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Mechanisms of Persistent NF-κB Activity in the Bronchi of an Animal Model of Asthma

Fabrice Bureau, Sylvie Delhalle, Giuseppina Bonizzi, Laurence Fiévez, Sophie Dogné, Nathalie Kirschvink, Alain Vanderplasschen, Marie-Paule Merville, Vincent Bours, and Pierre Lekeux

In most cells *trans*-activating NF-κB induces many inflammatory proteins as well as its own inhibitor, IκB-α, thus assuring a transient response upon stimulation. However, NF-κB-dependent inflammatory gene expression is persistent in asthmatic bronchi, even after allergen eviction. In the present report we used bronchial brushing samples (BBSs) from heaves-affected horses (a spontaneous model of asthma) to elucidate the mechanisms by which NF-κB activity is maintained in asthmatic airways. NF-κB activity was high in granulocytic and nongranulocytic BBSs. However, NF-κB activity highly correlated to granulocyte percentage and was only abrogated after granulocytic death in cultured BBSs. Before granulocytic death, NF-κB activity was suppressed by simultaneous addition of neutralizing anti-IL-1β and anti-TNF-α Abs to the medium of cultured BBSs. Surprisingly, IκB-β, whose expression is not regulated by NF-κB, unlike IκB-α, was the most prominent NF-κB inhibitor found in BBSs. The amounts of IκB-β were low in BBSs obtained from diseased horses, but drastically increased after addition of the neutralizing anti-IL-1β and anti-TNF-α Abs. These results indicate that sustained NF-κB activation in asthmatic bronchi is driven by granulocytes and is mediated by IL-1β and TNF-α. Moreover, an imbalance between high levels of IL-1β- and TNF-α-mediated IκB-β degradation and low levels of IκB-β synthesis is likely to be the mechanism preventing NF-κB deactivation in asthmatic airways before granulocytic death.


Chronic airway inflammation, associated with persistent overexpression of many proteins involved in immune and inflammatory responses, is a characteristic feature of asthma (for review, see Ref. 1). Protein overexpression depends on increased gene transcription, suggesting that activation of some transcription factors underlies asthma pathogenesis. Transcription factors that are thought to be involved in asthma are NF-κB, AP-1, NF-AT, cAMP response element binding protein, STATs, and GATA-3 (Refs 2 and 3; for review, see Ref. 4). All of the inflammatory genes overexpressed in asthma, such as those encoding proinflammatory cytokines, chemokines, adhesion molecules, and inflammatory enzymes, contain κB sites for NF-κB within their promoter (for review, see Ref. 5), suggesting that these genes are controlled predominantly by NF-κB and that NF-κB could be of particular importance in the initiation and the perpetuation of allergic inflammation. This assumption is reinforced by the fact that glucocorticosteroids, the most potent treatment for asthma, strongly inhibit NF-κB in vitro (6, 7).

The NF-κB family is composed of five structurally related DNA-binding proteins, called p50, p52, p65/RelA, c-Rel/Rel, and RelB (for review, see Ref. 8). The most common form of NF-κB is a heterodimer composed of p50 and p65 subunits, although the different family members can associate in various homo- or heterodimers through a highly conserved N-terminal sequence, called the Rel homology domain. DimORIZATION of various NF-κB subunits produces complexes with different DNA-binding specificities and *trans* activation potentials. In most cell types, inactive NF-κB complexes are associated with inhibitory proteins of the IκB family, which sequester NF-κB in the cytoplasm. The members of the IκB family are IκB-α, IκB-β, IκB-ɛ, p100, p105, and Bcl-3, where the most common IκB protein is IκB-α (8, 9). p105 and p100 are the precursors of p50 and p52, respectively. Following various stimuli, such as viruses, bacteria, pro-oxidants, and proinflammatory cytokines, IκB proteins are first phosphorylated, ubiquitinated, and then rapidly degraded by the proteasome, allowing NF-κB nuclear translocation and transcriptional initiation of NF-κB-dependent genes (10).

Macrophages of induced-sputum and bronchial epithelial cells from stable asthmatic patients exhibit increased NF-κB activity compared with cells from healthy patients (11). Mice deficient in p50 or c-Rel are unable to develop eosinophilic airway inflammation when sensitized and challenged with OVA (12, 13). In bronchial brushing samples (BBSs) recovered in heaves-affected horses, a spontaneous animal model of asthma (for review, see Ref. 14), NF-κB complexes are mainly atypical p65 homodimers (15). p65 homodimer activity drastically increases in BBSs from heaves-affected horses challenged with moldy hay, which contains the allergens responsible for the disease (i.e., proteins borne by

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spheres of *Aspergillus fumigatus*, *Faenia rectivirgula*, and *Ther-
moactinomyces vulgaris*). Interestingly, this increased activity is
maintained at high or moderate levels for at least 21 days after
glomer eviction from the horses’ environment. In this model it has
also been demonstrated that p65 homodimer activity found in
BBSs is highly correlated to the degree of lung dysfunction and to
the level of ICAM-1 expression (15). Although these in vivo ob-
servations confirmed that NF-kB is likely to play a crucial role in
allergic inflammation and in subsequent airway obstruction, no
study was devoted to the mechanisms by which NF-kB activity is
maintained in lung cells even when the etiologic agent is absent.

In the present report, we describe studies aimed at identifying
the mechanisms by which NF-kB activity is regulated in cells ob-
tained by bronchial brushing in heaves-affected horses after aller-
gen evicition, and we propose a cellular and molecular model that
accounts for the persistent NF-kB activity observed in the bronchi
of this animal model of asthma.

### Materials and Methods

#### Experimental animals

Nine horses (564 ± 18 kg; 18.6 ± 1.8 years; mean ± SD) with a history
and clinical signs of heaves were used. These horses typically developed
acute airway obstruction (crisis) when housed in a barn and fed moldy hay,
and they entered clinical remission once pastured or stabilized in a controlled
environment. One month before the experiment they underwent a thorough
clinical examination, including an electrocardiogram, arterial blood gas
analysis, hematometry, endoscopy of the airways, tracheo-bronchial lavage,
and pulmonary scintigraphy. This confirmed that they suffered from heaves
and were free from any other health problems. Six healthy horses (605 ±
99 kg; 7.6 ± 2.6 years) were used as controls. Experimental horses did not
receive any bronchoscopic maneuvers during the month preceding the
experiment.

Bronchial cells of heaves-affected horses were obtained by bronchial
brushing on two separate occasions: 24 h after the onset of a crisis and then
21 days after removal from the causative environment. To obtain crisis,
the horses were stabilized and subjected to a natural challenge with moldy hay.
The horses were considered to be in crisis when their breathing mechanic
variables were within the following limits: maximal difference in pleural
pressure ≥2.00 kPa, total pulmonary resistance ≥0.2 kPa·l·s⁻¹·m⁻², and dy-
namic compliance ≥8 l·Pa⁻¹. These respiratory mechanic variables were
calculated from simultaneous measurements of esophageal pressure, air
flow, and tidal volume (for more technical details, see Ref. 16). Eviction of
the antigenic agents was obtained by pasturing the horses or stabling them
with dust-free bedding and feed. Healthy horses were investigated twice at
the 21-day interval. The protocol was approved by the ethics committee of
the University of Liege.

#### Bronchial brushings

Horses were premedicated i.v. with 0.01 mg/kg romifidine (Sedivet; Boehr-
inger Ingelheim, Ingelheim, Germany). Bronchoscopy was performed with
a 9-mm diameter bronchoscope (Pentax, Breda, The Netherlands) using a
transnasal approach. The brushing was performed in 10 different places,
from the main bronchi to the fourth generation airways, by inserting a
cytology brush (Cook Veterinary Products, Eight Mile Plains, Australia)
into the different segments. Bronchial cells were obtained using 20 gentle
upward and downward strokes of the brush against the airway walls. Care
was taken to avoid bleeding. Bronchi were not irrigated with physiological
serum before brushing, to conserve leukocytes and to ensure that samples
were representative of the cellular changes occurring within the bronchi of
diseased horses.

#### Cell processing

After retraction of the brush into its protective sheath and its removal from
the bronchoscope channel, collected cells were dislodged by shaking the
brush into 15-mL conical tubes containing ice-cold RPMI 1640 medium
(Life Technologies, Merelbeke, Belgium) supplemented with 1% glu-
tamine, 10% FBS, 50 μg/mL gentamicin, and 10 μg/mL amphotericin B. The
harvested cell suspension was vortexed and filtered through gauze to
remove mucus. The cells were then centrifuged at 800 × g for 5 min, and
the pellet was resuspended in LHC-8 complete medium without hydrocor-
tisone (Biofluids, Rockville, MD) supplemented with 10 μg/mL amphoteric-
in B. The cells were then incubated at 37°C in a 5% CO₂-95% air mixture
for different times before protein extraction. The minimal culture time be-
fore protein extraction was 3 h. Cell density was assessed by the use of a
hemocytometer, and cell viability was evaluated by propidium iodide ex-
clusion (5 μg/mL of culture medium). Cell differentials were performed on
cytospin preparations stained with Diff-Quick (Dade Behring, Diestingen,
Germany). Where necessary, polymorphonuclear cells were separated from
the other cells using Histopaque centrifugation (specific gravity, 1.077;
Sigma, Bornem, Belgium).

### Cytoplasmic and nuclear protein extraction

Cytoplasmic and nuclear protein extracts were prepared as previously de-
scribed (17). Cytoplasmic extract contained 10 mM HEPES (pH 7.9), 10
mM KCl, 2 mM MgCl₂, 0.1 mM EDTA, 0.2% (v/v) Nonidet P-40, and 1.6
mM/g protease inhibitors (complete: Roche, Mannheim, Germany). The
pelleted nuclei were resuspended in 20 mM HEPES (pH 7.9), 1.5 mM
MgCl₂, 0.2 mM EDTA. 0.63 M NaCl, 25% (v/v) glycerol, and 1.6 mg/mL
protease inhibitors (nuclear buffer), incubated for 20 min at 4°C, and cen-
trifuged for 30 min at 12,000 × g (Eppendorf centrifuge 5415C; Eppendorf
Scientific, Hamburg, Germany). Protein concentrations were quantified with
the Micro bichinonic acid protein assay reagent kit (Pierce, Rockford, IL).

### Anti-IκB Abs

The anti-IκB Abs used were 1) a mouse mAb directed against IκB-α (a gift
from Katrina wood, University of Oxford, Oxford, U.K.); 2) a rabbit poly-
clonal Ab recognizing an NH₂-terminal peptide of mouse IκB-β (Santa
Cruz Biotechnology, Santa Cruz, CA); 3) a mouse mAb directed against
aa 1-444 of the human p50 subunit (Upstate Biotechnology). Santa Cruz, CA); 4) a mouse mAb directed against aa
1-12 of the human p50 (Upstate Biotechnology). Immunoblot experi-
ments performed with cytoplasmic extracts prepared from equine lympho-
cyes showed that all these Abs are equine reactive.

### Plasmids

The pRe/CMV-hemagglutinin (HA)-IκB-β expression vector was provided by
Alain Israel and Robert Weil (Institut Pasteur, Paris, France). The empty
pRe/CMV plasmid was purchased from Invitrogen (San Diego, CA). Construc-
tions were linearized with ScaI before coupled in vitro transcription and
translation.

### In vitro transcription and translation of IκB-β

Linearized pRe/CMV and pRe/CMV-HA-IκB-β plasmids were in vitro
transcribed and translated simultaneously using the TntT7 Coupled
Wheat Germ Extract System (Promega, Madison, WI) according to the
manufacturer’s instructions. The reactions were performed either with or
without 200 mM 3′-Sadenosine in the transcription-translation mix. The
[32P]labeled DNA was analyzed by autoradiography (Gel Doc 2000; Bio-
Rad, Hercules, CA). To confirm specificity, competition assays were performed
with a 50-fold excess of unla-
bled wild-type and mutated probes. The sequences of the oligonucleotides
Bound reactions were performed for 30 min at room temperature with 5
μg of nuclear proteins in 20 mM HEPES (pH 7.9), 10 mM KCl, 0.2 mM
EDTA, 20% (v/v) glycerol, 1% (w/v) acetylated BSA, 3 μg of poly(dI-dC)
(Amersham Pharmacia Biotech, Aylesbury, U.K.), 1 mM DTT, 1 mM
PMSF, and 100,000 cpm of [32P]labeled double-stranded oligonucleotide
probes. Probes were prepared by annealing the appropriate single-stranded
oligonucleotides (Eurogentec, Liege, Belgium) at 65°C for 10 min in 10
mM Tris, 1 mM EDTA, and 10 mM NaCl, followed by slow cooling to
room temperature. The probes were then labeled by end-filling with the
Klenow fragment of *Escherichia coli* DNA polymerase I (Roche), with
France). Labeled probes were purified by spin chromatography on Seph-
adex G-25 columns (Roche). DNA-protein complexes were separated from
unbound probe on 4% native polyacrylamide gels at 150 V in 0.25 M Tris,
0.25 M sodium borate, and 0.5 mM EDTA, pH 8.0. Gels were vacuum
dried and exposed to Fuji x-ray film (Tokyo, Japan) at ~80°C for 12 h.
The amount of specific complexes was determined by photodensitometry of the
autodensitography (Gel Doc 2000; Bio-Rad, Hercules, CA). To confirm spec-
ificity, competition assays were performed with a 50-fold excess of unlabeled
wild-type and mutated probes. The sequences of the oligonucleotides
used in this work are as follows: wild-type palindromic κB probe (19),
5′-TTGGCAACGCGGAGAATTCCTTCTCCTTAGTTT-3′; and mutated palindromic κB probe, 5′-TTGGCAACGCGGAGAATTCCTTCTCCTTAGTTT-3′.
For experiments performed with in vitro translation products, 2 μl of each reaction was incubated with the nuclear extracts obtained from BBSs for 30 min either before or after incubation with the radiolabeled probe.

**Immunoblots**

Protein extracts (10 μg) were added to a loading buffer (10 mM Tris-HCl (pH 6.8), 1% (w/v) SDS, 25% (v/v) glycerol, 0.1 mM 2-ME, and 0.03% (w/v) bromophenol blue), boiled, and run on a 10% SDS-PAGE gel. After electrophoresis, proteins were transferred to polyvinylidene difluoride membranes (Roche) and blocked overnight at 4°C with 20 mM Tris (pH 7.5), 500 mM NaCl, 0.2 (v/v) Tween 20 (Tris-HCl/Tween), and 5% (w/v) dry milk. The membranes were then incubated with the nuclear extracts obtained from BBSs incubated for 1 h with the first Ab (1/250 dilution for IL-1β and TNF-α, 1/2000 dilution for NF-κB) in the case of nuclear extracts (data not shown).

After washing, the membranes were incubated with peroxidase-conjugated rabbit anti-mouse IgG (1/2000 dilution) for 45 min. The membranes were washed and revealed with enhanced chemiluminescence detection method (ECL kit; Amersham Pharmacia Biotech). Equal loading of protein on the gels was confirmed in all experiments by probing the blots for either α-tubulin (Santa Cruz Biotechnology) in the case of cytoplasmic extracts or Oct-1 (Santa Cruz Biotechnology) in the case of nuclear extracts (data not shown).

**Neutralization experiments**

Neutralizing Abs directed against recombinant human IL-1β and TNF-α were purchased from Sigma. Anti-IL-1β and anti-TNF-α Abs were used at 3 or 8 μg/ml. These were incubated for 180 min before protein extraction with 3-h cultured BBSs obtained from heaves-affected horses, 21 days after allergen eviction.

**Statistical analysis**

Linear associations between variables were assessed by the use of standard least-square linear regressions. Correlation coefficients (r) were presented as measures of linear association for regression relationships. Significant differences of the slopes from zero were determined using Student’s t test. The differences between mean values were estimated using Student’s t tests for unpaired data. p < 0.05 was considered significant.

**Results**

**Cell number, type, and viability**

The number of harvested cells averaged 19.9 ± 7.0 (mean ± SD) million cells/animal (range, 11–34 million). Differential cell counts showed a significant increase in the percentage of granulocytes in BBSs obtained from heaves-affected horses compared with healthy horses (Table I). The viability of harvested granulocytes (92.5 ± 4.2%), as determined by propidium iodide exclusion, was significantly greater than the viability of the other cells present in BBSs (24.2 ± 9.3%). Accordingly, total cell viability measured in BBSs from heaves-affected horses was significantly higher than that measured in BBSs from healthy horses (Table I). We previously reported lower percentages of granulocytes in BBSs from heaves-affected horses (i.e., 3.4 ± 0.7% during the crisis and 1.8 ± 1.3% 21 days after allergen eviction), and cell viability that was not significantly different between healthy and diseased horses (15). This could be imputed to the fact that bronchi were irrigated with physiological serum before brushing in this earlier study. Indeed, bronchial irrigation partly eliminates granulocytes, which are present in large quantities in diseased horses at the surface of the airway epithelium.

**Positive correlation between the percentage of granulocytes and NF-κB activity in BBSs**

Consistent with our previous studies (15), NF-κB activity was much greater in nuclear extracts prepared from BBSs of heaves-affected horses during crisis (Fig. 1A, lanes 4, 6, and 8), when compared with extracts from BBSs of healthy horses (Fig. 1A, lanes 1–3). Twenty-one days after the eviction of the causative agents, NF-κB activity was maintained at high or moderate levels in BBSs from diseased horses (Fig. 1A, lanes 5, 7, and 9). As the percentage of granulocytes and NF-κB activity simultaneously increased in BBSs from heaves-affected horses, notably during crisis, correlations between the percentage of granulocytes in BBSs and the intensity of NF-κB DNA binding, as measured by photo- densitometry, were calculated. These regression analyses were conducted with the results obtained from four separate EMSAs. Correlation coefficients between the percentage of granulocytes and the intensity of specific NF-κB bands were 0.96 (p < 0.001, first gel), 0.97 (p < 0.001, second gel), 0.93 (p < 0.01, third gel), and 0.89 (p < 0.01, fourth gel). These significant correlations were all positive. Results obtained from a representative gel are shown in Fig. 1.

**NF-κB activity is increased in both granulocytes and nongranulocytic cells contained in BBSs from heaves-affected horses**

The strong correlation between the intensity of NF-κB activity and the percentage of granulocytes present in BBSs suggested that the increased NF-κB activity observed in BBSs from heaves-affected horses was restricted to polymorphonuclear cells. To verify this hypothesis, BBSs were recovered in heaves-affected horses 21 days after allergen eviction, and the granulocytes were separated from the other BBS cells using Histopaque centrifugation. Because BBSs contained many clusters made of various cell types, the use of specific immunological methods, such as flow cytometry, to separate the granulocytes from the other BBS cells was inadequate. Histopaque centrifugation only allowed partial cell separation. Two fractions were obtained: a granulocyte-enriched fraction, in which the percentage of granulocytes averaged 62.1 ± 12.2%, and a bronchial epithelial cell (BEC)-enriched fraction, in which the percentage of granulocytes averaged 9.5 ± 9.9%. EMSAs performed with total and enriched samples obtained simultaneously from the same heaves-affected horses showed identical NF-κB activities (Fig. 2), indicating that NF-κB activity is similarly increased in both granulocytes and nongranulocytic cells contained in BBSs from diseased horses.

**Granulocytic death and NF-κB deactivation are concomitant in cultured BBSs from heaves-affected horses**

NF-κB activity was maintained at high or moderate levels in the bronchi of heaves-affected horses 21 days after allergen eviction, indicating that NF-κB activity in asthma-like diseases does not necessarily require the continuous presence of the etiologic agent. To determine whether increased NF-κB activity is also sustained ex vivo, three BBSs obtained simultaneously from the same

| Table I. Characteristics of the cells recovered by bronchial brushing in healthy and heaves-affected horses |
|--------|--------|--------|--------|--------|
|        | BECs (%) | Macrophages (%) | Lymphocytes (%) | Granulocytes (%) | Viable Cells (%) |
| Healthy Horses | 96.6 ± 1.1 | 0.5 ± 0.3 | 0.3 ± 0.7 | 2.6 ± 0.9 | 25.3 ± 8.7 |
| AFFECTED Horses |       |        |        |        |        |
| During crisis | 54.9 ± 7.2* | 1.3 ± 0.8 | 0.6 ± 0.3 | 43.2 ± 5.3* | 57.2 ± 5.5* |
| After the crisis | 72.3 ± 10.1* | 0.9 ± 0.6 | 0.4 ± 0.4 | 26.4 ± 9.8* | 43.2 ± 7.3* |

* Significantly different from values recorded in healthy horses with p < 0.05.
heaves-affected horses (n = 9) 21 days after the crisis were cultured for 3, 24, or 48 h before assessment of NF-κB binding activity. Nuclear extracts prepared from BBSs cultured for 3 and 24 h demonstrated identical NF-κB activities (examples are given in Fig. 3A, lanes 1, 2, 4, and 5). BBSs cultured for 48 h displayed NF-κB activities that were either similar to those observed at 3 and 24 h (n = 5; an example is provided in Fig. 3A, lane 6) or drastically decreased (n = 4; an example is given in Fig. 3A, lane 3).

At 24 h, granulocyte viability and other BBS cell viability averaged 83.4 ± 10.5 and 20.3 ± 11.2%. At 48 h, these values reached 55.3 ± 24.3 and 18.5 ± 10.3%, respectively. The large variance observed in granulocyte viability at 48 h was due to the fact that the rates of granulocytic death were not consistent among the BBSs. At this time point, granulocytic viability was indeed maintained at high levels in BBSs from five horses (an example is given in Fig. 3B, lane 6), whereas granulocytic viability considerably decreased in BBSs from the four other horses (an example is provided in Fig. 3B, lane 3). Reduced granulocytic viability was always accompanied by a drastic decrease in NF-κB activity (an example is given in Fig. 3, A and B, lane 3). Conversely, when granulocytic viability was maintained at high levels, NF-κB activity was also sustained at high levels (an example is given in Fig. 3, A and B, lane 6). The link between granulocytic death and abolishment of NF-κB activity in cultured BBSs from heaves-affected horses was confirmed by regression analyses, which were conducted with the results obtained from three separate EMSAs. Correlation coefficients between granulocyte viability and the intensity of NF-κB bands were 0.93 (p < 0.01, first gel), 0.89 (p < 0.01, second gel), and 0.87 (p < 0.05, third gel). Correlation coefficients between the viability of the other BBS cells and NF-κB activity were also calculated. These correlation coefficients were not significant and ranged from 0.29–0.39. Results obtained from a representative gel are shown in Fig. 3. These data show that sustained NF-κB activity in cultured BBSs requires the presence of living granulocytes.

**IkB-β is the most prominent IkB protein found in equine BBSs**

In most cells stimulation leading to IkB-α proteolysis and nuclear translocation of NF-κB also results in the subsequent rapid NF-κB-dependent induction of IkB-α (20, 21). The reaccumulation of IkB-α following its loss allows a fast repression of NF-κB activity, thereby ensuring a transient NF-κB response. In cultured BBSs from heaves-affected horses, NF-κB activity was maintained as long as living granulocytes were present (Fig. 3), suggesting that mutual regulation of NF-κB and IkB-α is impaired in these samples. Two hypotheses could account for this observation: either IkB-α is degraded as soon as it is resynthesized, preventing NF-κB deactivation, or IkB-α is not produced in the BBSs obtained from heaves-affected horses. To explore these hypotheses, the presence of all IkB proteins in cytoplasmic and nuclear extracts prepared from 3-h cultured BBSs of healthy and heaves-affected horses 21 days after allergen eviction was assessed by immunoblots.

Only very low amounts of IkB-α were detected in cytoplasmic and nuclear extracts obtained from BBSs of both healthy and heaves-affected horses (Fig. 4). Similarly, p100 was undetectable (data not shown). Only small amounts of p105, the precursor of
with immunoblot in cytoplasmic extracts obtained from cells of healthy and heaves-affected horses (data not shown). On the contrary, significant amounts of IκB-β were observed in cytoplasmic extracts from BBSs of healthy horses, while considerably lower amounts of cytoplasmic IκB-β were observed in BBSs obtained from heaves-affected horses (Fig. 4). IκB-β was not detectable in the nuclear extracts from BBSs of healthy and heaves-affected horses (Fig. 4). These results indicate that IκB-β, rather than IκB-α, is the most prominent IκB protein found in the BBSs of horses and suggest that IκB-β is degraded in BBSs from heaves-affected horses.

Neutralizing anti-IL-1β and anti-TNF-α Abs inhibit the persistent IκB-β degradation and NF-κB activation in BBSs from heaves-affected horses

NF-κB stimulates the production of IL-1β and TNF-α. These proinflammatory cytokines induce the degradation of the IκB proteins and the subsequent activation of NF-κB, thus initiating autoregulatory feedback loops (for review, see Ref. 22). To determine whether these loops were involved in the sustained IκB-β degradation and NF-κB activation observed in the BBSs of heaves-affected horses before granulocytic death, neutralizing anti-IL-1β and/or anti-TNF-α Abs were added to the medium of 3-h cultured BBSs obtained from heaves-affected horses 21 days after allergen eviction. The final Ab concentrations was either 3 or 8 μg/ml. Cytoplasmic and nuclear extracts were prepared from treated BBSs 180 min after Ab addition and were subsequently analyzed for IκB protein expression by immunoblot and for NF-κB binding activity by EMSA. Neither the addition of anti-IL-1β Abs (3 or 8 μg/ml) nor the addition of anti-TNF-α Abs (3 or 8 μg/ml) was able to reduce IκB-β degradation and NF-κB activation in BBSs (Fig. 5A). Conversely, when added simultaneously, the anti-IL-1β and anti-TNF-α Abs (3 μg/ml of each Ab) drastically increased the cytoplasmic and nuclear amounts of IκB-β (Fig. 5B) and markedly decreased the NF-κB activity in BBSs (Fig. 5A), indicating that autoregulatory feedback loops involving both IL-1β and TNF-α are at least partly responsible for the sustained IκB-β degradation and NF-κB activation in the cultured BBSs from diseased horses. The cytoplasmic and nuclear amounts of IκB-α, p100, and p105 were not altered by the addition of neutralizing Abs to the culture medium.
IkB-β prevents p65 homodimer DNA binding and removes bound p65 homodimers from kB sites

We had previously demonstrated that active NF-κB complexes found in BBSs of heaves-affected horses were mainly p65 homodimers, rather than classical p65-p50 heterodimers (15). Knowing that IkB-β is the most prominent IkB protein present in equine BBSs (Fig. 4) and that the appearance of IkB-β in the nucleus of BBS cells and related deactivation is concomitant (Fig. 5), we hypothesized that IkB-β is able to prevent p65 homodimer DNA binding and to displace bound p65 homodimers from their kB sites. To verify this hypothesis, the effects of recombinant IkB-β on p65 homodimer DNA binding were investigated using EMSAs. First, either the linearized plasmid vector pRc/CMV or this same linearized plasmid containing the IkB-β cDNA insert (pRc/CMV-HA-IkB-β) was used as DNA templates in a coupled in vitro transcription/translation reaction system. The [35S]methionine-labeled translated products were analyzed by electrophoresis in a 10% polyacrylamide-SDS gel and by autoradiography. A major translated product was observed in the gel and by autoradiography. A major translated product was analyzed by electrophoresis in a 10% polyacrylamide-SDS gel and by autoradiography. A major translated product was observed in the gel.

**FIGURE 5.** Effects of neutralizing anti-IL-1β and/or anti-TNF-α Abs on NF-κB activity (A) and IkB-β expression (B) in BBSs. Three-hour cultured BBSs obtained from heaves-affected horses 21 days after allergen eviction were incubated for 180 min with neutralizing anti-IL-1β Ab (8 μg/ml), neutralizing anti-TNF-α Ab (8 μg/ml), or both neutralizing Abs (3 μg/ml each for each Ab). Cytoplasmic and nuclear extracts were prepared from treated BBSs and were subsequently analyzed for NF-κB binding activity by EMSA (A) and for IkB-β expression by immunoblot (B). This experiment is representative of three similar experiments.

**Discussion**

The chronic inflammation of the airways described in asthma is due to persistent overexpression of multiple inflammatory proteins, including proinflammatory cytokines, chemokines, adhesion molecules, and inflammatory enzymes (1, 4). Despite their crucial role in asthma pathogenesis, the precise mechanisms responsible for this sustained gene overexpression have been poorly investigated and remain obscure. We previously demonstrated that NF-κB activity, which plays a key role in the transcriptional initiation of many inflammatory genes, drastically increases in the bronchi of heaves-affected horses after allergen exposure and is maintained at high or moderate levels in the bronchi of diseased horses for at least 21 days after allergen eviction from their environment (15). Here, we show that this increased NF-κB activity also persists for at least 24–48 h in cultured BBSs obtained from heaves-affected horses 21 days after allergen evasion. As NF-κB activity usually returns to basal level within a few hours after the removal of external stimuli (23), our observations indicate that NF-κB activity is aberrantly sustained in the bronchial cells of heaves-affected horses after allergen evasion and strongly suggest that this persistent transcriptional activity could account for the maintained expression of inflammatory genes in asthmatic bronchi.

The accumulation of active granulocytes in the airways is thought to be of particular importance in the development of clinical asthma (24). Indeed, several investigators have demonstrated a positive and significant correlation between eosinophil profusion in the airways and lung dysfunction in asthmatic patients (25, 26). Furthermore, the resolution of eosinophilic inflammation, which
depends upon eosinophil apoptosis, is associated with clinical improvement of asthma (27). In the present study, the levels of NF-κB activity were high or moderate in granulocytes and non-granulocytic cells contained in BBSs obtained from heaves-affected horses 21 days after allergen removal. However, NF-κB activity strongly correlated with the percentage of granulocytes present in BBSs and was completely abrogated after granulocytic death, suggesting that the sustained NF-κB activation that occurs in the Airways of heaves-affected horses is mainly driven by the inflammatory cells that remain or appear in the bronchi after allergen evocation. As the level of NF-κB activity in the bronchi is closely related to the degree of pulmonary dysfunction (15), our results also provide a new insight into the molecular mechanisms by which the granulocytes impair lung function. First, the level of NF-κB activity in the granulocytes probably determines the amounts of broncho- and vasoactive inflammatory mediators released by these cells. Second, one may assume that activated granulocytes also secrete cytokines that are able to initiate the NF-κB-dependent synthesis of inflammatory mediators by other bronchial cells. Granulocytic death and clearance would be prerequisites for the cessation of the direct and indirect effects of these cells on lung function.

The physiological half-life of the circulating neutrophil, the most abundant granulocyte, is only 6 h (for review, see Ref. 28). In the present study, granulocyte viability was much longer, indicating that protective mechanisms delay inflammatory cell death in asthmatic Airways. TNF-α, which was produced by BBSs from heaves-affected horses, induces apoptosis of ex vivo cultured neutrophils at early time points, but inhibits apoptosis after culture for 18 h (29). This delayed protective effect is lost when protein synthesis is inhibited, indicating that TNF-α induces anti-apoptotic proteins that protect neutrophils which avoid early death (for review, see Ref. 30). It is likely that inflammatory cells that invade the site of inflammation are those that express anti-apoptotic proteins and are subsequently protected from death. This possibility might explain the prolonged survival observed in granulocytes from heaves-affected horses, even in the presence of TNF-α. A second hypothesis could account for the increased survival of inflammatory cells from asthmatic bronchi. Indeed, many cytokines present at inflammatory sites, such as GM-CSF, are able to delay granulocyte apoptosis (for review, see Ref. 30). These anti-apoptotic cytokines could counteract the cytotoxic effects of TNF-α.

An intriguing question concerns the maintenance of NF-κB activity in the bronchi before granulocytic death. A hallmark of many NF-κB-dependent genes that are switched on in inflammatory diseases is that their expression can be induced by the proinflammatory cytokines IL-1β and TNF-α (31). Activated granulocytes generate high amounts of IL-1β and TNF-α (32–34). IL-1β and TNF-α activate NF-κB, which, in turn, induces the expression of these proinflammatory cytokines, thus initiating autoregulatory feedback loops (for review, see Ref. 22). Accordingly, we postulated that these autoregulatory feedback loops might be involved in the granulocyte-mediated persistent NF-κB activity in bronchial cells of heaves-affected horses after allergen evocation. Addition of both neutralizing anti-IL-1β and anti-TNF-α Abs to cultured BBSs from heaves-affected horses resulted in the suppression of NF-κB activity, confirming our hypothesis. These findings are consistent with previous data from Lentsch et al. (35), who showed that NF-κB activity occurs in a time course similar to that for the production of IL-1β and TNF-α during IgG immune complex-induced lung injury. Interestingly, when either the anti-IL-1β or anti-TNF-α Abs was added individually to the medium of cultured BBSs, each was incapable of reducing NF-κB activity. These results indicate that either cytokine is independently able to maximally stimulate the signaling pathway leading to NF-κB activation in the cultured BBSs from heaves-affected horses and emphasize that the antagonization of a single cytokine would probably have a minor effect on allergic inflammation.

1xβ-β, rather than 1xβ-α, was the most prominent 1xβ protein found in BBSs from healthy and heaves-affected horses. Moreover, the appearance of 1xβ-β in the nucleus of BBS cells from heaves-affected horses was accompanied by NF-κB deactivation, as observed after treatment with anti-IL-1β and anti-TNF-α Abs. Finally, recombinant 1xβ-β was able to prevent DNA binding by p65 homodimers, which are the most abundant NF-κB complexes found in equine BBSs (15) and was able to remove bound p65 homodimers from their κB sites. Because BBSs contained mainly BECs in healthy horses and BECs plus granulocytes in heaves-affected horses, our results unambiguously demonstrate that the inhibition of p65 homodimers by 1xβ-β regulates NF-κB-dependent gene expression in equine bronchial epithelial cells and in equine bronchial granulocytes. However, large amounts of 1xβ-α are observed in equine blood granulocytes (our unpublished observations), suggesting a shift in 1xβ protein expression during granulocyte migration and activation. Although unexpected, our results are in accordance with those of Lentsch et al. (35, 36), who demonstrated that deactivation of NF-κB complexes predominantly composed of p65 by secretory leukocyte protease inhibitor is associated with increased levels of 1xβ-β, but not 1xβ-α, in a rat model of IgG immune complex-induced alveolitis. Furthermore, previous in vitro studies have demonstrated that 1xβ-β (37) as well as 1xβ-ε (38) preferentially inhibit the p65 homodimeric form of
NF-κB. As no equine reactive anti-IκB-α Ab is available, the inhibitory function of IκB-α in BBSs from heaves-affected horses was not investigated.

IκB-β has been demonstrated to be involved in the persistent NF-κB activity observed in some cells, including activated 70Z/3 pre-B cells (39), WEHI 231 mature B cells (40), HIV-1-infected myeloid cells (41), and T cells infected by the human T cell leukemia virus type 1 (42). In human T cell leukemia virus type 1-infected T cells, the persistent NF-κB activity results from the continuous degradation of IκB-β by the virally encoded Tax protein (42). In the other cells stimulation results in the degradation of IκB-β, which is rephosphorylated in a hypophosphorylated form that sustains NF-κB activation (39–41). Indeed, hypophosphorylated IκB-β interacts with NF-κB without masking its nuclear localization signal and its DNA binding site, thus acting as a chaperone for NF-κB nuclear entry and activity. Furthermore, hypophosphorylated IκB-β prevents NF-κB resequatation by IκB-α. However, IκB-β was not associated with active p65 homodimers in the nucleus of BBS cells obtained from heaves-affected horses, indicating that hypophosphorylated IκB-β is not involved in the sustained NF-κB activity found in these cells.

Rapid NF-κB-dependent resynthesis of IκB-α establishes an autoregulatory loop by which NF-κB activation is self-limited (Refs. 20 and 21; for review, see Ref. 43). Conversely, NF-κB activation does not induce IκB-β overexpression, indicating that NF-κB complexes exclusively released from IκB-β are not inhibited by an autoregulatory feedback mechanism (18). This observation led Thompson et al. (18) to anticipate that activation of NF-κB would probably be persistent in tissues lacking IκB-α, because no feedback inhibition through increased synthesis of IκB-α would occur in these tissues. In the present report, IκB-α was lacking, and IκB-β was continuously degraded by proinflammatory cytokines in BBSs from heaves-affected horses, providing the first in vivo example of the mechanism of sustained NF-κB activity theoretically described by Thompson et al. (18).

Taken together, our results allow us to propose a cellular and molecular model that accounts for the persistent NF-κB activity occurring in the bronchi of heaves-affected horses after allergen eviction (Fig. 7). In this model the NF-κB activity is maintained as long as there are living granulocytes in the airways. Before granulocyte death, NF-κB activity is maintained in all bronchial cells by granulocyte-dependent autoregulatory feedback loops involving the proinflammatory cytokines IL-1β and TNF-α. IκB-β is expressed at a basal level unaffected by cell stimulation with IL-1β and TNF-α, is rapidly degraded, does not reach the nucleus, and is subsequently unable to stop the cytokine-mediated NF-κB activation.

After granulocyte death, the autoregulatory feedback loops involving IL-1β and TNF-α are strongly attenuated, allowing IκB-β-dependent NF-κB deactivation in nongranulocytic cells.

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