

The Allergen Der p1 Induces NF- κ B Activation through Interference with I κ B α Function in Asthmatic Bronchial Epithelial Cells

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Received June 9, 1997

Asthma is an inflammatory disease of the airways due to an interaction between genetic and environmental factors, and allergy represents the most important predisposing trait. Here, we investigated why and how the allergen most often implicated in the pathogenesis of asthma and other allergic diseases causes the expression of the genes for proinflammatory cytokines in airway epithelium. We found that Der p1 promotes activation of transcriptional factor NF- κ B by interference with the function of its cytoplasmic inhibitor I κ B α . This is the first report on the effect of an allergen on transcriptional factors. Our results improve the understanding of the mechanisms involved in allergic diseases and suggest potential therapeutic utility of NF- κ B blockers. © 1997 Academic Press

Asthma is a chronic inflammatory disease of the airways, where bronchial epithelial cells play an important effector role (1-4). Asthmatic bronchial epithelial cells produce a number of cytokines which potentiate the activity of antigen-presenting cells and/or participate in the recruitment of inflammatory cells in the airways, including granulocyte/macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-8, tumor necrosis factor α (TNF α) and regulated upon activation in normal T cells expressed and secreted (RANTES) (1,2,4).

The mechanisms of this upregulation of cytokine expression in asthmatic epithelium may depend on an aberrant response of epithelial cells to the allergens encountered at the mucosal surface because, differing

from bronchial epithelial cells of healthy subjects and cells of allergic individuals without asthma, bronchial epithelial cells of allergic patients with asthma are unable to inactivate certain allergens (5). Thus, in bronchial epithelial cells of asthmatic patients allergic to house dust mites, Der p1, the major allergen from the mite *Dermatophagoides Pteronyssinus* (6), moves from the apical to the basolateral surface, where it can have access to antigen-presenting cells. By contrast, bronchial epithelial cells of non-asthmatic subjects sensitized to the same allergen inactivate Der p1 by 30 minutes of exposure and do not transport it. This aberrant permeability of asthmatic bronchial epithelial cells to the allergen is accompanied by increased expression of the mRNA for some of the cytokines already known to be expressed *in vivo* by these cells (5), but how Der p1 can induce cytokine gene transcription is unknown.

GM-CSF, IL-8, TNF α (7) and RANTES (8) have all been shown to be regulated by transcriptional factor NF- κ B. We therefore tested the hypothesis that Der p1 induces transcription of the genes for those cytokines because it activates transcription factor NF- κ B, and that this mechanism operates *in vivo* because NF- κ B activation is detectable in the bronchial epithelium of asthmatic patients naturally exposed to the allergen.

NF- κ B is a heterodimeric protein whose subunits belong to the Rel proto-oncoprotein family (9). In most cell types, including human bronchial epithelial cells (8), the predominant inducible form of NF- κ B is composed of 50kD (p50) and 65kD (RelA, formerly p65) subunits (10,11). In unstimulated cells, the p50/RelA complex is sequestered in the cytoplasm by interaction with inhibitory proteins, including I κ B α . I κ B α specifically masks the nuclear translocation signal of RelA, and in so doing prevents the nuclear translocation of the NF- κ B complex (9-11). Upon stimulation, I κ B α is rapidly phosphorylated, released from the complex and

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subjected to proteolytic degradation. Liberated NF- κ B dimers are allowed to translocate to the nucleus, where they bind to DNA, through specific NF- κ B sites located in the promoter and/or enhancer elements of target genes, and activate transcription (9-11).

Our results indicate that in primary cultures of asthmatic bronchial epithelial cells Der p1 induces transcription of the genes for cytokines GM-CSF and RANTES by promoting nuclear translocation of NF- κ B through the phosphorylation and degradation of I κ B α . We also report NF- κ B activation in concomitance with increased expression of GM-CSF and RANTES transcripts in freshly isolated bronchial epithelial cells of asthmatic patients sensitized to Der p1 and naturally exposed to the allergen. Our data therefore suggest that it may be possible to control allergic asthma by developing drugs which specifically block or prevent activation of this transcriptional factor in bronchial epithelium.

MATERIALS AND METHODS

Isolation and cultures of bronchial epithelial cells. For the experiments on the *in vitro* effect of Der p1, cells were obtained from 16 asthmatic patients allergic to *Dermatophagoides Pteronyssinus* (6 males, 10 females; mean age 32.5 years, range 19-43 years) by endobronchial brushing on bronchoscopy, according to a standard protocol (1,2,5). Purified epithelial cells (1) were seeded onto collagen-coated 24-well culture plates (10⁶/well), in 2% Ultraser G (IBF Biotechnics, Savage, MD) and antibiotics (1,2). Before testing, cells were incubated for 8 days to allow disappearance of the spontaneous cytokine expression (1) which is known to occur *in situ* (1,3,4). For the experiments testing cytokine mRNA expression and NF- κ B activation during natural exposure to Der p1, two groups of subjects were selected. The first group was composed of 10 asthmatic patients allergic to *Dermatophagoides pteronyssinus* (4 females, 6 males; mean age 29.6 years, range 20-36 years). All of them were receiving an inhaled β_2 -receptor agonist (salbutamol) on demand, as bronchodilator, and 5 were also taking an inhaled glucocorticoid (beclomethasone dipropionate, 250-500 μ g daily). Nonetheless, all were symptomatic with daily episodes of wheezing and shortness of breath. The second group was composed of 8 non-asthmatic subjects allergic to *Dermatophagoides Pteronyssinus* (5 females, 3 males; mean age 30.2 years, range 21-42 years). They were receiving no medication and were tested as controls. Bronchial epithelial cells were obtained by the procedures reported above but, in this case, they were immediately processed for northern blot analysis and electrophoretic mobility shift assay (EMSA). This study was approved by the appropriate institutional review boards and all the subjects gave informed consent.

Determination of natural exposure to Der p1. To document that natural exposure to Der p1 had indeed occurred in the asthmatic and non-asthmatic donors of bronchial epithelial cells tested to evaluate the effect of that exposure, dust samples were taken from mattress and pillows every 2 days for the 8 days preceding bronchoscopy, using a standard collecting instrument (ALK Laboratories, Denmark). Dust samples were extracted and analyzed by an enzyme-linked immunosorbent assay for Der p1, according to the manufacturer's recommendation (ALK Laboratories). All the subjects had been exposed to more than 10 μ g Der p1/g dust, a concentration representing a risk factor for acute asthma in allergic individuals (12).

Exposure of cell cultures to Der p1 *in vitro*. Der p1 was purified from lyophilized mite extracts by affinity chromatography, using the

monoclonal antibody 4C1 (Indoor Biotechnologies Ltd, Deeside, UK). Cultured cells were exposed to 1 μ g/ml Der p1 (5) for various periods of time. All reagents and the Der p1 solution were free of endotoxin contamination.

EMSA. The DNA probes were double-stranded oligonucleotides containing the NF- κ B sites present in the GM-CSF (from -76 to -85) and RANTES (at position -32) gene promoter regions (5'-CAC-AGTTCAGGTAGTTCCCCGCT-3' and 5'-ACTCCCCTTAGGGGATGCCCTCA-3', respectively) (8). The reagents for the binding reaction were 5 μ g nuclear extract proteins and 10⁵ cpm ³²P-labelled NF- κ B probe in 15 μ l of binding buffer (50 mM NaCl, 1 mM dithiothreitol, 10 mM Tris HCl, pH 7.5, 5% glycerol and 4 μ g poly-dIdC) (8). Reagents were incubated at 21°C for 30 minutes. The DNA/protein complexes were resolved by 5% polyacrylamide gel electrophoresis and localized by autoradiography (8). Specificity of the binding was evaluated by adding 20-fold excess of unlabelled double-stranded oligonucleotides to the binding mixture 10 minutes before the addition of the labelled probe. For supershift analysis, a polyclonal antibody to human RelA (sc-109, Santa Cruz Biotechnology, Inc., CA), 2 μ g, was added in the EMSA binding mixture after 10 minutes of incubation, followed by reincubation for additional 20 minutes and gel electrophoresis (8).

Western blot analysis of I κ B α . Proteins in total cell extracts were separated by SDS-PAGE and transferred onto a membrane filter. Immunoreactive I κ B α was detected by using a rabbit anti-human I κ B α antibody (Rockland, Boyertown, PA), at a dilution of 1:100 in blocking buffer, and a peroxidase-conjugated donkey anti-rabbit immunoglobulin.

Cell transfection with the expression vector for HA-I κ B α and western blot. Bronchial epithelial cells were transfected by electroporation (13) with a construct made up by inserting 3 copies of the HA epitope tag in front of the translation initiation codon of human I κ B α cDNA cloned in pCMV4 (14). Then, cells were stimulated for 30 and 60 minutes with 1 μ g/ml Der p1, and total cell extracts were separated by 10% SDS-PAGE, transferred onto nitrocellulose membranes and analyzed for the expression of HA-I κ B α fusion protein by western blot analysis, using a monoclonal antibody against the HA epitope (anti-HA, Boehringer Mannheim, Germany).

Polymerase chain reaction (PCR). This was used to analyze the expression of GM-CSF and RANTES mRNA in Der p1-exposed cultured bronchial epithelial cells. After extraction of total RNA, the mRNA was reverse-transcribed into cDNA and first-strand cDNA was amplified with oligonucleotides specific for GM-CSF and RANTES by the PCR procedure, as described in detail (1,5). The cDNA for β -actin was also amplified as control. The sequences of oligonucleotide 5' and 3' primers were as follows: GM-CSF, ATGTGGCTGCAGAGCCTGCTGC and CTGGCTCCCAGCAGTCAAAGGG, predicted size 424 bp; RANTES, GCTGTCTATCCTCATTTGCTAC and TCTCCATCCTAGCTCATCTC, predicted size 260 bp; β -ACTIN, GTGGGGCGCCCCAGGCACCA and CTCCTTAATGTCACGCACGATTTC, predicted size 570 bp. A 30-cycle PCR was performed in a DNA Thermal Cycler (Perkin Elmer Cetus Co., Norwalk, CT) at 94°C for 1 minute, 54°C for 2 minutes, and 72°C for 3 minutes. PCR products were analyzed by electrophoresis on 2% agarose gels, and visualized by ethidium bromide staining.

Northern blot analysis. This was used to analyze the expression of mRNA for GM-CSF and RANTES in freshly isolated bronchial epithelial cells of allergic patients with asthma and allergic subjects without asthma. After isolation by the acid guanidium thiocyanate-phenol-chloroform extraction method, RNA (10 μ g) was separated on agarose gels, blotted onto nitrocellulose membranes, and hybridized to specific ³²P-labelled probes corresponding to human GM-CSF and RANTES sequences, as described (8,15).

RESULTS

To evaluate the functional role of NF- κ B in allergen-induced cytokine expression, we exposed primary cul-

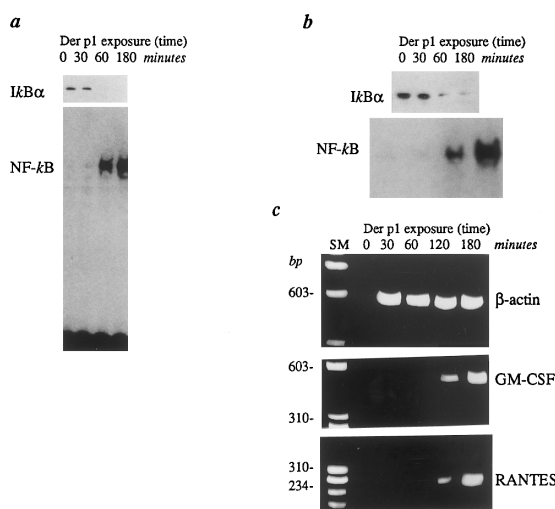


FIG. 1. Effect of Der p1 exposure on NF-κB activation and on transcription of GM-CSF and RANTES genes in cultured bronchial epithelial cells of asthmatic patients allergic to *Dermaphagoides Pteronyssinus*. Cells were stimulated for the indicated periods of time with 1 μg/ml Der p1. **a.** IκBα immunoreactivity and NF-κB binding to the probe for the NF-κB site in the GM-CSF gene (representative of 5 experiments). **b.** IκBα immunoreactivity and NF-κB binding to the probe for the NF-κB site in the RANTES gene (representative of 5 experiments). **c.** Ethidium bromide-stained gels showing GM-CSF, RANTES and β-actin PCR products (representative of 6 experiments) (SM, size marker: *HaeIII* digestion of ϕX174 DNA, Promega Co., Madison, WI).

tures of bronchial epithelial cells from allergic asthmatic patients to 1 μg/ml Der p1 for various periods of times. Then, cell extracts were analyzed for IκBα immunoreactivity and for binding of NF-κB to the oligonucleotides corresponding to the NF-κB binding sites of human GM-CSF and RANTES promoters. In extracts from unstimulated cells an antibody to IκBα specifically detected a single band of the appropriate size (37 kD). Between 30 and 60 minutes after the addition of Der p1 to the cultures, IκBα disappeared from cells and this phenomenon coincided with the appearance of NF-κB DNA-binding activity (Fig. 1a and Fig. 1b), suggesting that the two events were causally related. Induced NF-κB DNA binding activity was followed by transcription from the GM-CSF and RANTES genes (Fig. 1c). After 3 hours of allergen exposure, NF-κB DNA-binding activity was still evident, in absence of any IκBα immunoreactivity and at a time when GM-CSF and RANTES transcripts were fully detectable (Fig. 1a-c).

To examine whether and how Der p1 induced phosphorylation and degradation of IκBα, cultured asthmatic bronchial epithelial cells were transiently transfected with influenza hemagglutinin (HA)-tagged cDNA expression vector encoding human IκBα. After stimulation of the transfectants with Der p1, phosphorylation and degradation of the transfected HA-IκBα

fusion protein were analyzed by immunoblotting analysis with an antibody against the HA epitope tag. Der p1 exposure firstly caused the appearance of a second HA-IκBα band in the immunoblot (band indicated by the arrow in Fig. 2). This more slowly migrating HA-IκBα molecule has been shown to represent HA-phosphorylated IκBα (14). After further stimulation, HA-IκBα immunoreactivity disappeared, indicating IκBα degradation.

We next evaluated whether NF-κB activation is also detectable, in association with cytokine gene transcription, during natural exposure to Der p1 in the airway epithelium of asthmatic patients sensitized to *Dermaphagoides Pteronyssinus*. We found an increased expression of GM-CSF and RANTES transcripts in freshly isolated bronchial epithelial cells of those patients, as compared to the cells from allergic subjects without asthma (Fig. 3a). This was associated with a strong NF-κB-DNA binding activity which was absent in the control cells (Fig. 3b). A rabbit anti-RelA antiserum decreased the mobility of the upper NF-κB/DNA complex, causing the supershift shown in Figure 3c (lane 3), whereas a control rabbit immunoglobulin did not show any effect on complex formation and mobility (lane 4). This identified the transcriptional activator RelA of NF-κB dimers as major component in the retarded protein/DNA complex.

DISCUSSION

This is the first study describing the effect of an allergen on transcriptional activators and, in particular, on the activation of transcriptional factor NF-κB. Nonetheless, it does not elucidate how Der p1 induces phosphorylation and degradation of IκBα, thereby allowing NF-κB translocation to the nucleus and DNA binding. This allergen seems to require much more time than other known stimuli to achieve an effect, particularly when compared to TNFα or IL-1 (14,16). It took more than 30 minutes before there was a decay of the NF-

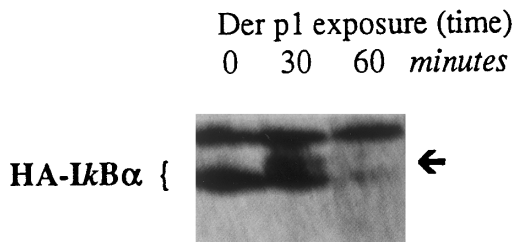


FIG. 2. Effect of Der p1 exposure on the phosphorylation and degradation of HA-IκBα fusion protein in bronchial epithelial cells of asthmatic patients. Cells were transfected with the HA-tagged IκBα cDNA cloned in pCMV4 and then exposed for the indicated periods of time to 1 μg/ml Der p1. The arrow indicates the band representing the HA-phosphorylated IκBα molecule in the immunoblot (representative of 3 experiments).

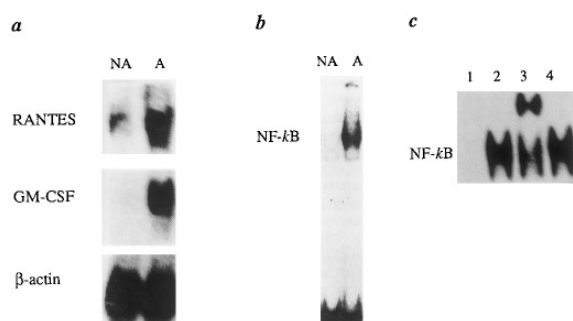


FIG. 3. Expression of mRNA for RANTES and GM-CSF, and NF- κ B-DNA binding activity in freshly isolated bronchial epithelial cells from asthmatic patients (A) and non-asthmatic (NA) subjects allergic to *Dermatophagoides Pteronyssinus* and naturally exposed to Der p1. **a.** Northern blot analysis showing RANTES and GM-CSF transcripts. The mRNA for β -actin is also shown as control (representative of 5 experiments). **b.** EMSA with the DNA probe corresponding to the NF- κ B site of the GM-CSF promoter region (representative of 5 experiments). **c.** Supershift analysis. Lane 1, free probe; lane 2, NF- κ B/DNA complexes from nuclear extracts of bronchial epithelial cells of one asthmatic patient; lane 3, same sample after the addition of an anti-RelA antibody; lane 4, same sample after the addition of control rabbit immunoglobulin (representative of 3 experiments).

kB inhibitor in Der p1-stimulated cells, whereas I κ B α phosphorylation and degradation have been previously observed in various cell types after 5-15 minutes of exposure to those cytokines or phorbol esters. Despite these kinetic differences, however, the elimination of I κ B α was associated with the appearance of NF- κ B DNA-binding activity in the same way as shown with other stimuli (16).

Exposure to Der p1 has been strongly associated with the prevalence of asthma and the risk of developing asthma in sensitized individuals (17-19). Our results improve the understanding of the mechanisms implicated in the genesis of airway inflammation in allergic asthmatics exposed to this allergen and help explain why only some allergic subjects develop asthma.

Inhaled glucocorticoids are the most effective drugs for asthma treatment (20), but side effects limit their use. They control airway inflammation by blocking the influx of inflammatory cells and their activation, effects at least in part due to their ability to downregulate the expression of proinflammatory cytokines in bronchial epithelium (1,3,21). One of the proposed mechanisms of action of glucocorticoids is the inhibition of induced NF- κ B activation (22-25). Some of the patients we tested to evaluate the effect of natural Der p1 exposure were actually taking inhaled glucocorticoids, and still we were able to detect cytokine gene transcription and strong NF- κ B DNA-binding activity. This might be due to the use of low doses, as all treated patients had symptoms indicating poor control of airway inflammation. Thus, if inhaled glucocorticoids do interfere with induced NF- κ B DNA-binding activity in asthma, they need to be administered at high doses and on a regular

basis to achieve that effect. Our results suggest that it may be possible to treat this disease more effectively and safely by using drugs which specifically block or prevent NF- κ B activation.

At the time of testing the effect of natural exposure to Der p1, all the asthmatics were receiving an inhaled β_2 -receptor agonist as bronchodilator, whereas the controls were taking no medication. This can not account for the observed differences in cytokine expression and NF- κ B activation because treatment of asthmatic patients for 8 days with that bronchodilator is unable to affect cytokine gene expression and cytokine release in bronchial epithelium (26).

ACKNOWLEDGMENTS

We thank all the subjects who volunteered to participate in this study, which was supported through grants from the Italian National Research Council, Cariplo Foundation, and Ferring Srl, Milano, Italy.

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