

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Background: Overexpression of the transcriptional regulatory factor activator protein 1 might contribute to T-cell glucocorticoid (GC) refractoriness in GC-resistant asthma.

Objective: We sought to address the hypothesis that clinically GC-resistant asthma is accompanied by failure of systemic GCs to inhibit phosphorylation of c-jun and c-jun N-terminal kinase (JNK) in bronchial mucosal cells.

Methods: We performed enumeration of total (CD45⁺) leukocytes and cells expressing c-fos and total and phosphorylated c-jun and JNK in bronchial biopsy sections from 9 GC-sensitive and 17 GC-resistant asthmatic patients taken before and after oral prednisolone (40 mg/1.72 m² body surface area daily for 14 days) using specific antibodies, immunohistochemistry, and image analysis.

Results: At baseline, mean total (CD45⁺) mucosal leukocytes, total cells expressing phosphorylated c-jun and JNK, and mean percentages of cells in which these molecules were phosphorylated were similar in both groups, whereas mean total numbers of c-fos-immunoreactive cells were increased in the GC-resistant asthmatic subjects ($P = .04$). After prednisolone, the mean total cells expressing phosphorylated c-jun and JNK and the mean percentages of cells in which these molecules were phosphorylated were significantly reduced in the GC-sensitive ($P \leq .02$) but not the GC-resistant asthmatic subjects. Mean total CD45⁺ leukocytes and c-fos-immunoreactive cells were not significantly altered in either group.

Conclusion: Clinical GC responsiveness in asthma is accompanied by reduced phosphorylation of bronchial mucosal c-jun and JNK, a phenomenon not seen in resistant patients.

Clinical implications: Dysregulation of activator protein 1 activation leading to clinical GC resistance might reflect

identifiable environmental influences and is a target for future therapy. (J Allergy Clin Immunol 2006;118:368-75.)

Key words: Asthma, glucocorticoid, activator protein 1, therapy, resistance

Glucocorticoids (GCs) form the mainstay of treatment for asthma, although a minority of individuals, termed GC-resistant asthmatic patients, do not show improvement in their FEV₁ values after administration of GC therapy at therapeutic dosages. In previous studies we have shown that lectin-induced proliferation of peripheral blood T cells is inhibited by dexamethasone at therapeutic concentrations *in vitro* in GC-sensitive but not GC-resistant asthmatic patients¹ and that this is a reproducible abnormality.² We have further shown that GC-exposed PBMCs from GC-resistant compared with GC-sensitive asthmatic patients have fewer activated GC receptors available for DNA binding³ but show increased DNA binding of the transcriptional regulatory protein activator protein 1 (AP-1) after phorbol ester stimulation.⁴ Furthermore, phorbol ester stimulation of cells from GC-sensitive patients induced a GC-resistant phenotype, which was associated with direct interaction between the activated GC receptor and AP-1, as detected by means of coimmunoprecipitation.⁵ Binding of the GC receptor to other proinflammatory transcriptional activators was unaffected. These data are consistent with the hypothesis that mononuclear inflammatory leukocytes from GC-resistant asthmatic subjects inappropriately overexpress components of AP-1, which has the functional consequence of sequestering the activated GC receptor, thus causing refractoriness to GC-induced inhibitory responses.

AP-1 is comprised of variable heterodimers of jun (c-jun, jun B, and jun D) and fos (c-fos, fos B, Fra-1, and Fra-2), although the most stable complex in activated cells is the c-fos/c-jun heterodimer,⁶ which transactivates a number of asthma-relevant cytokine genes, including *IL5* and *GM-CSF*.^{7,8} AP-1 is activated through the transcriptional regulation of c-fos⁹ and the phosphorylation of c-jun, which is the end result of the action of a cascade of kinases.¹⁰ C-fos expression is increased by a wide variety of growth factors and mitogens through complex signaling pathways involving activation of mitogen-activated protein kinase and calcium-dependent

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Abbreviations used

AP-1: Activator protein 1
ERK: Extracellular signal-related kinase
GC: Glucocorticoid
JNK: Jun N-terminal kinase
MEK: Mitogen-activated protein/ERK kinase
SAPK: Serum-activated protein kinase

mechanisms.^{11,12} C-jun is phosphorylated by jun N-terminal kinase (JNK), one of a group of intracellular kinases that are also known as the serum-activated protein kinases (SAPKs). In addition to activation of c-jun, JNK can also increase its transcription, as shown, for example, by the ability of JNK inhibitors, such as SP600125, to abolish AP-1 activation through inhibition of c-jun transcription in animal models.¹³ JNK is in turn activated by phosphorylation by JNK kinase, a member of the mitogen-activated protein kinase/extracellular signal-related kinase (ERK) kinase (MEK) family of serine-threonine kinases.

The reason why AP-1 is overexpressed in inflammatory leukocytes of GC-resistant asthmatic subjects is not clear, but because GCs have been reported to diminish AP-1 activity by inhibiting JNK phosphorylation,^{14,15} we surmised that this process might be deficient in GC-resistant asthmatic patients. Therefore we hypothesized that clinically GC-resistant asthma is accompanied by failure of GCs to inhibit phosphorylation of c-jun and JNK within cells of the bronchial mucosa. Consequently, we have measured the expression of c-fos and total and phosphorylated c-jun and JNK in bronchial mucosal cells in groups of GC-sensitive and GC-resistant asthmatic subjects before and after a course of systemic GC therapy.

METHODS

Patients

Adult subjects were recruited from the outpatient populations of Guy's and King's College Hospitals, London, United Kingdom. All subjects met the diagnostic criteria for asthma as defined by the American Thoracic Society,¹⁶ had FEV₁ values of 30% to 70% of the predicted value, and demonstrated 15% or greater reversibility of FEV₁ in response to inhaled β_2 -agonist (nebulized salbutamol, 5 mg). Atopy was defined as a positive skin prick test response to one or more of a panel of common aeroallergens in the presence of valid negative (diluent) and positive (histamine) controls. Current smokers and exsmokers of 5 pack-years or longer were excluded. All exsmokers had ceased smoking 12 or more months before the study. Asthmatic subjects who had required a course of systemic GC therapy for disease exacerbation in the 2 months before enrollment were also excluded. All asthmatic subjects had stable disease in at least the 2 weeks before enrollment, as defined by an unchanged requirement for regular medication, and remained clinically free of infection throughout the study period. The study patients were taking inhaled, short-acting β_2 -agonist as required and inhaled GC therapy regularly, although a proportion were taking concomitant inhaled long-acting β_2 -agonist therapy (Table I). Female patients were advised to take contraceptive precautions during the period of the study. All patients provided written informed consent

to participate in the study, which was approved by the Research Ethics Committees of Guy's and King's College Hospitals.

Trial of oral GC therapy

On day 0, FEV₁ and reversibility of FEV₁ in response to bronchodilator (nebulized salbutamol, 5 mg) were measured according to standard American Thoracic Society protocols.¹⁷ Subjects were then asked to take oral prednisolone at a dose adjusted for body surface area (40 mg/1.73 m²) as a single dose at 8 AM daily for 2 weeks (days 1–14). On day 14, spirometry was repeated at the same time of day. Patients were asked to discontinue any long-acting β_2 -agonist 48 hours before and throughout the study and short-acting β_2 -agonist 4 hours before spirometry on days 0 and 14. After the trial of oral prednisolone therapy, any long-acting β_2 -agonist therapy was resumed. Patients were classified as GC resistant if the change in FEV₁ between days 0 and 14, expressed as a percentage of the baseline value, was 15% or less and GC sensitive if the change in FEV₁ was greater than 15%. This division is essentially arbitrary but along the lines of an international consensus definition.¹⁸ By using these criteria, 9 patients were classified as GC sensitive and 17 as GC resistant.

Fiberoptic bronchoscopy

Fiberoptic bronchoscopy was performed on all patients on days 0 and 14 of the study after measurement of spirometry according to American Thoracic Society guidelines. Before each procedure, a peripheral venous blood sample was obtained and allowed to clot on glass for estimation of plasma adrenocorticotrophic hormone concentrations. Patients were premedicated with intravenous midazolam (2–10 mg) and atropine (500 μ g). Before bronchoscopy, salbutamol (5 mg) and ipratropium bromide (500 μ g) were administered by using an air-driven nebulizer with a mouthpiece. Topical anesthesia during bronchoscopy was achieved with xylocaine (10%) to the oropharynx, lignocaine (4%) to the vocal chords, and lignocaine (2%) to the tracheobronchial tree delivered in 2-mL aliquots, with the total dose of lignocaine not exceeding 6 mg/kg body weight. Oxygen was delivered throughout the procedure with nasal prongs, and blood pressure and oxygen saturation (measured by means of pulse oximetry) were monitored throughout the procedure. Up to 10 endobronchial biopsy specimens were taken from segmental and subsegmental bronchi in the right lower lobe with reusable biopsy forceps (FB-15C-1; Olympus, Medical Systems Corp, Tokyo, Japan). Nebulized salbutamol and ipratropium were administered for a second time, and the subjects were observed for 2 hours before being escorted home. The bronchoscopic procedures were well tolerated by all of the asthmatic patients.

Preparation of specimens

Bronchial biopsy specimens were coded and processed as previously described.^{19,20} Briefly, biopsy specimens were embedded in OCT compound (Bayer, Basingstoke, United Kingdom), snap-frozen in precooled isopentane, and stored at -70°C until cryostat sectioning. Sections of nominal 5- μ m thickness were freshly cut and thaw mounted onto poly-L-lysine-coated slides (BDH, Poole, United Kingdom). After air drying, sections were fixed in acetone (BDH) for 15 minutes, dried again, and stored foil wrapped in airtight containers at -70°C until used.

Immunohistochemistry and image analysis

Primary antibodies for immunostaining were used at preoptimized dilutions as follows: murine monoclonal anti-CD45 (panleukocyte marker, 200 μ g/mL, 1:100 dilution); rabbit polyclonal anti-c-fos (H-125, not cross-reactive with fos B or Fra-1/2, 200 μ g/mL, 1:50); rabbit polyclonal anti-c-jun (H-79, not cross-reactive with jun B or

TABLE I. Demographic and clinical characteristics of GC-sensitive and GC-resistant asthmatic patients

	GC-sensitive	GC-resistant	P value*
N (male patients)	9 (8)	17 (11)	
Age (y)	48 ± 3	53 ± 3	.31
Atopy (% total)	6 (67)	9 (53)	
Daily inhaled GC dosage (BDP equivalent µg)	1430 ± 487	1048 ± 153	.35
Pretrial inhaled LABA therapy (% total)	5 (56)	7 (41)	
Baseline FEV ₁ (L)	1.94 ± 0.20	1.89 ± 0.16	.84
Baseline FEV ₁ (% predicted)	58.9 ± 5.1	58.7 ± 4.6	.97
β ₂ -agonist reversibility (%)	33.3 ± 6.2	23.6 ± 2.9	.12
Oral GC FEV ₁ reversibility (L)	0.62 ± 0.10	0.001 ± 0.04	.001†
Oral GC FEV ₁ reversibility (%)	39.4 ± 13.4	0.38 ± 2.51	.02†
Serum ACTH day 0 (ng/L)	18.3 ± 2.31	17.2 ± 1.9	
Serum ACTH day 14 (ng/L)	5.7 ± 0.63	5.4 ± 0.59	.77†

Oral GC FEV₁ Reversibility (%) = $(FEV_{1day14} - FEV_{1day0}) / FEV_{1day0} \times 100\%$. GC-resistant asthma is defined as oral GC reversibility of 15% or less.

Data are expressed as means ± SEM.

BDP, Beclomethasone dipropionate; LABA, long-acting β₂-agonist; ACTH, adrenocorticotrophic hormone.

Student 2-sample *t* test comparing *absolute values at baseline or †changes in each group after oral prednisolone.

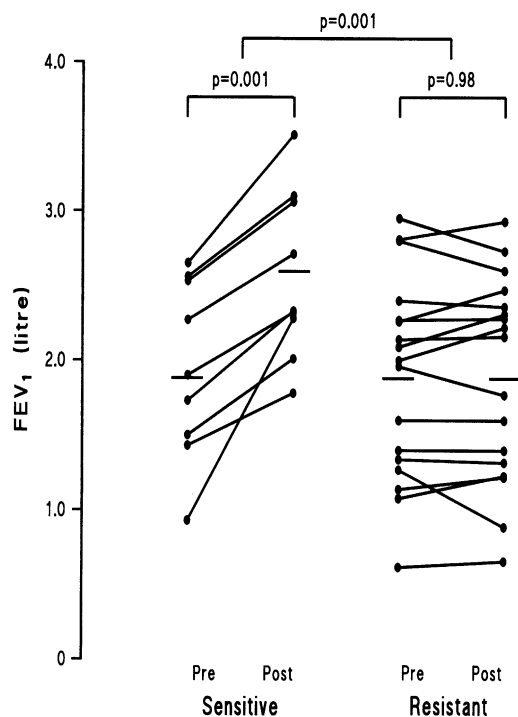


FIG 1. FEV₁ measurements in the GC-sensitive (n = 9) and GC-resistant (n = 17) asthmatic patients before (Pre) and after (Post) oral prednisolone therapy (Student paired *t* test within groups; Student 2-sample *t* test between groups). Bars show means.

jun D, 200 µg/mL, 1:200); murine monoclonal anti-phosphorylated c-jun (KM-1, not cross-reactive with unphosphorylated c-jun, 200 µg/mL, 1:50); murine monoclonal anti-JNK (F-3, 200 µg/mL, 1:50); and murine monoclonal anti-phosphorylated JNK (G-7, not cross-reactive with unphosphorylated JNK, 200 µg/mL, 1:50). All antibodies were from Santa Cruz Biotechnology (Autogen Bionuclear, Wiltshire, United Kingdom), except anti-CD45, which was from Dako (High Wycombe, United Kingdom). Biotinylated secondary

antibodies (swine anti-rabbit, 1:200; rabbit anti-mouse, 1:400) were from Dako.

The staining procedure was as previously described.^{19,20} Briefly, endogenous biotin was first blocked by means of incubation of the sections with 0.01% avidin and then 0.01% biotin (Dako, Glostrup, Denmark) for 10 minutes each. Nonspecific binding was then blocked by means of incubation of the sections with 10% normal rabbit or swine serum (for rabbit or swine secondary antibodies, respectively). Sections were next incubated with primary antibody for 90 minutes (except in the case of c-jun, JNK, and phosphorylated JNK, where incubation was overnight). After blocking of endogenous peroxidase, sections were incubated with the appropriate biotinylated secondary antibody for 30 minutes and then avidin-biotin complex (ABC; Dako) for a further 30 minutes. The signal was developed with Fast 3',3'-diaminobenzidine (Sigma-Aldrich, Dorset, United Kingdom), and then the slides washed in tap water and counterstained with Mayer's hematoxylin (Sigma). Positive cells were stained brown. Omission or substitution of the primary antibody with an irrelevant species- and isotype-matched antibody was used as a negative control. Stained cells in coded sections were counted by 2 independent observers (interobserver variation coefficient, <7%) in the entire sub-mucosal areas of the biopsy specimens (ie, excluding the epithelium, glands, and muscle) with an Olympus BX40 microscope coupled with a Zeiss Vision KS300 imaging system (Carl Zeiss, Hamburg, Germany), which measured these areas automatically. Data were expressed as the numbers of immunoreactive cells per square millimeter of mucosa. Insufficient sections were available from biopsy specimens from a few of the patients to complete analysis with all of the antibodies (as indicated in the figure legends).

Statistical analysis

The data were analyzed with the aid of 2 commercially available packages (Minitab for Windows; Minitab, State College, Pa, and GraphPad Prism; GraphPad Software Inc, San Diego, Calif). The latter software was used to confirm that all groups of data shown in Figs 1 through 4 conformed to a Gaussian distribution, legitimizing parametric analysis. The software uses Kolmogorov-Smirnov analysis of deviation from a Gaussian distribution by using the Dallal and Wilkinson approximation to the Lilliefors method. Analysis was therefore parametric throughout, with the Student paired and 2-sample *t* tests as appropriate to compare differences in absolute

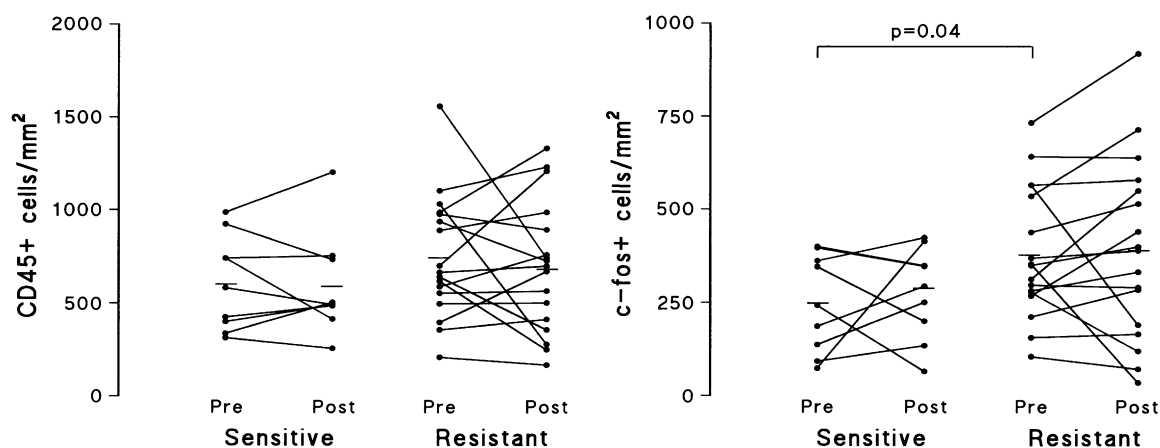


FIG 2. Total CD45⁺ leukocytes (*left panel*) and total cells expressing c-fos immunoreactivity (*right panel*) per unit area of mucosa in bronchial biopsy specimens from GC-sensitive (*n* = 9) and GC-resistant (*n* = 17) asthmatic patients before (*Pre*) and after (*Post*) oral prednisolone therapy (Student 2-sample *t* test). Bars show means.

cell counts and changes after oral prednisolone therapy in each group. Correlation was by means of linear regression. A *P* value of less than .05 was considered statistically significant.

RESULTS

All classified patients successfully completed the trial of prednisolone. The GC-sensitive and GC-resistant asthmatic patients were well matched in terms of age, sex, atopic status, pretrial medication, pretrial lung function, and β_2 -agonist reversibility (Table I). By definition, they showed highly significant differences in their FEV₁ responses to the trial of oral prednisolone (Table I and Fig 1). Serum adrenocorticotrophic hormone concentrations were suppressed in all patients after oral prednisolone, which is consistent with compliance with the therapy. The degree of suppression did not significantly differ in the clinically sensitive and resistant patients (Table I).

Total (CD45⁺) leukocytes

Data on total (CD45⁺) leukocytes are shown in Fig 2. Mean total numbers of mucosal CD45⁺ leukocytes were similar in the GC-sensitive and GC-resistant asthmatic patients and did not change significantly after oral prednisolone therapy (sensitive: 605 $\pm$ 85 to 593 $\pm$ 92 cells/mm², *P* = .83; resistant: 748 $\pm$ 81 to 693 $\pm$ 85 cells/mm², *P* = .53).

C-fos immunoreactivity

Data on c-fos immunoreactivity are shown in Fig 2. Mean total numbers of mucosal c-fos-immunoreactive cells did not significantly change in either the GC-sensitive or the GC-resistant asthmatic patients after oral prednisolone (sensitive: 248 $\pm$ 44 to 275 $\pm$ 41 cells/mm², *P* = .62; resistant: 380 $\pm$ 42 to 390 $\pm$ 59 cells/mm², *P* = .80). The GC-resistant patients did, however, have significantly greater numbers of cells

expressing c-fos at baseline than the GC-sensitive patients (*P* = .04).

Total and phosphorylated c-jun

Data on total and phosphorylated c-jun are shown in Fig 3. Mean total numbers of mucosal cells expressing immunoreactivity for phosphorylated c-jun did not significantly differ in the GC-sensitive and GC-resistant asthmatic patients at baseline. In association with oral prednisolone therapy, however, there was a significant decrease in the mean numbers of cells expressing phosphorylated c-jun in the GC-sensitive but not the GC-resistant patients (sensitive: 117 $\pm$ 30 to 54 $\pm$ 15 cells/mm², *P* = .02; resistant: 148 $\pm$ 27 to 173 $\pm$ 35 cells/mm², *P* = .29). The mean change was also significantly higher in the GC-sensitive patients (*P* = .01). The mean total numbers of mucosal cells expressing immunoreactivity for c-jun did not significantly change in both the GC-sensitive and GC-resistant asthmatic patients after prednisolone. Consequently, the mean percentage of the total c-jun-immunoreactive cells in which the c-jun was phosphorylated was significantly reduced in the GC-sensitive but not the GC-resistant patients (sensitive: 61% $\pm$ 7% to 37% $\pm$ 5%, *P* = .002; resistant: 46% $\pm$ 5% to 56% $\pm$ 6%, *P* = .09), and again the mean reduction was also significantly higher in the GC-sensitive patients (*P* < .0001).

Total and phosphorylated JNK

Data on total and phosphorylated JNK are shown in Fig 4. Mean total numbers of mucosal cells expressing immunoreactivity for phosphorylated JNK did not significantly differ in the GC-sensitive and GC-resistant asthmatic patients at baseline. In association with oral prednisolone therapy, however, there was a significant decrease in the mean numbers of cells expressing phosphorylated JNK in the GC-sensitive but not the GC-resistant patients (sensitive: 58 $\pm$ 10 to 31 $\pm$ 2 cells/mm², *P* = .01; resistant: 76 $\pm$ 14 to 68 $\pm$ 19 cells/mm², *P* = .55). The

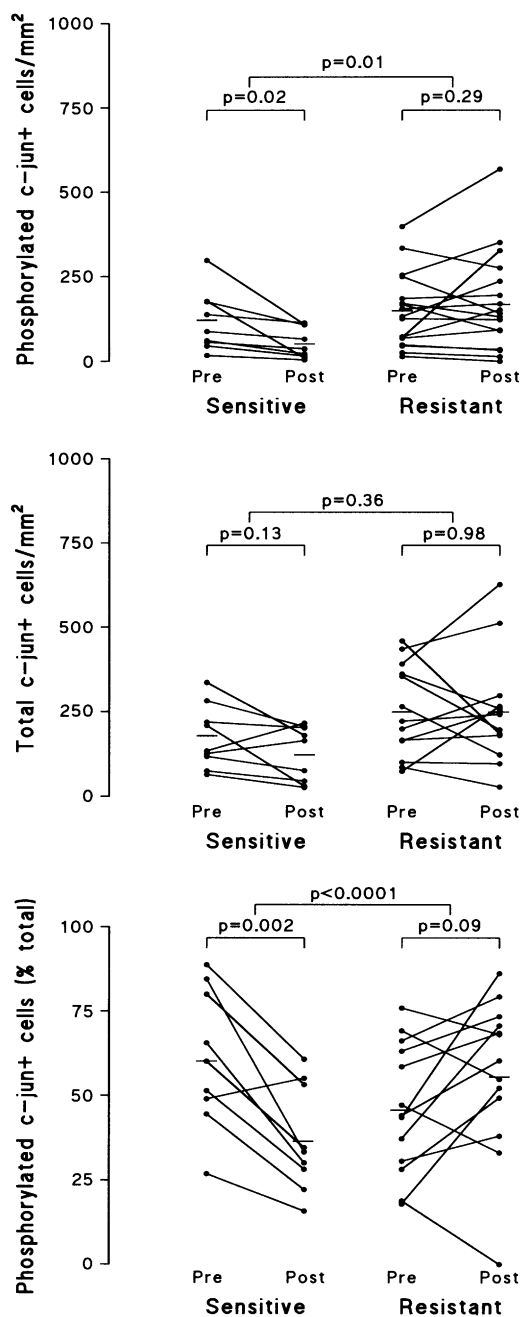


FIG 3. Absolute numbers of cells expressing phosphorylated c-jun (top) and total c-jun (middle) immunoreactivity and percentages of total c-jun-immunoreactive cells showing c-jun phosphorylation (bottom) per unit area of mucosa in bronchial biopsy specimens from GC-sensitive (n = 9) and GC-resistant (n = 13) asthmatic patients before (Pre) and after (Post) oral prednisolone therapy (Student paired *t* test within groups; Student 2-sample *t* test between groups). Bars show means.

mean total numbers of mucosal cells expressing immunoreactivity for JNK did not significantly change in the GC-sensitive asthmatic patients but showed a significant decrease in the GC-resistant patients after prednisolone. Consequently, the mean percentage of the total JNK-immunoreactive cells in which the JNK was phosphorylated

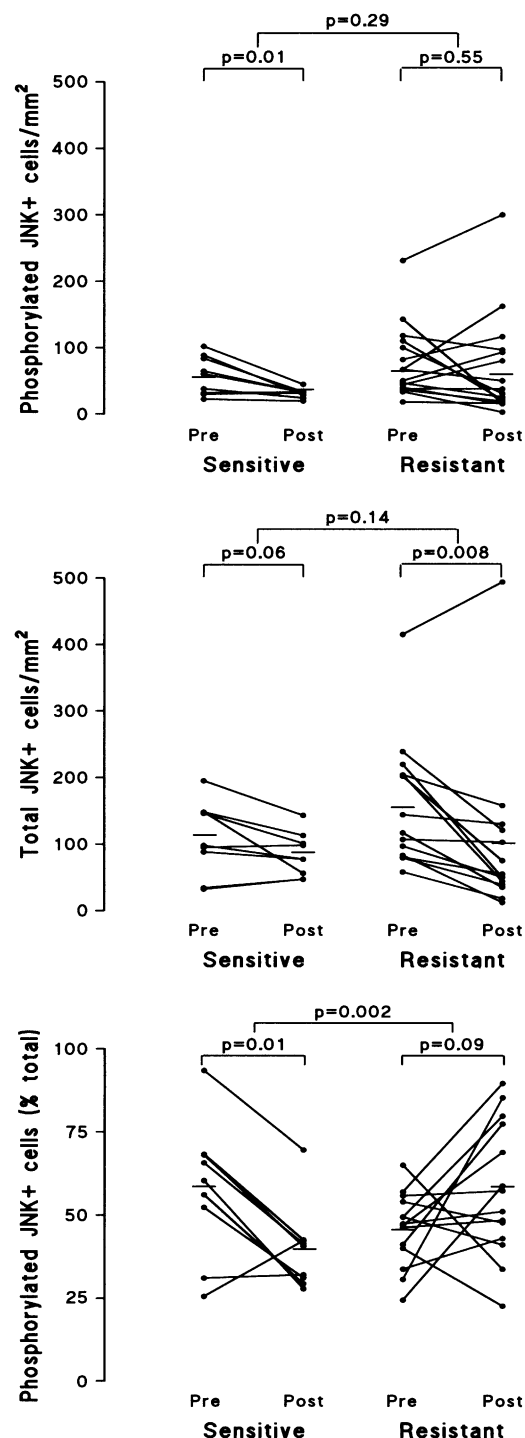


FIG 4. Absolute numbers of cells expressing phosphorylated JNK (top) and total JNK (middle) immunoreactivity and percentages of total JNK-immunoreactive cells showing JNK phosphorylation (bottom) per unit area of mucosa in bronchial biopsy specimens from GC-sensitive (n = 9) and GC-resistant (n = 13) asthmatic patients before (Pre) and after (Post) oral prednisolone therapy (Student paired *t* test within groups; Student 2-sample *t* test between groups). Bars show means.

was significantly reduced in the GC-sensitive but not the GC-resistant patients (sensitive: $58\% \pm 7\%$ to $40\% \pm$

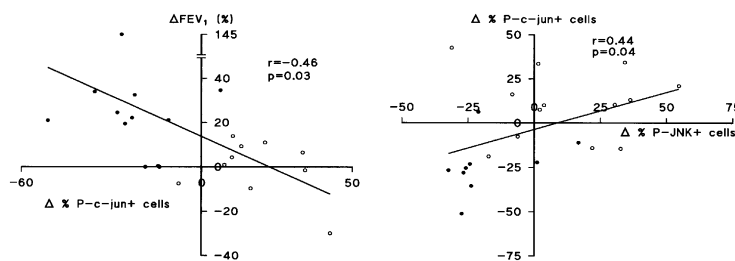


FIG 5. *Left panel*, Correlation between percentage changes in FEV₁ and changes in the percentages of c-jun-immunoreactive cells in which the c-jun is phosphorylated in the GC-sensitive (filled circles, n = 9) and GC-resistant (open circles, n = 13) asthmatic patients after oral prednisolone. *Right panel*, Correlation between changes in the percentages of c-jun-immunoreactive cells in which the c-jun is phosphorylated and JNK-immunoreactive cells in which the JNK is phosphorylated in the GC-sensitive (filled circles, n = 9) and GC-resistant (open circles, n = 14) asthmatic patients after oral prednisolone (Pearson linear regression).

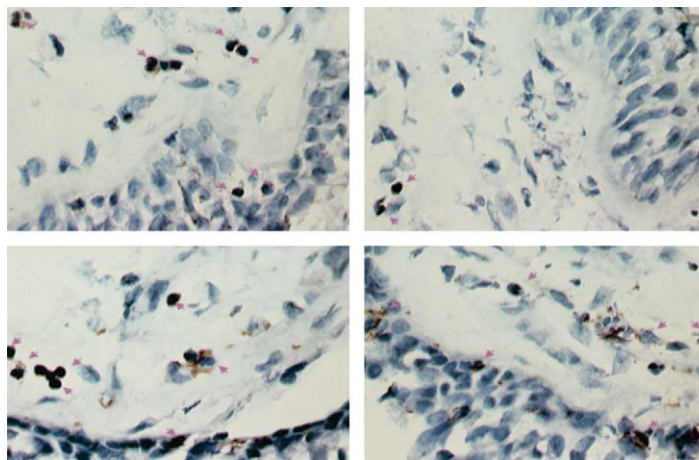


FIG 6. Example of stained sections showing cells immunoreactive for phosphorylated JNK before (left panels) and after (right panels) prednisolone therapy in one GC-sensitive (top) and one GC-resistant (bottom) asthmatic patient. Arrows point to stained cells.

4%, $P = .01$; resistant: $46\% \pm 3\%$ to $58\% \pm 5\%$, $P = .09$). The mean change was also significantly higher in the GC-sensitive patients ($P = .002$).

Correlation with changes in lung function

Data on correlation with changes in lung function are shown in Fig 5. In the asthmatic patients considered as a single group, a negative correlation was observed between the percentage changes in FEV₁ and the changes in the percentages of bronchial mucosal cells expressing phosphorylated c-jun before and after oral prednisolone therapy. A positive correlation was observed between the changes in the percentages of cells expressing phosphorylated JNK and the changes in those expressing phosphorylated c-jun.

A typical photomicrograph of sections stained with phosphorylated JNK using the described protocol is shown in Fig 6.

DISCUSSION

This is the first systematic study of the expression of AP-1 components in the asthmatic bronchial mucosa,

although one previous report²¹ documented increased mucosal expression of c-fos in asthmatic patients compared with control subjects. The data lend further support to the hypothesis that clinically GC-resistant asthma is associated with altered regulation of the expression and activation of the components of AP-1 within bronchial mucosal cells. GC-resistant compared with GC-sensitive asthmatic patients showed increased baseline bronchial mucosal cellular expression of c-fos and evidence of impaired downregulation of the MEK/JNK/c-jun phosphorylation cascade after systemic GC therapy. These data are consistent with our previous studies showing increased c-fos expression in blood mononuclear cells from GC-resistant asthmatic patients⁵ and impaired downregulation of phosphorylation of c-jun and JNK by oral prednisolone therapy in invading inflammatory cells in a cutaneous, tuberculin-induced model of acute inflammation in these patients.²² In the present study the changes observed in association with oral prednisolone therapy were not associated with significant changes in the mean total numbers of mucosal leukocytes, which likely formed the majority of the cells expressing AP-1 components, suggesting that the changes do not reflect significant influx or egress of cells from the

site. Precise phenotyping of the mucosal leukocytes expressing AP-1 components was not practicable because of a lack of suitable antibodies for double staining and in any case would not have modified the conclusions. In contrast to the lack of downregulation of JNK phosphorylation in the GC-resistant asthmatic patients, these patients did show downregulation of total JNK protein after oral prednisolone, a phenomenon not observed in the GC-sensitive patients. The mechanism of this observation is unclear.

The fact that in association with oral prednisolone therapy reduction in JNK phosphorylation correlated with reduction in c-jun phosphorylation, which in turn correlated with changes in FEV₁, strongly underlines the possible functional significance of the observations with regard to regulation of airways caliber, with GC-resistant asthmatic patients lying at the extreme end of a spectrum of GC responsiveness. According to the correlation in Fig 5, variation in c-jun phosphorylation accounts for about 20% of the observed variation in FEV₁ response, indicating, unsurprisingly, that other factors consequent on systemic GC therapy also regulate this response. Although the mechanism of this relationship is unclear, it is possible to speculate that increased activation of c-jun and expression of c-fos might increase AP-1 activity within mucosal inflammatory cells, thus increasing the transcription of asthma-relevant cytokines, such as IL-4 and IL-5,^{7,8} while sequestering the ligand-bound GC receptor, preventing its binding to DNA³⁻⁵ and exerting its transrepressive effects. In line with this, systemic GC therapy failed to inhibit expression of IL-4 and IL-5 mRNA in bronchoalveolar lavage fluid T cells from GC-resistant asthmatic patients, as it did in GC-sensitive patients.²³ There might be other as-yet-unidentified mechanisms whereby expression of AP-1 components regulates airways caliber in asthma.

The relationship between expression of AP-1 components and the inhibitory effects of GCs in asthma is likely complex and in dynamic equilibrium. On the one hand, as shown in previous reports^{14,15,22} and as further evidenced in the present study, GCs inhibit phosphorylation of c-jun and JNK through inhibitory effects on the JNK/SAPK signaling pathway. On the other hand, c-jun has been shown to downregulate transcription of the GC receptor gene by acting on its promoter,²⁴ which could result in fewer functional GC receptors in airways cells, whereas AP-1 binds to the GC receptor directly, with mutual inhibition of the activities of both moieties. Whereas in most asthmatic patients systemic GCs exert a net anti-inflammatory effect, this equilibrium appears to be destabilized in favor of excessive production of AP-1 components in GC-resistant patients, with consequent GC hyporesponsiveness in inflammatory cells. We hypothesize that external environmental influences might contribute to this phenomenon. For example, it is emerging that oxidative stress influences JNK phosphorylation through activation of JNK/SAPK signaling²⁵ and inhibition of phosphatases.²⁶ Smoking is one possible source of oxidative stress (not applicable to the patients in this study) that has been shown to reduce GC responsiveness in patients with asthma.²⁷ Dietary antioxidant intake could conceivably exert an influence.

Altered regulation of the expression and activation of AP-1 components is likely not the only mechanism for refractoriness to GC therapy in asthma. Impaired nuclear translocation of the ligand-bound GC receptor in blood mononuclear cells has been linked with GC hyporesponsiveness.^{28,29} In the latter study²⁹ stimulation of T cells with enterotoxin superantigen produced by *Staphylococcus aureus* rendered them resistant to GC inhibition *in vitro*, and this was accompanied by phosphorylation of both the mitogen-activated ERK signaling protein and the GC receptor itself, as well as its impaired nuclear translocation. Treatment of cells with MEK/ERK inhibitors abrogated all of these effects. The authors inferred that activation of the MEK/ERK signaling pathway renders T cells resistant to GC inhibition by means of direct ERK-mediated phosphorylation of the GC receptor, which impairs its nuclear translocation. Other signaling kinases can also directly phosphorylate the GC receptor. For example, incubation of blood T cells with exogenous combination of IL-2 and IL-4 *in vitro*, which has previously been shown to reproduce a reduction in the ligand binding affinity of the GC receptor observed directly *ex vivo* in T cells from GC-resistant asthmatic patients and induce GC resistance *in vitro*,³⁰ was accompanied by phosphorylation of the GC receptor, which in this case was inhibited by a specific p38 mitogen-activated protein kinase inhibitor, which also abrogated the effects of exogenous IL-2/IL-4 in reducing the ligand binding affinity of the GC receptor and inducing GC resistance.³¹ The authors inferred that p38 mitogen-activated protein kinase, activated by exogenous cytokines, phosphorylates the GC receptor and thereby reduces its ligand binding affinity. Activated JNK has also been reported to phosphorylate the GC receptor, enhancing its nuclear export after ligand dissociation.³² These studies underline the complexity of interaction of kinase signaling pathways in regulating GC responsiveness in individual target cells and once more emphasize the probable importance of local environmental influences (oxidative stress, local cytokine milieu, and exogenous superantigens) in regulating this responsiveness.

Further understanding of the interactions between GCs, c-fos, and the components of the MEK/JNK/c-jun cascade and the influence of the bronchial mucosal environment on these interactions will likely inform more logical therapeutic approaches to the problem of GC-resistant asthma.

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